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Membranes and signalling systems. Berridge, M.J. AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, U.K.

When cells respond to external signals they usually have to encode the information into a form which can be interpreted by the internal effector systems. For the control of growth, cells employ a variety of transducing mechanisms some of which are multi-purpose in that they are also used for regulating other cellular processes such as contraction and secretion whereas others have a more specific role in mitogenesis. An example of the former is the phosphoinositide signalling system which controls two important intracellular signal pathways initiated by the second messengers diacylglycerol (DG) and inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$)[1]. The receptors directly coupled to tyrosine kinases represent a separate transduction mechanism more specifically associated with the control of cell growth. Either of these two separate transducing mechanisms can activate cell growth but usually the mitogenic stimulus is greatly potentiated if both pathways are activated simultaneously. There are indications that the two pathways interact with each other suggesting that cell growth is regulated by an intracellular network of mitogenic signals. The bifurcating phosphoinositide signalling pathway has been implicated in the action of many mitogenic signals including fertilization and the action of growth factors such as bombesin, vasopressin, angiotensin II, thrombin, antigens and platelet-derived growth factor (PDGF). Just how this pathway functions to stimulate cell proliferation is still unclear but there are indications that both the protein kinase C and calcium pathways can induce the transcription of early response genes such as *fos* and *jun*. These messenger pathways are initiated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$). Injecting an antibody against this lipid can block cell proliferation in response to certain growth factors (bombesin, PDGF) but not others (FGF, insulin)[2]. Also, pertussis toxin blocks the mitogenic action of thrombin on lung fibroblasts but has no effeect on the action of EGF[3]. Experiments of this kind confirm the existence of separate mitogenic pathways. The mode of action of PDGF is beginning to provide fascinating insights into the complex interactions which exist between these mitogenic pathways. In addition to initiating the tyrosine phosphorylation cascade, PDGF can also stimulate inositol lipid metabolism at two levels. It can stimulate the hydrolysis of $PtdIns(4,5)P_2$ apparently through a direct phosphorylation of phospholipase C perhaps by a mechanism similar to that described for EGF[4]. However, when the tyrosine kinase insert is removed, proliferation is blocked but $PtdIns(4,5)P_2$ hydrolysis is unchanged suggesting that the latter is not connected with the initiation of cell growth[5]. Removing the insert does prevent the PDGF receptor from stimulating the kinase which phosphorylates PtdIns to give $PtdIns3P$[6]. It is this type I kinase which is also activated by *c-src* suggesting that this novel lipid plays some key role in cell proliferation[6,7]. Unravelling the interactions of these different mitogenic pathways holds the key to our understanding of the membrane events which regulate how cells grow.

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Prospects for pharmacological intervention with signal transduction. Rink, T.J. Smith Kline & French Research Ltd., The Frythe, Welwyn, Herts. AL6 9AR, U.K.

In considering this vast topic it may be helpful to consider two broad classifications for modulation of biological signals that might, and in a few cases actually appear to, control cancerous growth. First, certain cancers may depend on constitutive or abnormal stimulation through recognised, specific "first messenger" pathways. Thus interference with these pathways may produce selective control. Examples would include: the current practice of blockade of oestrogen receptors in some breast cancers, and removal or blockade of androgen drive in prostatic cancer; and the putative utility of blockade of tissue or organ-specific growth factors that may sustain particular cancers, e.g. the possible involvement of bombesin in small cell carcinoma of the lung. A second approach is to inhibit cellular signal pathways that may be over-/or inappropriately expressed in cancerous cells, or to activate cellular messenger systems that may serve to regulate growth. Examples here could be the possible utility of sub-type selective inhibitors of protein kinase-C (predicated partly on the tumour promoting effects of C-kinase activators), or activation of the cAMP pathways in view of the growth control effects of this signal pathway in certain tissues. Since mitogens activate not only the C-kinase pathway but also Ca^{2+} mobilisation one could speculate that blockade of, for instance, receptor-mediated Ca^{2+} entry might selectively interfere with abnormal proliferation. An interesting recent development has been the identification of inhibitors of certain receptor-associated tyrosine kinases, which appear to reduce proliferative responses to receptor stimulation. Most of these approaches remain to be tested, not least because suitably potent and specific pharmacologic agents are not yet available. Moreover one might argue that interference with cellular signal transduction systems shared by numerous cell types would be unlikely to show the requisite specificity. On the other hand, inhibition of common signal pathways could functionally antagonise responses dependent on multiple mediators. Two responses to this challenge are: 1, pharmacological agents may have much more impact on pathologically activated pathways than on normal control systems; and the emerging plethora of sub-types of various components of signalling such as C-kinase isozymes, multiple G-proteins and PI kinases offer a prospect for selectivity: and, 2, an encouraging precedent is seen in the "calcium antagonists" which, against expectation, can be highly selective therapeutically because of their specificity among the structural and functional diversity of the voltage-gated Ca^{2+} channels.

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Physical aspects of membrane structure and function

Chapman, Dennis
Department of Protein & Molecular Biology
Royal Free Hospital School of Medicine
Rowland Hill Street, London NW3 2PF

This talk will outline our present views of biomembrane structure based upon information deduced by the application of a range of physical techniques to both model and natural biomembranes. Some of these techniques will be briefly described to illustrate the type of information which has been obtained. The techniques will include methods such as FTIR spectroscopy, laser flash photolysis, fluorescence and nmr and esr spectroscopy. The organisation of both the lipid and protein components will be discussed. Concepts such as lipid fluidity, its modulation by cholesterol phase transitions, protein-lipid interactions and the consequences of lipid asymmetry will be briefly indicated. The type of information about membrane proteins which is revealed by these physical techniques will also be illustrated. The deduction of the types of secondary structure present in membrane protein, both of a qualitative nature and for a quantitative assessment will be outlined. The deduction of the presence of water pores in some proteins and measurements of the dynamics and movement, both lateral and rotational, of these proteins will be illustrated. Some examples of the membrane proteins which will be discussed will be the Ca-AtPase, the glucose transporter protein, bacteriorhodopsin, rhodopsin and the H^+/K^+-ATPase. Recent studies of related systems including calcium binding proteins such as calmodulin, parvalbumin and troponin C will also be discussed as well as studies of the structure of a number of signal polypeptides.

4

Biophysical parameters influencing adriamycin cytotoxicity.

Thomas R. Tritton, Department of Pharmacology, University of Vermont School of Medicine, Burlington, VT 05405 USA.

Defining the mechanism of action of adriamycin has proven to be a difficult task because of the wide variety of biological responses elicited by this drug. The major explanations offered for adriamycin-induced cytotoxicity include DNA intercalation, generation of reactive oxygen species, production of alkylating moieties, stabilization of the topoisomerase II/DNA cleavage complex and damage to the plasma membrane. All but the last of these mechanisms require intracellular events to occur to bring about drug action. The hypothesis being explored at this meeting is that cell surface signalling mechanisms may be involved in initiating or modulating the intracellular effects. In this presentation we will discuss the structural basis for adriamycin's ability to interact with and change the organization of membranes, which in turn allows such signals to be transduced.

Phospholipid selectivity. We have measured the ability of adriamycin to interact with liposomal bilayers of varying composition by studying both membrane fluidity and the facility of membranes to fuse with each other. The drug increases fluidity (measured by light scattering) in membranes of all compositions tested except those containing cardiolipin, where adriamycin causes a decrease in fluidity. Similarly, membrane fusion (measured by proton NMR) is also differentially responsive to adriamycin when cardiolipin is present. Thus, this phospholipid confers specificity on a bilayer with respect to it's ability to interact with adriamycin.

Membrane location. Identification of the location of adriamycin in membrane bilayers has been approached in three ways. First, we have used iodide ion quenching of the drug's fluorescence as a yardstick of penetration into the bilayer. The basis for such experiments is that collisional quenching of a fluorophore depends on direct contact between the emitting species and the quenching reagent. Use Stern-Volmer formalism we are able to deduce that adriamycin is partially, but not completely, buried in the membrane. Also, the presence of cardiolipin creates a second, more deeply buried site within the bilayer. A second type of quenching experiment uses paramagnetic (nitroxide) quenching of the drug's fluorescence to quantify the location; by this approach we can calculate that the average distance from the adriamycin chromophore to the center of the bilayer is 6.2 Å in the solid phase and 3.8 Å in the fluid phase. The third approach to membrane location involves measuring the angular distribution of polarized fluorescence intensities in oriented membranes. This technique allows calculation of the angle of the molecule with respect to the membrane coordinate system. Adriamycin takes on an angle of ~55°, which is particularly disruptive to the ordered packing of the acyl chains. This finding begins to offer a structural explanation for the extraordinary ability of adriamycin to damage membrane signal transduction and other functions.

Structure-activity relationships. We have quantified the binding affinity of a series of anthracyclines with liposomal membranes and compared the results to biological data on cytotoxicity and uptake. The results allow the development of a set of rules about structure-activity relationships and suggest that to obtain maximal activity an anthracycline would have the following three properties: (1) a high affinity constant for neutral membranes; (2) low intracellular uptake; (3) the presence of a nonionizable aminosugar. Thus, purely physical measurements, taken in conjunction with an understanding of the biology of a drug, can yield insights into fundamental pharmacologic problems.

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Nuclear magnetic resonance studies of anti-cancer drug-membrane interactions

Ben de Kruijff, Klaas Nicolay and Frits de Wolf, Centre for Biomembranes and Lipid Enzymology (CBLE) and Institute of Molecular Biology and Medical Biotechnology (IMB), University of Utrecht, Padualaan 8, 3584 CH Utrecht, NL

Lipids in biological membranes play a variety of different structural and functional roles.One particular aspect concerns their polymorphic phase properties. The bilayer organization of membranes clearly is the basic structural organization of the lipid part of biological membranes. However, in every biological membrane lipids occur which by themselves do not preferably organize in bilayers (non-bilayer lipids). The abundant and regulated presence of non-bilayer lipids in membranes, the morphology of specialized membrane systems and the functional abilities of these lipids strongly suggest that these lipids, and the structures they can form, play important roles in different aspects of membrane function, such as fusion, transbilayer transport of proteins and functional integration of proteins in membranes (1).

Several anti-cancer drugs have a high affinity interaction with specific lipids or lipid classes. Prominent examples are members of the anthracycline and ellipticine family. These positively charged amphipatic drugs have a strong interaction with acidic membrane phospholipids. As a result of this interaction dramatic changes in lipid organization and consequently in membrane function can take place. In case of doxorubicin the prominent and specific interaction with the mitochondrial lipid cardiolipin results in marked changes of organizational properties of the lipid (2), a block of protein import (3) and mitochondrial respiration (4). This latter property is most likely involved in cardiotoxicity of the drug as in vivo NMR studies on rat hearts witnessed a significant drop in energy-rich phosphates upon doxorubicin administration (5).

Our recent NMR studies on interactions between doxorubicin and phosphatidic acid, a key negatively charged lipid in signal transduction at the level of the plasma membrane revealed the strength of this interaction and its large consequences for acyl chain order (6). Members of the ellipticin family of anti-tumor drugs also have high affinity interaction with negatively charged lipids. However, the consequences of the interaction for lipid organization are different in that these drugs appear not to be able to cause phase separation in mixed systems (7). These results indicate that interactions between anti-tumor drugs and membrane lipids might play an important role in their anti-neoplastic action and toxicity.

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Ether Lipid-membrane biophysical interaction.

A. Noseda, L. Diomede, M. Salmona, P.L. Godwin, J.G. White and E.J. Modest. Mario Negri Institute for Pharmacological Research, Milan, Italy, and Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC, USA.

Ether lipid (EL) analogs of platelet activating factor (1-octadecyl-2-acetyl-sn -glycero-3-phosphatidylcholine, PAF) inhibit malignant cell growth. This cytotoxic activity is mediated through an interaction with the plasma membrane. In fact, EL accumulate at the plasma membrane level and interfere with membrane biochemistry and biophysics. However, their mechanism of action has not been fully understood. Reports have appeared on ether lipid biochemical effects on membrane-associated metabolic systems such as inhibition of protein kinase C and Na/K ATPase activities, phosphocholine synthesis, decrease of arachidonic acid release and alteration of phospholipid turnover.

We have focused our attention on EL membrane biophysical interaction and studied the effects of EL on model and biological membranes of malignant and normal cells. Our studies involved four different, but complementary approaches: 1) scanning and transmission electron microscopy (SEM and TEM); 2) differential scanning calorimetry (DSC); 3) electron spin resonance (ESR); and 4) fluorescence polarization (FP).

SEM and TEM studies, aimed at elucidating the structural features of the interaciton between EL and the plasma membrane, show that the ether lipids 1-octadecyl-2-methyl-glycero-3-phosphocholine, ET-18-OMe, and 1-thiohexadecyl-2-ethyl-glycero-3-phosphocholine, ET-16S-OEt, can strongly affect the morphology of leukemic cell (HL60, K562, L5178Y) membranes in vitro and in vivo. Membrane perturbations consisted in ruffling, blebbing, cell shedding and the formation of holes in the membranes. Holes covered up to 13% of the cell surface at the highest concentration used and averaged a diameter of 1.5 um. The onset and progression of these membrane alterations correlate well with drug toxicity in vitro. L5178Y cells obtained from ET-18-OMe treated mice showed the same membrane lesions.

DSC studies indicate that EL partition into phospholipid model membranes and alter their physical properties as indicated by the decrease in PC containing vesicle transition temperature and by the decrease in this same parameter and changes in the calorimetric enthalphy of PE containing vesicles. A significant correlation is also present between EL cytotoxicity against cancer cells in vitro and their ability to partition into phospholipidic domains. A variety of oxyether, thioether and amidoether analogs were used.

ESR and FP investigations allow the use of whole cells probed with 5-nitroxystearate and diphenylhexatriene (or trimethylammoniumdiphenylhexatriene) for ESR and FP experiments, respectively. Membrane fluidity increase are detectable when cancer cells are exposed to subtoxic doses of EL, thus indicating that membrane perturbations occur at a very early stage and precede cytotoxicity.

Cholesterol might play also a role in determining EL uptake and/or cytotoxicity. Preliminary data show that different cholesterol concentrations in model membrane can modulate EL uptake differently. We are expanding this line of research in an attempt to define the possible importance of this finding for EL toxicity.

In summary, the plasma membrane appears as the target for EL action since 1) its morphology is deeply altered when exposed to EL in vitro and in vivo; 2) EL fluidize the plasma membrane of whole cells. Membrane fluidity is an important parameter for cell integrity and for the correct functionality of membrane enzymes; 3) EL ability of partitioning into phospholipidic domains and altering their physical properties might explain their fluidizing effect and be an important requirement for cytotoxicity; 4) membrane components modulating EL uptake might be important in determining EL toxicity and selectivity.

The possible implications of EL membrane interaction for cancer chemotherapy will be also discussed.

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Growth Factors and Their Receptors. Moses, H.L., Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

The development of aberrations in growth control is obviously central to the process of carcinogenesis. Major growth regulatory molecules are the polypeptide growth factors that stimulate cell proliferation through binding to specific cell surface receptors. Their importance in cancer has been emphasized by recent data linking growth factors and oncogenes. Many of the oncogenes (and their normal counterparts, proto-oncogenes) have been shown to code for growth factors, growth factor receptors or proteins important in the growth factor- receptor- response-pathway. Increased availability of growth factors or constitutive activation of the growth factor- receptor- response-pathway through mutation, amplification or rearrangement of proto-oncogenes involved in this pathway is probably involved in the carcinogenic process leading to abnormal proliferation. That negative regulators of cell proliferation are also important in normal growth control and the abnormal proliferation characteristic of cancer cells has gained widespread acceptance only in the last few years.

The concept of both positive and negative regulation of cell proliferation by diffusible peptides is illustrated by studies with the so-called transforming growth factors (TGF). Two TGF's have been purified, cloned and well-characterized, one a potent mitogen (TGFα) and the other a potent growth inhibitor (TGFß) for most cell types. TGFα is a 5.6 kD polypeptide that has sequence and structural homology with EGF and appears to mediate all of its biological effects through binding to the EGF receptor. TGFα has been reported to be produced by some cancer cells and to be present in embryonic, but not adult tissue. TGFα has been widely regarded as the embryonic form of EGF inappropriately expressed in cancer cells. Recent studies have demonstrated TGFα production by normal human neonatal and adult keratinocytes in cell culture and *in vivo* implying that TGFα autocrine activity is probably a normal phenomenon. Autoinduction of TGFα was observed in the cultured keratinocytes.

TGFß1 is the prototype of a superfamily of genes involved in growth control, extracellular matrix production and development. This family consists of a large number of developmental genes more distantly related to TGFß1 and isolated from drosophila, xenopus and mammals and three closely related genes isolated from mammals (TGFß1, TGFß2 and TGFß3) that are synthesized as large precursors and show 70-80% sequence similarity at the amino acid level in the mature region of the molecule which is the carboxy-terminal 112 amino acids. The regulation of action of the TGFß's is complex. Their mRNA's are autoregulated in cultured cells and vary in abundance in different tissues during embryologic development. The proteins are released in a latent form, and activation of the latent forms is also an important regulatory step. This activation involves dissociation of non-covalent bonding between the mature peptides and the glycosylated N-terminal peptides of pre-pro-TGFß's. Plasmin can activate latent TGFß1 by cleaving within the N-terminal glycopeptide. The TGFß's are potent inhibitors of proliferation of most epithelial cells. The mechanism of growth inhibition involves specific reduction of expression of a few genes, including c-*myc*. TGFß reduction of c-*myc* involves both cis-acting negative transcriptional elements and attenuation.

Both increased autocrine stimulation by TGFα and other growth stimulatory factors or decreased autocrine inhibition by TGFß could lead to an increased proliferative potential and thereby contribute to neoplastic transformation and other disease processes.

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Studies of the Relationship of structure to functions for the epidermal growth factor receptor help probe - its role in cancer cells.

Waterfield, Michael - Ludwig Institute for Cancer Research
91 Riding House Street, London W1, England

The interaction of Epidermal Growth Factor (EGF) or Transforming Growth Factor α (TGFα) with their specific cell surface receptor on target cells can trigger a mitogenic effect. Altered receptor expression - either over expression of normal receptors or expression of a truncated receptor encoded by the viral oncogene (v-erb B) can contribute to the conversion of normal to transformed cells. The EGF receptor consists of 3 domains - an extracellular ligand binding domain, a single transmembrane hydrophobic domain and a cytoplasmic domain. The only known intrinsic activity stimulated by ligand binding is a protein tyrosine kinase activity which can autophosphorylate the receptor at a number of sites (P1-P4) and also can phosphorylate several non-receptor proteins where role in signal transduction is unclear. In some but not all cells an increase in inositol phosphatides follows ligand binding and recent evidence suggests that this could be mediated by interaction and perhaps tyrosine phosphorylation of specific phospholipase Cs. However, in most cell types the activation of PI breakdown does not seem to follow ligand binding and account for signal transmission. A number of different mechanisms involving ligand induced conformational changes in the receptor external domain with the consequential activation of the receptor kinase, (presumably also by conformational changes) have been proposed. The overwhelming evidence suggests that ligand binding induces receptor dimerisation with inter molecular phosphorylation by the activated kinase being a consequence of the apposition of the internal domains, these being brought together as a consequence of the oligomerisation of the external domains (Schlesinger 1988). As part of a study to test this model and also to provide potential antagonists or inhibitors of the receptor activation process we have produced both the ligands EGF and TFGα and the receptor from normal and mutant cDNAs expressed in a variety of cell systems. Using the baculovirus vectors developed by Summers (and in some cases modified by McCormac) we have expressed EGF, TGFα, intact receptor and receptor domains in insect cells. The secreted external domain encoded by a truncated cDNA can be produced in mg. quantities and detailed biophysical studies have been carried out with the domain. The 115kd domain binds EGF and TFGα with the same affinity as solubalised receptor. Centrifuge studies show that this domain will not oligomerise in the presence of ligand. Detailed CD and florescence analysis however reveals a number of conformation changes induced by the ligands. The definition of such changes as associated with particular structural features of the receptor (e.g. specific tryptophan residue etc) are being probed by mutagenesis studies. The relevance of such studies to mechanistic models for the signal transduction processes will be discussed. Studies of the structural changes required to generate an autonomously functioning receptor which are derived from expression of truncated and mutated receptors will be discussed.

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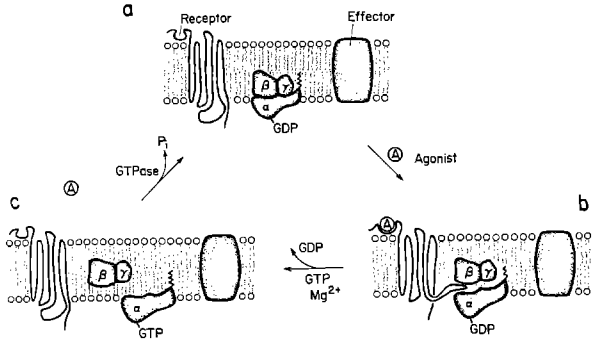
The role of G proteins in transmembrane signalling.

David E. Clapham, Mayo Foundation, Rochester, MN 55905.

Guanine nucleotide binding proteins (G proteins) couple more than 70 receptors to enzymes such as adenylyl cyclase, phospholipase C, cGMP phosphodiesterase, or ion channels. The mechanism of action of G proteins has largely been based on three model systems: the stimulation and inhibition of adenylyl cyclase, the stimulation of cGMP phosphodiesterase, and the activation of a cardiac potassium-selective ion channel. G proteins may arbitrarily be divided into two broad classes by their molecular weights. The classical, higher molecular weight G proteins are heterotrimers consisting of α, β, and γ subunits. The α subunit has intrinsic GTP binding and hydrolysis activity and in most cases is the effector arm of the system. For cardiac K^+ channels, both α and $\beta\gamma$ subunits initiate channel activity: $\beta\gamma$ does so via stimulation of phospholipase A_2 and generation of arachidonic acid and its metabolic products. In other cases, α subunits activate enzyme activity. Figure 1 summarizes the commonly accepted G protein cycle.



A second class of G protein-like molecules (MW 20D-26KD) are less well understood. These low molecular weight G proteins may exist in the membrane as monomers (α) and have similarities to ras and rho oncogene products. Roles in cytoskeletal function and exocytosis have been proposed. Current questions in signal transduction include: How is specificity of action of G protein subunits maintained, how does G protein structure relate to function, and how are G proteins organized in the cell membrane? Recent data will be summarized.

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All p21 ras proteins are polyisoprenylated but only H-ras and N-ras are palmitoylated.

C.J. Marshall[1], J.F. Hancock[1], A.I. Magee[2], J. Childs[2], [1]Section of Cell and Molecular Biology, Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, [2]National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA.

We have carried out a detailed investigation of the post-translational modifications that are required to localise the p21 ras proteins to the inner-surface of the plasma membrane where they are active. These studies show the post-translational modifications are complex and can be divided into two steps. The first step takes place in the cytosol and involves the removal of the three C-terminal amino acids from the primary translational product, pro p21, polyisoprenylation on cysteine 186 and carboxyl methylation presumably on this same residue. These modifications result in an increase in hydrophobicity of the processed protein and enable the protein to weakly associate with the membrane fraction. Polyisoprenylation appears to be an essential step in post-translational modification since blocking polyisoprenylation by inhibitors of mevalonic acid synthesis blocks all processing steps. The second step of processing is palmitoylation. Mutational analysis together with peptide mapping shows that palmitoylation does not occur on cysteine 186 as previously thought but takes place on cysteine residues a few amino acids upstream of cysteine 186. Point mutation of these upstream cysteine residues to serine results in an absence of palmitoylation. However, such proteins still retain some biological activity as measured by transformation assays and are associated with the membrane fraction. The major form of the Kirsten ras protein in mammalian cells which uses exon 4B does not contain a cysteine residue upstream of cysteine 186. We have shown that this protein is not palmitoylated and is more weakly associated with the plasma membrane than the palmitoylated forms of N-ras and Harvey-ras. By examination of the C-terminal sequences in ras related proteins, the C-termini of these proteins can be classified into two types, those which are processed like N- and H-ras and are palmitoylated and those which are processed like Kirsten ras 4B and are not palmitoylated. We hypothesise that the CAAX motif found at the C-termini of many proteins is involved in the post-translational modification steps of polyisoprenylation, C-terminal proteolysis and carboxyl-methylation.

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Inositol phospholipids and mitogenic signalling: targets for medicinal chemistry. Downes C.P., Macphee C.H., Stephens L.R., Hawkins, P.T., Milliner K.J., Ward J.G. and Young R.C.Y. Smith Kline & French Research Limited, The Frythe, Welwyn, Hertfordshire, England AL6 9AR.

A variety of hormones, neurotransmitters and growth factors stimulate receptor-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce two powerful intracellular signal molecules: inositol 1,4,5-trisphosphate (Ins1,4,5P_3) which acts by releasing Ca^{2+} from internal stores; and 1,2 diacylglycerol which is a stereospecific activator of protein kinase C. Both Ca^{2+} and protein kinase C (which is a target for several classes of tumor promoter) have been implicated as mitogenic signals. Moreover at least one oncogene (mas) encodes a receptor that directly stimulates inositol phospholipid hydrolysis. Recent studies in our laboratory have demonstrated an unexpected complexity of myo-inositol metabolites which include intermediates in the removal of Ins (1,4,5)P_3 and participants in pathways leading to the synthesis of inositol polyphosphates such as inositol pentakisphosphate and inositol hexakisphosphate. The latter two compounds are apparently ubiquitous components of mammalian cells, but little is known of their functions. One recently discovered myo-inositol metabolite is a novel phospholipid, phosphatidylinositol 3-phosphate (PtdIns3P). It is synthesised by a highly specific PtdIns 3-kinase which differs from the previously described PtdIns 4-kinase by being insensitive to inhibition by adenosine and being strongly inhibited by non-toxic detergents such as Triton X-100. More importantly, PtdIns 3-kinase appears to associate specifically with activated tyrosine kinases such as the activated platelet derived growth factor receptor and the products of viral oncogenes including $pp60^{v\text{-}src}$ and the middle T antigen/$pp60^{c\text{-}src}$ complex. This suggests that PtdIns3P, or one of its metabolites, may play an important part in mitogenic signalling. The branch point in inositide metabolism created by the co-occurrence in cells of PtdIns 3- and 4- kinases, thus represents an exciting prospect for therapeutic intervention in cancer chemotherapy. We have approached the development of medicinal chemistry in this field by exploring the substrate requirements of partially purified PtdIns kinases. The results indicate distinctions between both the ATP and phosphatidylinositol recognition sites of PtdIns3- and 4-kinases and demonstrate the stereospecific nature of the latter's interaction with its lipid substrate.

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THE BIOCHEMISTRY AND BIOLOGY OF PAF-ACETHER AND RELATED COMPOUNDS. E. Ninio, INSERM U.200, 32 rue des Carnets, 92140 Clamart, France.

Phospholipids, for years thought to be merely an inert component of cell membranes, are now considered as important intermediates of the transmembrane signalling pathways. The discovery of paf-acether (paf, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) showed that an ether-linked phospholipid was able to mediate a cell-to-cell message. Numerous cell responses to paf are mediated by specific binding sites present in the plasma membranes. Thanks to the discovery of paf antagonists such as natural plant extracts (ginkgolides, kadsurenone), paf structural analogs (CV 6209, SRI 63-072) and triazolobenzodiazepine (WEB 2086) it is possible to distinguish between specific and non-specific effects of paf on its targets. The list of paf actions on cells, yet uncomplete, is as follows: platelet activation, neutrophil aggregation, chemotactic activation and lysosomal enzyme and superoxide release, modulation of lymphocyte surface antigens and of interleukin formation ; some molecular effects like increase of intracellular Ca^{2+} concentration and protein kinase C activation in intact cells, initiation of glycogenolysis in liver and increase of DNA synthesis in bone marrow cells. The paf precursor and/or metabolite, lyso paf, is inactive in most systems. However it inhibits the protein kinase C activity in vitro and induces chemotactism of eosinophils. Lyso-phosphatidylcholine, an analog of paf, possess a biphasic action on protein kinase C since it stimulates it at low whereas it is inhibitory at higher doses. Up to now lyso-phosphatidylcholine as well as other lyso compounds were merely considered as detergents to underline their lytic and undesirable properties. The cells do not store these kind of molecules. The enzymes responsible for the reacylation of lyso molecules are numerous, such as acyltransferases and CoA-dependent and independent transacylases. However, some of these lyso phospholipids "escape" from the reacylation with long chain fatty acids and are transformed into paf or its acyl-acetyl-analog by a specific acetyltransferase. In some types of cells (neutrophils, monocytes and mast cells) stimulated with specific agonists, lyso paf is generated time- and dose-dependently and its formation is well correlated with that of paf. A rapid stimulation of acetyltransferase due to the phosphorylation of an inactive proenzyme is certainly responsible for the lyso paf acetylation into paf. The second route of paf biosynthesis is called the de novo pathway and involves the enzyme CDP-choline phosphotransferase acting on alkyl-acetyl-glycerol. This route contributes mainly to the constitutive paf formation in organs such as kidney, lung and uterus. Paf biosynthesis is regulated at different levels in cells and organs. Besides the control through the anabolic pathways most precisely via activation of phospholipase A_2 and acetyltransferase, we recently found that the final output of paf production depended on the level of the paf-degrading enzyme, acetylhydrolase. Mouse macrophages maintained in culture for 24 hr express 5-fold increase in their acetylhydrolase quantity. The role for paf in health and disease becomes evident since its implication in human asthma, shock, ischemia and allergy is strongly suggested. Paf was isolated from inflammatory sites and from peritumoral tissue. Paf probably initiates the early events in the onset of inflammatory diseases and contributes to the perpetuation of this process due to its chemoattractant properties for a variety of cells. Paf analogs are widely used as paf antagonists (CV, SRI) but also as cytotoxic and cytostatic drugs in anticancer therapy. Recently, 1-O-octadecyl-2-methyl analog of paf was shown to decrease the epidermal growth factor binding in human breast cancer cell line. It is reasonable to postulate that the phospholipid environment of many receptors and enzymes is responsible for the attenuation or activation of their activities. Moreover, components of phospholipids (after hydrolysis induced by cell activation), like fatty acids, phosphoinositols, diacylglycerol, alkyl-acyl-glycerol or even complete phospholipids as paf, may serve as inter- or intracellular mediators and thus to participate to the so-called transmembrane signalling process.

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Interaction of Adriamycin with inositol lipid metabolism. Hickman, J.A. and Thompson, M.G. CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, U.K.

The antitumour drug Adriamycin normally inhibits cell growth and brings about cell death in the G_2/M phase of the cell cycle. It is therefore unlikely that this membrane-active drug interferes with those events at the plasma membrane which are involved in the G_1/S transition but rather with those concerned with the preparation for cytokinesis. We have chosen to study the effects of Adriamycin on the membrane and cytoskeleton by using the human erythrocyte as a model system. After a 10 minute incubation with Adriamycin (10uM) the calcium-induced morphological transition of erythrocytes from the discocyte to the echinocyte form was inhibited (Chahwala and Hickman, Cancer Res. 45, 4986, 1985). The morphology of the human erythrocyte is controlled by complex interactions between the membrane and elements of the cytoskeleton. The inositol lipids appear to play an important role in this control: calcium loading and ATP depletion induce morphological changes concomitant with a fall in the amount of phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$). Several groups have recently suggested that $PtdInsP_2$ is sequestered into discrete pools in the erythrocyte membrane. We showed Adriamycin inhibited a fall in what appeared to be one such "pool" as it inhibited the ATP-depletion-induced shape change (Thompson et al., Cancer Res. 47, 2799, 1987). The mechanism of this inhibition is unclear: as an aminoglycoside it may, as has been suggested for the antibiotic neomycin, physically complex with the negatively charged lipid. Alternatively, it may interfere with the control of $PtdInsP_2$ breakdown. In order to examine this latter idea, we have investigated the regulation of PtdIns metabolism in the erythrocyte, and the effects of Adriamycin upon it. In erythrocyte membrane vesicles, the addition of 1-10uM GTP or GTPYS stimulated the production of inositol phosphates, whereas calcium-stimulated iP_3 release was inhibited by GTP or GTPYS (>100uM). This suggests that both stimulatory and inhibitory G proteins may control $PtdInsP_2$ metabolism. Preliminary data suggest that low concentrations (10^{-7}-10^{-8}M) Adriamycin caused a rise in iP_3 production from membrane vesicles. It remains to be established whether this effect is mediated *via* the putative G protein system described above.

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Membrane-targeted ether lipid analogs with anti-neoplastic activity. E.J. Modest, A. Noseda, L.W. Daniel, and C. Piantadosi. Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC, USA; Boston University School of Medicine, Boston, MA, USA; School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA; Mario Negri Institute for Pharmacological Research, Milan, Italy.

Membrane-targeted, DNA-noninteractive ether lipid analogs of platelet activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) provide a new approach to the chemotherapy of cancer. The prototype analog is 1-octadecyl-2-methylglycero-3-phosphocholine (ET-18-OMe, **1**). These analogs express their antitumor properties (1) by direct cytotoxic or cytostatic action, (2) by macrophage activation, and (3) by differentiation of malignant cells. They interact with the plasma membrane but not with DNA: they do not interfere with DNA synthesis or function and are nonmutagenic. The ether lipid analogs also inhibit protein kinase C (PKC), interfere with phosphatidylcholine biosynthesis, cause morphologic damage to malignant cell membranes, alter the physical properties of neoplastic cell membranes and model membranes, affect calcium channels, and are useful as selective bone marrow purging agents in leukemia.

To date the majority of biochemical, pharmacologic, and clinical studies, in our laboratories and elsewhere, have been carried out with ether phospholipid analogs (Type A), represented by **1** or its 1-thioether analogs. We are now studying a second class of ether lipid analogs without phosphorus substitution in the molecule (Type B), represented by 1,2-octadecylidine glycerol-3-N,N-dimethyl-3'-hydroxypropyl ammonium bromide (**2**) and N-(3-hexadecyloxy)propyl pyridinium bromide (**3**).

We have studied the ether phospholipid analogs as inhibitors of HL60 and K562 human leukemic cells and BG1 human ovarian carcinoma cells in culture. Some of the new Type B nonphosphorus ether lipid analogs have unusually high levels of activity against HL60-derived PKC or HL60 cell growth. For example, **2** has an IC_{50} of 0.4 μM against PKC compared with 4.0 μM for **1**, and **3** has an ID_{50} of 0.8 μM against HL60 cells compared with 2.1 μM for **1**.

1 has been reported to inhibit the growth of a number of primary and metastatic transplantable murine tumors. In the L5178Y mouse leukemia, we have found i.p. or s.c. administration of **1** or 1-*S*-hexadecyl-2-ethylthioglycero-3-phosphocholine (ET-16S-OEt, **4**) (50 mg/kg/day) to effect significant retardation of ascites and 40-89% increase in life span. In two solid tumor systems, M5076 ovarian reticulosarcoma and Lewis Lung carcinoma, i.p. administration of **1** or **4** (25 mg/kg/day x 21) gave 25-50% reduction in tumor size with no increase in life span. Pharmacoanalytic methods are under development for both Type A and Type B ether lipids by GC/MS and supercritical fluid chromatography.

Combination of the membrane-interactive ether phospholipids with DNA-interactive agents gave promising results in vitro: **1** and **4** were used with adriamycin, cisplatin, and 4-hydroperoxycyclophosphamide against the BG1 tumor, and with araC against the L5178Y leukemia. Studies combining ether lipid analogs with hyperthermia are in the early stages. These are interesting approaches in view of the strictly membrane-directed action of the ether lipids.

We are engaged in detailed structure-activity correlations of both classes of ether lipid analogs, including not only inhibition of malignant cells and PKC, but also membrane interactions and other parameters. [Supported in part by NIH grants CA 41314, CA 43297, CA 42216, and BRSG RR 05404; ACS grant RD 262; and Assoc. Ital. per la Ricerca sul Cancro].

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MEMBRANE-TOXIC LIPIDS AND CRYOPRESERVATION FOR PURGING OF AUTOLOGOUS BONE MARROW GRAFTS.

Berdel W.E., Okamoto S., Olson A.C., Winton E.F., Danhauser-Riedl S., Steinhauser G., Rastetter J. and Vogler W.R.
Emory University School of Medicine, Atlanta, Georgia 30322, USA and Department of Medicine I, Technische Universität München, 8000 Munich, FRG.

Autologous bone marrow transplantation (ABMT) represents an effective supportive treatment for high dose radio- and chemotherapy with marrow-toxic protocols in some malignant diseases. However, one problem of this approach is the reimplantation of residual malignant disease possibly present in the marrow. Studies to assess the applicability of ether lipids for purging of residual malignant myeloid and lymphoid cells from bone marrow for the treatment of leukemia and lymphoma with ABMT after ablative chemo- and radiotherapy show that 4 hours of incubation with the 1-O-alkyl ether lipid ET-18-OCH_3, and subsequent cryopreservation can eliminate $\geq$ 3-log clonogenic cells of the malignant myeloid HL-60 and KG-Ia as well as the lymphoblastoid Li-A, Daudi and histiocytic Su-DHL-4 cell lines under conditions which allow recovery of more than 50% of the normal human hematopoietic progenitors. The myeloid K-562 cell line revealed higher resistance span. The thioether phospholipid BM 41.440 was not superior to ET-18-OCH_3 and combinations of ET-18-OCH_3 with the ether linked lipoidal amine CP-46,665 offered no advantage over ET-18-OCH_3 alone. These results were obtained with experimental protocols similar to the clinical purging procedure.
In a clinical phase I study in patients with acute leukemia to determine the safety and efficacy of ABMT using remission marrows treated with ether lipids in vitro, bone marrow cells were harvested from 11 patients in remission and assayed for marrow progenitor cells (CFU-GEMM assay) before and after a 4 hour incubation with ET-18-OCH_3 (50 µg/ml) and after controlled rate freezing and rapid thawing. The mean ± S.D. of total colonies was 37.9 ± 17.9 (10^5 cells/plate) for unpurged samples and 36.5 ± 18.1 for purged samples. In 3 instances there was a significant decrease in total colonies after purging, freezing and thawing. A significant increase of total colonies was observed in 2 patients. Two patients have reconstituted their bone marrow after marrow-ablative therapy and ABMT with purged (50 µg/ml) remission marrow within this ongoing study.

DFG Be 822/2-5, 822/3-1

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"Alkylphosphocholines as Anticancer Drugs." Clemens Unger, University Medical Clinic, Goettingen, West Germany.

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Early signals in the mitogenic response. Rozengurt, E. Imperial Cancer Research Fund, P O Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Quiescent cultures of Swiss 3T3 cells can be stimulated to recommence DNA synthesis by polypeptide growth factors, neuropeptides and various pharmacological agents that act via multiple signal transduction pathways (1). Neuropeptides of the bombesin family provide potent mitogens to elucidate these pathways and may act as autocrine growth factors for small cell lung cancer (SCLC) cells. The peptides bind to specific receptors which have been characterized by radioligand binding and sensitivity to antagonists and identified as glycoproteins of Mr 75,000 - 85,000 by chemical cross-linking (2,3). Following binding, bombesin elicits a cascade of early molecular events including stimulation of phosphorylation of the acidic Mr 80,000 cellular protein (80K) which is a major substrate of protein kinase C; Ca^{2+} mobilization mediated by $Ins(1,4,5)P_3$; Na^+ and K^+ fluxes; transmodulation of EGF receptor; enhancement of cAMP accumulation and expression of the proto-oncogenes c-fos and c-myc (3,4).

Results using membrane preparations and permeabilized 3T3 cells indicate that G proteins play a role in the transduction of the mitogenic signal triggered by the binding of bombesin to its receptor. A pertussis toxin-insensitive G protein couples the bombesin receptor to the generation of a signal that activates protein kinase C (5) whereas a pertussis toxin-sensitive G protein mediates cross-talk between transmembrane signalling pathways (6).

Bombesin-mediated mitogenesis can be blocked by different antagonists (7) and by interrupting the signal-transduction process at various post-receptor levels. Thus, prolonged treatment with vasopressin causes heterologous desensitization to the mitogenic action of bombesin. This mitogenic block is not mediated by down-regulation of the bombesin receptor but by uncoupling the receptor from its signalling system (8). Loss of responsiveness to bombesin-stimulated DNA synthesis is also induced by exposure to phorbol esters (9) which causes down-regulation of protein kinase C (1,4). Vasopressin treatment does not induce down-regulation of this kinase indicating that these treatments block bombesin-stimulated DNA synthesis by different mechanisms. Current evidence indicates that murine 3T3 cells which express receptors for several mitogenic neuropeptides and are thus capable of multiple neuropeptide regulation, provide a model system relevant to SCLC. A detailed understanding of the signal transduction pathways in the model system may identify novel targets for therapeutic intervention.

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SIGNALLING PATHWAYS IN THE ACTION OF GROWTH FACTORS

W.H. Moolenaar, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam.

A major class of growth factors activates a specific receptor tyrosine kinase. Epidermal growth factor (EGF)-stimulated tyrosine kinase activity is essential, but not necessairily sufficient, for signal transduction. Using permeabilized cell systems, we have examined the possible involvement of a G protein in EGF action. Recent results will be presented.

Another class of mitogens activates a phosphoinositide(PI)-specific phospholipase C via a receptor-linked G protein. An intriguing member of this class is phosphatidic acid (PA). PA mitogenicity is not dependent on other mitogens and is blocked by pertussis toxin. PA evokes at least three separate signalling cascades: (i) activation of a pertussis toxin-insensitive G protein mediating PI hydrolysis with subsequent mobilization of Ca^{2+} and stimulation of protein kinase C; (ii) release of arachidonic acid in a GTP-dependent manner, but independent of prior PI hydrolysis; and (iii) activation of a pertussis toxin-sensitive G_i protein mediating inhibition of adenylate cyclase. The peptide bradykinin mimics PA in inducing responses (i) and (ii), but fails to activate G_i and to stimulate DNA synthesis. Our data suggest that the mitogenic action of PA occurs through G_i or a related pertussis toxin substrate and that, unexpectedly, the PI hydrolysis-protein kinase C pathway is neither required nor sufficient, by itself, for mitogenesis.

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Ion channels and calcium signaling in T-lymphocytes. Cahalan, M.D., Chandy, K.G., Grissmer, S., Lewis, R.S., Department of Physiology and Biophysics, University of California, Irvine, Irvine, California 92717.

T and B lymphocytes play fundamental and diverse roles in the immune response, including recognition of antigens, secretion of lymphokines and antibodies, and killing of foreign or virus-infected cells. The advent of patch-clamp recording techniques and video imaging of fluorescent ion-sensitive dyes has placed a new emphasis on the responses of individual cells in the immune system. The properties and possible functional roles of K^+, Cl^-, and Ca^{2+} channels in T lymphocytes will be reviewed. Three distinct types of voltage-gated K^+ channels have been identified in lymphoid cells. These subtypes of K^+ channels are distributed in a characteristic pattern among functional and developmental subsets of T cells. Abnormalities in the expression of K^+ channels are seen in several murine models of autoimmunity. Several lines of evidence point to a requirement for functional n-type K^+ channels in the activation of T and B lymphocytes by mitogens. The expression of type n K^+ channels is correlated with the degree of cell proliferation, and K^+ channel blockers inhibit T-cell mitogenesis in a parallel potency sequence. In addition to voltage-gated K^+ channels recent patch-clamp studies have identified Ca^{2+}-activated K^+ channels in lymphoid cells. A novel type of Cl^- channel with 1-2 pS conductance, opens during exposure to osmotic gradients that result in cell swelling. The acute modulation of these "mini" Cl^- channels by changes in extracellular tonicity and suggests that they underlie Cl^- efflux during a regulatory decrease in cell volume. Recently, we have described a mitogen-activated Ca^{2+} current in T cells using a combination of whole-cell or perforated-patch recording and single-cell Ca^{2+} measurements using fura-2. The particular advantage of this combined approach is that it allows the correlation of particular components of membrane current with the $[Ca^{2+}]_i$ rise in an individual cell. PHA treatment of Jurkat T cells induces a small Ca^{2+}-selective inward current after a delay of 100-300 sec, accompanied by a rise in $[Ca^{2+}]_i$. The absence of readily detectable current fluctuations during its induction suggests that, if the current is conducted by channels, they must have an extremely small conductance or exceedingly brief openings. The Ca^{2+} current may contribute to oscillations in $[Ca^{2+}]_i$ evoked by mitogenic stimuli.

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Cellular Ca^{2+} homeostasis and cancer drugs. Powis, G. Mayo Clinic & Foundation, Rochester, MN 55905, U.S.A.

Changes in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are part of intracellular signal transduction pathways that mediate the action of growth factors and mitogens. $[Ca^{2+}]_i$ is maintained at around 10^{-7} M while the extracellular Ca^{2+} concentration is 10,000-fold higher. The cell maintains exquisite control over $[Ca^{2+}]_i$ which rises transiently to no more than 10^{-6} M during intracellular signalling. Larger and more sustained increases in $[Ca^{2+}]_i$ are associated with cell toxicity. The initial transient increase in $[Ca^{2+}]_i$ probably acts as a trigger that initiates a sequence of events leading to cell proliferation. It is followed by an increased cellular influx and efflux of Ca^{2+} that may be important in sustaining the response. A fundamental event connected with cell transformation is the quantitative or qualitative alteration in the expression of proto-oncogenes associated with cell growth regulation. A major function of oncogenes is to code for components of signal transduction pathways. Inappropriate or modified expression of an oncogene product can lead to the constitutive activation of a signal transduction pathway and unrestrained or transformed cell growth. Blocking changes in $[Ca^{2+}]_i$ is one way it may be possible to prevent abnormal oncogene expression. Ca^{2+} for growth factor signalling can come from outside the cell or be released from internal stores by second messengers such as inositol-1,4,5-trisphosphate (IP_3) and arachidonic acid ($C_{20:4}$). Doxorubicin, 10^{-6} M, blocks changes in $[Ca^{2+}]_i$ caused by histamine in nerve cells, possibly by blocking IP_3 synthesis or by acting on voltage-dependent plasma membrane Ca^{2+} channels. Doxorubicin does not, however, block the $[Ca^{2+}]_i$ response to PDGF in Swiss 3T3 fibroblasts. Other cancer drugs do not appear to act on growth factor $[Ca^{2+}]_i$ signalling. Part of the $[Ca^{2+}]_i$ response to PDGF is mediated by plasma membrane Ca^{2+} channels that can be blocked by relatively high concentrations (10^{-5} M) of nifedipine. Agents that have been found to decrease the second messenger mediated release of Ca^{2+} from internal stores in saponin-permeabilized Swiss 3T3 cells are polysulfated compounds such as heparin, dextran sulfate (500 KDa) and suramin. The compounds appear to act by blocking the binding of IP_3 to its putative receptor on the endoplasmic reticulum. Ca^{2+} release by GTP and $C_{20:4}$ is not affected. These compounds are probably too large to enter the cell but provide leads to the types of compounds that might be used to control cell growth by this mechanism. The PAF analogue ether lipid ET-18-OCH_3 inhibits the ATP dependent uptake of $^{45}Ca^{2+}$ in permeabilized Swiss 3T3 cells with an EC_{50} of 35 μM. Incubation of the cells with 10 μM ET-18-OCH_3 for 18 hr before permeabilization inhibits IP_3 mediated Ca^{2+} release. In summary, inhibition of $[Ca^{2+}]_i$ signalling is a plausible target for new drug development that might lead to selective control of the growth of transformed cells. Some lead compounds are available that act by this mechanism and point towards a challenging new era of experimental chemotherapy. The work described here was supported by NIH grant CA42286.

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Effects of alkylating agents on K^+-transport. Grunicke H., Doppler W., Oberhuber H., and Maly K. Institute of Medical Chemistry and Biochemistry, University of Innsbruck, A-6020 Innsbruck, Austria

At therapeutic concentrations alkylating antitumor agents affect a variety of membrane functions. These include inhibiton of the transport systems for thymidine, amino acids and glucose. A rapid decrease of the influx of the potassium congener ^{86}Rb is one of the most striking effects after exposure to alkylating agents. As potassium fluxes seem to be intimately correlated with the regulation of cellular replication, the observed depression of the Rb-uptake by alkylating agents may contribute to the antiproliferative activity of these drugs. Therefore, the effects of alkylating agents on K^+-transport systems have been investigated in greater detail. The studies revealed that nitrogen mustard (HN2) inhibits the ouabain-sensitive and the furosemide-sensitive Rb-uptake of Ehrlich ascites tumor cells whereas the transport which is resistant to both inhibitors is not affected. At HN2-concentrations below 10 µM reduction in Rb-uptake is predominantely due to an interference with the furosemide-sensitive system. The depression of the furosemide sensitive Rb-transport system by HN2 is proportional to the reduction in cell-growth and is much less expressed in HN2-resistant cells. Mean cell volume is not affected by HN2-concentrations which depress the furosemide-sensitive Rb-uptake system. The inhibition of the furosemide sensitive Rb-transport by HN2 requires the uptake of the drug into the cell but is neither the result of an alkylation of DNA and a subsequent depression of protein synthesis nor simply due to a loss of cell viability. A selective complete block of the system by furosemide does not inhibit cellular replication. One has to conclude, therefore, that the depression of the furosemide-sensitive K^+-transport alone is not responsible for the antiproliferative activity of HN2. HN2 depresses the Na^+/H^+-antiporter within the same dose range. Since an activation of this as well as the other transport systems which are affected by alkylating agents are well known early events after stimulation by growth factors or other mitogens, the findings suggest that an early effect of alkylating agents is an interference with growth factors signal transduction. This conclusion is supported by findings which demonstrate that inhibitors acting at various steps in growth factor signal transduction, synergistically enhance the antiproliferative activity of alkylating and platinating agents. These observations may lead to new improved drug combinations in tumor chemotherapy.

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Mechanisms of EGF receptor protein tyrosine kinase activation. Haley J.D.[1], Allen H.[2], Hsuan J.J.[3], Foulkes J.G.[1] and Waterfield M.[3]. [1]Oncogene Science, Inc., 350 Community Drive, Manhasset, New York, 11030, USA; [2]Ludwig Institute for Cancer Research, 91 Riding House Street, London, W1 8BT, UK; [3]Dept. of Immunology, NIMR, Mill Hill, London, NW7 1AA, UK.

Inadvertent transduction of mitogenic signals through activation of cellular protein-tyrosine kinases is a major mechanism by which phenotypic cellular transformation has been shown to occur. In vivo, well studied examples of tyrosine kinase activation associated with cellular transformation include the phl/c-abl fusion of the Philadelphia chromosomal translocation in chronic myeloid leukemia and the single base change activation of c-neu in nitroso-methyl urea treated mice in neuroblastoma. Similarly, gene amplification and overexpression of putative growth factor receptors such as the epidermal growth factor receptor or the c-neu/c-erbB2 proto-oncogene have been implicated in progression and poor prognosis in human breast cancer. Finally, autocrine expression of growth factors themselves, for example, TGF-alpha, EGF or PDGF/sis can result in phenotypic transformation of mammalian fibroblasts and may be secreted by a wide variety of human tumor types. Clearly, inadvertent activation of such mitogenic pathways by ligand or by deregulation of the tyrosine kinase itself are likely targets in the ontology and progression of human tumors.

Activation of the EGF receptor (c-erbB) tyrosine kinase has been implicated in tumorgenesis, either by overexpression of the normal receptor in the presence of the EGF, or through expression of a truncated receptor lacking the EGF binding domain as in the viral oncogene v-erbB. In a series of experiments, normal and truncated human EGF receptors expression in Rat1 fibroblasts were analyzed for receptor tyrosine kinase activity and several transformation parameters in comparison with polyoma middle T and EJ-ras. Expression of a truncated EGF receptor lacking the extracellular ligand binding domain activate the intrinsic tyrosine kinase and induces transformation of immortalized rodent fibroblasts. The transformed phenotype becomes enhanced by further truncation of the C-terminal domain containing the tyrosine autophosphorylation sites P1 and P2. Overexpression of EGF receptors with an intact extracellular region results in EGF dependent transformation, which is reduced by C-terminal truncation. Transformation is dependent on the cellular receptor concentration and can be selected as a stable phenotype. We conclude that expression of receptors with a truncated EGF-binding domain alone is sufficient to transform mammalian fibroblasts, in contrast to chick fibroblasts transformed by v-erbB where additional deletion of C-terminal receptor sequences appears to be an absolute requirement.

The human erythroleukemic cell line K562 normally lacks the epidermal growth factor (EGF) receptor. We have expressed the EGF receptor in K562 cells by transfection of the receptor cDNA to investigate receptor function in erythroid cells. The tyrosine kinase activity of the EGF receptor in the K562 transfectants was compared to that in A431 cells. The K562 receptor kinase is shown to be activated in the absence of EGF and does not derive from ligand binding (eg., binding of TGF alpha in an autocrine mechanism). The K562 EGF receptor has one major, constitutive phosphorylation site in vivo, a threonine residue(s) that is not the site phosphorylated by protein kinase C at Thr 654. Possible mechanisms or activation of the K562 receptor kinase, for example, involving this threonine phosphorylation or tyrosine phosphatase activation will be discussed.

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The diversity of protein kinase C polypeptides. Parker P.J., Schaap D. and Marais R.M. Cell Regulation Laboratory, Ludwig Institute for Cancer Research, 91 Riding House St., London W1P 8BT.

Protein kinase C (PKC) has been a source of much interest in recent years, particularly following the observation that the phorbol ester class of tumour promoters activate this enzyme by mimicking the action of the putative natural second messenger diacylglycerol. Indeed phorbol dibutyrate and diacylglycerol have been shown to compete for binding to PKC. That activation of PKC is involved in cellular responses to phorbol esters is evidenced by the observation that they can mimic responses elicited by membrane permeable diacylglycerols. Furthermore the time course of down regulation of PKC induced by chronic phorbol ester treatment parallels the loss of phorbol ester responsiveness indicating that PKC expression is a necessary part of phorbol ester action.

The cDNA cloning of PKC has led to the identification of a family of related gene products. To date there exist six distinct genes ($\alpha, \beta, \gamma, \delta, \varepsilon, \zeta$) that give rise to at least seven polypeptides (β_1 and β_2 are derived by alternative splicing from the β gene). cDNA probes and monospecific antisera have been employed to show that these genes are differentially expressed in tissues and cell lines. The identification of multiple PKCs begs the question whether or not they are isoenzymes - catalysing the same phosphorylations - and to what extent their regulation is the same? In order to address these issues much effort has been directed towards the biochemical characterization of the different gene products and this has shown that there are differences both with respect to substrate specificity and effector dependence. This suggests that while certain PKC isotypes may show overlapping specificity, they are likely to fulfil unique roles in vivo.

The multiplicity of PKCs and their differential expression make these critical regulatory enzymes a possible target for intervention. As more information becomes available concerning the characteristics of these PKC isotypes so one might anticipate a rational approach to intervention. That such an approach is viable is in part evidenced by the derivation of a monoclonal antibody specific for one isotype (γ) that selectively interacts at/with the phorbol ester binding site of this protein, a functional site that is nevertheless conserved in other PKCs.

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Synthetic inhibitors of protein kinase C. Gross, J.L., Dexter, D.L., Herblin, W.F., Hesson, D.P., Jiang, J.B., Blaney, J.M., Ardecky, R.J., Behrens, C.H., Ganti, V.R., Pounds, J.S.* and Do, U.H.* Medical Products Dept., E. I. du Pont de Nemours & Co., Wilmington, DE 19898 and * Du Pont, NEN Products, Boston, MA 02118

Protein kinase C (PKC) is a family of phospholipid and calcium regulated kinases specifically involved in the transduction of extracellular signals. Because several classes of structurally distinct tumor promoters such as phorbol esters bind at the regulatory domain of PKC, this enzyme has been implicated in tumor promotion as well as in the control of cell differentiation and proliferation. PKC antagonists may thus be useful as therapeutic agents for cancer as well as for other proliferative disorders. Three approaches have been used to obtain and explore synthetic modulators of PKC activity: 1) computer modeling to generate novel compounds designed to specifically interact at the regulatory domain of PKC, 2) synthesis of [N,N-dimethyl-3H]N,N-dimethyl-staurosporine ([3H]DMS) to explore catalytic inhibitors of PKC, and 3) synthesis of novel PKC inhibitors identified as acting at the regulatory domain of PKC by competition with [3H]phorbol- 12,13-dibutyrate ([3H]PDBu). Using computer modeling and "ensemble" geometry, the 3-dimensional structures of natural products which bind to the regulatory domain of PKC were examined. By analyzing superimposable pharmacophores from the 3-dimensional structures of phorbol myristate acetate and dihydroteleocidin B, a triangle hypothesis was developed which defined three points of pharmacophore binding required for interaction with PKC. Based on this hypothesis, a series of benzyl alcohols was designed which bound to mouse brain PKC at concentrations (Ki=2 uM) similar to that observed for the natural endogenous activator of PKC, diacylglycerol (Ki=2.6 uM). Members of this series exhibited competitive kinetics for [3H]PDBu, were partial agonists of catalytic activity in a cell free system, and stimulated the activity of the protease plasminogen activator in cultured endothelial cells. Since the microbial alkaloid staurosporine is the most potent and selective catalytic inhibitor of PKC, [3H]DMS binding to PKC was analyzed. [3H]DMS bound to PKC reversibly and with high affinity. Unlike [3H]PDBu binding to PKC, [3H]DMS binding was not dependent on calcium or phospholipid. [3H]DMS bound to the catalytic domain of PKC since high concentrations of PDBu could not compete for [3H]DMS binding. [3H]DMS binding was displaced by the catalytic inhibitors staurosporine (Ki=4.7 nM), DMS (Ki=4.7 nM), K-252a (Ki=66 nM), and H-7 (Ki=79 uM). MgATP inhibited [3H]DMS binding only at high concentrations (Ki=2.5 mM), suggesting that [3H]DMS did not bind to PKC at the ATP site required for enzymatic catalysis. Two compounds with antitumor activity, adriamycin and the nucleoside analog sangivamycin competed for [3H]DMS binding with Ki's of 54 and 15 uM respectively, suggesting that this competition assay can detect potential anticancer agents. A series of bis-naphthalene sulfonamides was synthesized and evaluated for inhibition of [3H]PDBu binding and catalytic activity of PKC and for inhibition of human tumor cell growth in vitro. Several analogs in this series inhibited [3H]PDBu binding with Ki's of 0.5-1 uM. Those compounds which most potently inhibited [3H]PDBu binding were also the most effective inhibitors of PKC catalysis. The potency of [3H]PDBu competition correlated with their ability to inhibit the growth of the human melanoma RPMI-7272 line in culture. Two analogs in this series exhibited modest antitumor activity against B16 melanoma in mice, suggesting that agents designed as inhibitors of PKC activity may be useful as antitumor agents.

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Pharmacological activities of modulators of protein kinase C. Gescher, A. Cancer Research Campaign Experimental Chemotherapy Group, Aston University, Birmingham B4 7ET, UK.

Protein kinase C (PKC) is physiologically activated by diacylglycerols (DAGs) which are generated via the phospholipase C-mediated cleavage of polyphosphoinositides. Interest in the possibility of modulating PKC activity by exogenous agents was sparked by the finding that this enzyme is the major or the only cellular receptor via which tumour-promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) mediate their pleiotropic effects on cells. In many cell lines treatment with TPA affects growth, in some cases as a consequence of induction or inhibition of differentiation or of induction of mitogenesis. In view of the growth-modulatory efficacy of TPA the question can be asked: are there TPA agonists or antagonists which influence growth and differentiation in a selective, therapeutically exploitable fashion? The propensity of phorbol esters to activate PKC and to exert biological effects is governed by a tight structure-activity relationship and TPA is the most potent derivative. However some naturally occurring terpenoid compounds structurally unrelated to phorbol esters (e.g. mezerein, teleocidin, aplysiatoxin, ingenol 3-tetradecanoate) are potent agonists of TPA. Exogenously added DAGs at high concentrations mimic some but not all of the effects of TPA on cells. One of the most intriguing classes of compounds with PKC-activating properties are the bryostatins. They are a family of 17 structurally related macrocyclic lactones which were extracted from the marine bryozoan Bugula neritina by Pettit et al. at Arizona State University and found to exhibit antineoplastic activity against the murine P388 lymphocytic leukaemia model. Bryostatins are not simply another class of phorbol ester agonists. In many cell types they fail to mimic and indeed often antagonise the effects of phorbol esters.

The structures of compounds which have been shown to inhibit PKC are just as diverse as those exhibited by PKC activators. Examples of naturally occurring agents with PKC-inhibiting properties are dialkyl- and alkylacyl-glycerols, lipoidal amines such as palmitoylcarnitine and the antifungal microbial alkaloid staurosporine. In general PKC inhibitors are neither highly selective for PKC nor are they, except staurosporine, very potent enzyme inhibitors.

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"Inhibitors of Tyrosine Protein Kinase." Yosef Graziani, Ben Gurion University of the Negev, Beer Sheva, Israel.

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"Membrane Signals in Hemopoietic Cell Differentiation."
T. Michael Dexter, Holt and Christie Radium Institute, Manchester, UK.

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The effects of retinoids on leukemic cell signalling during differentiation. Geny B., Ladoux A., Chomienne C., Huang M. and Abita J.P. INSERM U.204, Hôpital Saint-Louis, 75475 Paris FRANCE.

The human promyelocytic leukaemia cell line HL-60, which can be induced to differentiate terminally either into granulocyte-like or into monocyte-like cells, constitute an ideal model to study the intracellular signalling pathways through which the effects of the differentiation inducers may be transduced. In this presentation we will focus mainly on the results obtained with all-trans-retinoic acid (RA) one of the most potent agent which induces the granulocytic differentiation of HL-60 cells.

1) RA inhibits in a dose and time dependent manner the formation of inositol phosphates and of diacylglycerol when added to intact cells. The inhibition begins after 10 min and reaches a maximum at 45 min. Studies with cells permeabilized with streptolysin O and with membrane preparations have revealed that RA might interfere with the coupling between phospholipase C and its regulatory G protein. 2) RA provokes a stimulation of the plasma membrane Na^+-K^+ATPase activity which reaches a maximum (170% of control) after 7-8h. This stimulation is preceded by an increase in the intracellular concentration of Na^+ and accompanied by an increase inthe intracellular concentration of K^+. If HL-60 cells are first treated with low concentrations of ouabain so that the activation of the sodium pump happens earlier (3h), the differentiation induced by a submaximal concentration of RA is accelerated and potentiated. 3) The intracellular pH (pHi) of HL-60 cells is regulated by two plasma membrane located systems: an amiloride sensitive Na^+/H^+ exchanger and a Na^+ dependent HCO_3^-/Cl^- exchange system those properties are different from that of normal granulocytes. RA produces a cell alkalinization which develops over 48h to attain a maximum of 0.35 pH unit (pH 7.03 to pH 7.38), which precedes by about 24h the appearance of the differentiated phenotype and which is the result of a 2 fold increase in the activity of the Na^+/H^+ exchanger. On the other hand, we have shown that the monocytic differentiation of HL-60 cells as well as that of the other human leukemic cell line U-937 are preceded also by an increase in pHi but of only 0.15-0.2 pH unit. 4) RA leads to a time-dependent decrease in the capacity of adenylate cyclase (AC) of HL-60 cells to be hormonally stimulated and to synthesize cyclic AMP (cAMP) in intact cells. Studies with membrane preparations have shown that AC of RA-treated cells is not stimulated by forskolin, is less succeptible to stimulation by GTP and is inhibited by low concentrations of GTP in the presence of forskolin. Recent experiments suggest that the changes in AC activity are the results of changes in the synthesis of the regulatory proteins G_s and/or G_i. On the other hand, RA-induced differentiation is markedly potentiated by agents which increase intracellular levels of cAMP, an effect which may be due to an increase and a translocation of a cAMP dependent protein kinase. 5) RA treatment causes also, before the appearance of the differentiated phenotype, an increase in cytosolic calcium-activated phospholipid-dependent protein kinase (PKC) activity. Both these later events result in changes in protein phosphorylation which will be dicussed.

All these, and other data, indicate that the differentiation process of leukemic cells involves a number of different signalling pathways functionning sequentially and/or in parallel, and that RA might act directly at the level of the plasma membrane. The fact that the effects induced by RA are also produced by some synthetic analogues but not by others has permitted the study of a structure-activity relationship and the definition of the parts of the molecule necessary for its differentiating activity.

Finally, the role of the recently evidenced nuclear RA receptors in the differentiation process will be examined as well as the use of RA in vivo in an alternative approach to the therapy of promyelocytic leukaemia.

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Organ specificity of cancer metastasis is determined by cell surface properties: adhesive, invasive and growth properties of unique tumor cells and host cell properties at particular organ sites. Garth L. Nicolson, Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030 USA.

The sites of distant metastases in many clinical cancers and animal tumors are nonrandom, and their distributions cannot be rationalized by anatomical or mechanical hypotheses based on the simple lodgment or trapping of tumor cell emboli in the first capillary bed encountered [1, 2]. Evidence will be presented in support of differences in tumor cell surface properties as an explanation for the nonrandom distributions of metastases, where the unique properties of particular tumor cells and the different characteristics of each organ microenvironment collectively determine the organ preference of metastasis. Using animal models for metastasis based on murine melanoma, large cell lymphoma and fibrosarcoma and rat mammary adenocarcinoma we have examined tumor cell and host organ properties, such as differential tumor cell adhesion to organ-derived microvessel endothelial cells or their subendothelial basement membrane-like matrix and organ parenchymal cells, differential tumor cell invasion of host organ tissues and extracellular matrix and their expression of degradative enzymes, and differential tumor cell responses to organ-derived growth-stimulatory and growth-inhibitory factors [3-10]. These appear to be collectively important in determining the organ specificity of metastasis.

In the interactions of tumor cells with microvessel endothelial cells we have identified several tumor cell and endothelial cell surface components involved in organ-preference of adhesion [3]. These include endogenous lectins, integrin-like adhesion components and other adhesion molecules [1, 2]. Malignant cells possess another set of cell surface adhesion components for basement membranes, including receptors for fibronectin, laminin, collagen, heparan sulfate proteoglycan, and other components [4, 5]. During invasion metastatic cells release and display on their cell surfaces specific degradative enzymes, such as metalloproteases, proteinases and endoglycosidases, that are used to dissolve basement membranes and other structures [6]. Malignant cells also respond to organ-derived chemotactic factors and make their own autocrine motility factors. In the case of rat mammary carcinoma cells, the autocrine motility factor is a M_r ~53,000 glycoprotein secreted by and bound to receptors on the highly metastatic cell clones [7].

Finally, we have found that highly metastatic cells differentially respond to organ-derived, secreted, paracrine growth factors which are present in dissimilar amounts in different organs [5, 8, 9]. One of the most potent of these paracrine growth factors for lung-colonizing epithelial tumor cells has been purified to homogeneity and shown to be a unique M_r ~66,000 glycoprotein that has been isolated from rodent and porcine lungs [10] and binds to a specific receptor on lung-metastasizing tumor cells. These and other tumor cell surface and host properties may eventually be used to predict and explain the unique metastatic distributions of certain malignancies.

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The cytoskeleton in metastasis. Mareel M., Van Larebeke N., and Bracke M. Laboratory of Experimental Cancerology, Dept. Radiotherapy and Nuclear Medicine, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium.

Metastasis can be dissected into invasion and uncontrolled growth; the expression of these two phenotypes may occur separately in time. The cytoskeleton of normal and malignant cells consists of microtubules, intermediate filaments and actin filaments; these elements of the cytoskeleton are structurally and functionally related. Numerous reports have implicated the cytoskeleton in activities of invading cells. We describe here the effects on invasion of selected drugs known to interact with cytoskeletal elements. The test system mostly consisted of confronting pairs of normal tissue and malignant cells in organ culture; histology and immunocytochemistry were the methods of analysis (1).

Microtubule inhibitors were antiinvasive both in organ culture and in experimental tumours in vivo. This was found for three kinds of microtubule inhibitors with similar functional effects : Colchicine-like drugs (colcemid, podophyllotoxin, and nocodazole; vinblastine, vincristine, and vindesine) inhibited tubulin assembly in the test tube and disassembled microtubules in living cells. Taxol enhanced microtubule assembly. At antiinvasive concentrations estramustine bound to MAPs and produced an unorganized network without affecting the overall assembly-disassembly equilibrium; at 10x higher concentrations this drug caused disassembly. Since all these drugs affected both spindle and interphase microtubules at similar concentrations, they inhibited both growth and invasion. That inhibition of growth was not responsible for the antiinvasive effect appeared from drugs (e.g. 5-fluorouracil) that were antiproliferative but permissive for invasion. From the analysis of cell motility we concluded that microtubule inhibitors were antiinvasive because they interfered with directional migration of cells (2,3).

Drugs affecting actin filaments were antiinvasive at least in vitro. These included cytochalasins, known to inhibit actin filament growth by binding to the barbed end, and dipyridamole, possibly affecting the actin cytoskeleton by an unknown mechanism. Experiments with these drugs were not continued because antiinvasive and toxic concentrations were too close to each other.

The expression of intermediate filaments of the keratin type has been reported to be sensitive to retinoic acid (4). In organ culture, retinoic acid showed antithetic effects on invasion, as demonstrated with variants from the human mammary cancer cell line MCF-7. Some variants e.g. MCF-7(AZ) were not invasive in control medium but became so upon addition of 10^{-6}M retinoic acid to the culture medium. By contrast, this compound inhibited the invasion of variants (e.g. MCF-7/6, obtained from H. Rochefort, Marseille, France) that expressed the invasive phenotype under control conditions. Preliminary observations have suggested that retinoic acid-mediated changes of intermediate filaments are translated into changes of those cell surface components that regulate cell-cell, and cell-substrate adhesion. Most of these cell surface components are integral membrane glycoproteins. It is interesting that, at least in some cell types, alkyl lysophospholipids have shown antithetic effects of invasion at concentrations that altered the carbohydrate moieties of cell-surface glycoproteins (5). The observations with retinoic acid and alkyl lysophospholipids suggest the possibility of modulating the expression of the invasive phenotype at distinct steps of a specific pathway.

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Cell signalling and metastasis. Hart, I.R., Imperial Cancer Research Fund Laboratories, Lincolns Inn Fields, London WC2A 3PX.

The required proliferation of tumour cells at both primary and secondary sites during metastatic development suggests that cell signalling systems could play an important role in regulating cancer spread. Moreover the majority of tumours are not initially metastatic but become malignant as a consequence of evolution and progression (Nowell, P.C. Science 194 : 23, 1976). This raises the possibility that responses to particular ligands and cell signalling mechanisms could vary within a single tumour type according to the stage of tumour development. These possibilites will be illustrated by reference to a series of murine and human cell strains and lines derived from melanocytes at various stages of transformation.

Non-tumorigenic, diploid melanocytes are capable of extended growth in tissue culture if protein kinase C (PKC) activators, such as 12-O-tetradecanoylphorbol 13-acetate (TPA) phorbol 12, 13 dibutyrate or mezerein, are included in the culture medium. Growth stimulation appears to be a consequence of PKC activation by such compounds rather than a result of their tumour-promoting capacity since similar effects are obtained with sapinotoxins A and D. Addition of cAMP elevating substances to such supplemented medium further enhances cellular proliferation, decreasing population doubling times and increasing saturation densities, though these agents alone are incapable of supporting in vitro growth. In contrast the addition of similarly supplemented medium to malignant melanoma lines inhibits proliferation.

Starting with a non-tumorigenic line of mouse melanocytes, dependent for their growth on the continuous presence of 200 nM TPA, it has proved possible to derive tumorigenic and metastatic clones. These transformed derivatives arose as a consequence either of transfection with viral, activated cellular - Ha-ras or polyoma middle T (PyMt) oncogenes or through irradiation with ultraviolet (UV-C) light. Transformation in all instances was associated with a change in growth behaviour from a dependency on the presence of TPA to inhibition of cellular proliferation upon addition of PKC-activators. These results suggest that the development of malignant melanomas could be as a consequence of molecular lesions in the PKC- mediated signalling pathway and raise the hope that correction of this defect could facilitate treatment of this disseminating cancer.

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On the role of the cell membrane in Ca^{2+}-mediated cytotoxicity and programmed cell death. Orrenius, S. Dept Toxicology, Karolinska Institutet, S-104 01 Stockholm, Sweden.

The cell membrane plays a critical role in the maintenance of a number of vital cell functions, including intracellular ion homeostasis and signal transduction. It is also an important target for cytotoxic chemicals which may interfere with signal transduction and affect ion fluxes through the cell membrane. The latter effect may result from the inhibition of ion extrusion or from the stimulation of ion influx. Thus, both alkylating and redox-active chemicals have been found to affect intracellular ion homeostasis through the inhibition of transport-ATPases in the plasma membrane, whereas cytotoxic T lymphocytes and natural killer (NK) cells appear to kill their target cells by stimulation of the influx of extracellular Ca^{2+}. A similar mechanism plays an important role in the elimination of thymocytes during the development of mature T cells. Progression of toxic cell injury in mammalian cells has been associated with the perturbation of intracellular Ca^{2+} homeostasis. Sustained increases in cytosolic Ca^{2+} are believed to mediate cell killing through the activation of Ca^{2+}-dependent processes catalyzed by proteases, phospholipases and endonucleases. Evidence for the involvement of proteases and phospholipases in toxic cell killing has come from studies with the reactive disulfide, cystamine, and the putative toxic metabolite of paracetamol, N-acetyl-p-benzoquinone imine. A third catabolic process found to be sensitive to a sustained elevation of cytosolic Ca^{2+}, is the cleavage of DNA mediated by Ca^{2+}-activated endonuclease(s). The involvement of endonuclease activity in cell killing has been investigated in immature thymocytes, most of which are normally eliminated by this process. In thymocytes, glucocorticoid hormones have been found to cause endonuclease activation and chromatin condensation characteristic of a process known as apoptosis. Endonuclease activation is, in turn, preceded by a sustained elevation of cytosolic Ca^{2+} caused by the glucocorticoid-induced synthesis of a heat-labile factor which facilitates influx of Ca^{2+} across the plasma membrane. A similar pattern of DNA damage, suggestive of endonuclease activation, is caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in immature thymocytes and can also be observed in cells exposed to oxidative stress or certain toxic chemicals. Thus, it appears that Ca^{2+}-mediated processes may play a role in both physiological and toxic cell killing and that the critical perturbation of intracellular Ca^{2+} homeostasis may result from the impairment of normal ion transport through the plasma membrane.

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Flow cytometric evidence of interaction of cancer chemotherapeutic agents with the plasma membrane of the murine erythroleukemic cell (MELC). Massaro, E. J.[1], Zucker, R. M.[2], Elstein K. H.[2], and Easterling, R. E.[1] [1]Environmental Protection Agency, Research Triangle Park, NC 27711, and [2]NSI Technology Services Corp., Research Triangle Park, NC, 27709.

Certain cancer chemotherapeutic agents, e.g. podophyllotoxin derivatives (VM-26, VP-16), anthracyclines (adriamycin, AD 32, AD 198), actinomycin D, and m-AMSA apparently interfere with topoisomerase II (Topo II) function inhibiting cell proliferation. Topo II inhibition has been proposed as the mechanism of action of these agents. However, adriamycin (ADR) has been reported to be a plasma membrane toxicant. We observed that AD 198 and VM-26 perturb the MELC plasma membrane at concentration x time values which block cell cycle traverse in G_2. Exposure to 1 μg/ml (~1.4 μM) AD 198 for 6 h increases cellular carboxyfluorescein (CF: from intracellular hydrolysis of carboxyfluorescein diacetate) fluorescence in ~20% of the MELC suggesting reduction of the rate of CF efflux. Flow cytometric (FCM) analysis reveals a linear increase in mean cellular CF fluorescence of up to 80% following 4 h exposure to 0.05 μg/ml (~0.08 μM) - 25 μg/ml VM-26. Following exposure to 5 μg/ml AD 198 for 6h or 18 h recovery in drug-free medium after 6 h exposure to 1 μg/ml, the cells exhibit resistance to detergent (NP_{40})-mediated cytolysis. Exposure to 0.001 - 0.1 μg/ml VM-26 for 24 h blocks cell cycle traverse in G_2, but has no effect on NP_{40}-mediated cytolysis. However, exposure at/above 0.5 μg/ml results in inhibition of cytolysis. Exposure to 0.01 μg/ml (~0.02 μM) - 0.25 μg/ml ADR for 6 h blocks cells in G_2. However, 18 h after reincubation in drug-free medium, the G_2 block of cells exposed to 0.01-0.025 μg/ml is reversed. In contrast, the block persists in cells exposed to higher doses. Cells exposed to 0.01-25 μg/ml ADR exhibit no resistance to NP_{40}-mediated cytolysis and exclude propidium iodide (are viable). (This is an abstract of a proposed presentation and does not necessarily reflect EPA policy.)

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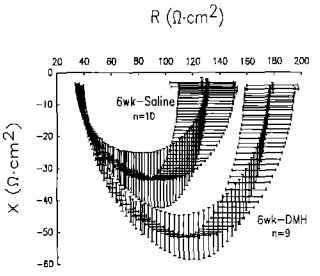
Altered impedance spectra in an animal model of colonic cancer. Thompson S.M. and Davies R.J. Department of Surgery, Univ. of California, San Diego, CA 92103

Recent studies suggest that while tumor formation in colorectal cancer may be a localized event, changes in proliferative index are evident throughout the colon. That is, there is an apparent "field defect" wherein alterations in the mucosa associated with the cancer are present at sites distant to the lesion. Because a primary function of distal colon is the transport of ions via conductive pathways in the apical and basolateral membranes, we compared the transepithelial impedance spectra of distal colons from CF1 mice which had received 6 weekly injections of either 1,2-dimethylhydrazine (DMH, 20 mg/kg) to induce colonic cancer or saline vehicle only. Mice were sacrificed at two time points, 7-10 days and 40 weeks, after their last injection. Colons were opened and number, size and position of any tumors noted. Sections of normal appearing mucosa from the most distal 1 cm of colon were mounted in a modified Ussing chamber and short-circuited. Impedance spectra were obtained under voltage clamp conditions using a composite waveform consisting of 106 discreet frequencies. At 7-10 days following DMH the entire colon appeared normal, however, the impedance spectrum was significantly altered from control as demonstrated by Nyquist plots showing the mean ± sem resistance (R) and reactance (X) at each frequency. Curve fits to individual spectra suggest that membrane resistance (R_m) is increased but capacitance (C_m) and thus, membrane area, are unchanged. At 40 weeks nearly all colons of DMH-treated mice had multiple polyps and tumors and were blanketed with microneoplasms. Under these circumstances both resistance and reactance were less than 40-wk controls. Compared to the early time point, both R_m and C_m tended to increase with age in controls, but R_m decreased while C_m remained constant in DMH-treated colons.



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Nuclear magnetic resonance investigations of tumorigenicity in human glioma cell lines. Metz K.R.*, Geder L.#, Mikus J.L.#, Gaydos A.J.#, and Kreider J.W.+ Radiology Dept.*, New England Deaconess Hospital, Harvard Medical School, Boston, MA 02215 and Depts. of Neurology#, Microbiology#+, and Pathology+, Pennsylvania State University College of Medicine, Hershey, PA 17033

Seven human glioma cell lines were investigated using high-resolution natural abundance ^{13}C and ^{31}P NMR spectroscopy. Cell lines studied ranged from highly aggressive FG and D-54MG to HMCN-1 which exhibits low tumorigenicity. The ^{13}C NMR spectra of all of these lines contained peaks arising mainly from lipids, particularly phospholipid head groups and the backbone carbons of fatty acyl chains. In addition, peaks in the 70-75 ppm region were observed and assigned to cellular myo-inositol. The intensities of these signals were strongly correlated with the aggressiveness of the cell line as measured by the colony-forming potential in soft agar and by the tumorigenicity in nude mice. When the cells were grown in Ham's F-12 medium containing differentiation-inducer dimethylsulfoxide (DMSO), the aggressiveness was reduced and a parallel decrease in NMR-detectable myo-inositol was found. The level of cellular inositol may be related to the flux through the polyphosphoinositide cycle which is known to be implicated in tumor cell growth. NMR measurements show promise for non-invasive determinations of tumor aggressiveness *in vivo*.

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Doxorubicin has structural effects on acidic phospholipids involved in signal transduction. de Wolf, F.A., Maliepaard, M., Berghuis, I. and de Kruijff, B. Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

We investigated the effect of doxorubicin (DOX) binding on DOX fluorescence and on the order and dynamics of the acyl chains in model membranes composed of dioleoylphosphatidic acid (DOPA) or dioleoylphosphatidylserine (DOPS) as representative acidic phospholipids involved in plasma membrane signal transduction. Dioleoylphosphatidylcholine (DOPC) was used as a control zwitterionic phospholipid. Saturation binding to large unilamellar was observed with a stoichiometry of 0.1, 2.5 and 1.5 mol DOX per mol DOPC, DOPA and DOPS, respectively at pH 7.4 . The apparent (estimated) Kd was 20-25 μM both for DOPA and DOPS, but the binding was cooperative.

Addition of small unilamellar vesicles of DOPA to a DOX solution caused strong fluorescence quenching, which was reversed by increasing the vesicle concentration, most likely due to dilution of ADM in the membranes. Under these conditions, the chromophores of DOX were fully inaccessible to the aqueous fluorescence quencher iodide. ^2H NMR studies on DOPA, specifically ^2H-labeled at the 11-position of both acyl chains, revealed a dramatic decrease of the quadrupolar splitting indicating a large acyl chain disordering effect of the drug. DOX did not affect acyl chain ordering in the corresponding DOPC system. ^{31}P NMR and small angle X-ray scattering analysis of the DOX-DOPA system revealed that the overall bilayer structure and headgroup conformation at the level of the phosphate groups was unaffected by DOX.

These data demonstrate a high affinity interaction between DOX and PA or PS. They indicate that DOX binding to these lipids is partly electrostatic and that DOX can penetrate the membranes resulting in a strongly perturbed acyl chain packing.

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Epidermal growth factor stimulates the anchorage-independent growth of human squamous cell carcinomas overexpressing its receptors. Lee K., Shigeno C., *Hatanaka M., and Konishi J. Dept Nuclear Medicine and Radiology, Faculty of Medicine, *Dept Serology and Immunology, Institute for Virus Research, Kyoto University, Shogoin, Sakyo-ku, Kyoto 606, Japan

Overexpression of the receptors for epidermal growth factor (EGF) has been shown to occur at a high incidence both in primary squamous cell carcinomas and in established cell lines such as A431, while EGF has been shown to inhibit the in vitro growth of these cells. Thus, functional roles of EGF receptor overexpression in squamous cell carcinomas remain to be elucidated. We have previously demonstrated that EGF effects on the growth of A431 cells are reciprocal, depending on the culture conditions; EGF stimulates the anchorage-independent growth and inhibits the anchorage-dependent growth (Exp Cell Res 173:156, 1987). To examine whether the reciprocal responsiveness to EGF is a property common to cells overexpressing EGF receptors, we studied the effects of EGF on the anchorage-dependent and -independent growth of three other human squamous cell carcinoma lines (Ca9-22, HSC-2, and NA) in which both gene amplification and overexpression of EGF receptors have been previously described (Cancer Res 46:414, and 46:1648, 1986). While EGF inhibited the anchorage-dependent growth of these three cell lines, it stimulated their anchorage-independent growth. EGF caused a dose-dependent promotion of soft agar colony formation in these cells; few colonies were made without EGF. Also, EGF dose-dependently enhanced the DNA synthesis of these cells in soft agar culture. The effective EGF doses that stimulated the anchorage-independent growth were the same as those required to inhibit the anchorage-dependent growth. These results, thus, indicate that the proliferative responses to EGF are characterized by a preference for the anchorage-independent, rather than -dependent growth, in cells overexpressing EGF receptors. Moreover, EGF has been shown to stimulate the in vivo growth of three of the four cell lines studied (A431, Ca9-22, and NA) (Int J Cancer 40:706, 1987). Taken together, our data suggest that in vitro EGF responsiveness of these cells in soft agar culture, but not that in monolayer culture, better correlates with the in vivo EGF responsiveness, similar to the correlation between the acquisition of the anchorage-independence and in vivo tumorigenicity (J Cell Biol 82:1, 1979). Overexpression of EGF receptors may predict in vivo responsiveness to possible anti-EGF therapy against squamous cell carcinomas.

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"EXPRESSION OF c-erbB-2 (NEU, HER-2) IN HUMAN PANCREATIC ADENOCARCINOMAS"

Maguire H. C. Jr., Williams T. M., Hellman M.E., Weiner D. B. and Greene M. I.

The c-erbB-2 gene specifies a transmembrane phosphoglycoprotein that has about 50% homology with the epidermal growth factor receptor; a ligand for this putative growth factor receptor has not yet been identified. In breast carcinomas, increased c-erbB-2 protein has been associated with factors portending a poor prognosis. We have surveyed over 120 human cancer cell lines for increased expression of c-erbB-2 protein, using a polyclonal antisera generated by repeated immunization of rabbits with a synthetic peptide (residues 1240-1255) at the C terminal end of the molecule; for immunization, the peptide was coupled to Keyhole Limpet Hemocyanin (KLH). Cell lines were evaluated by immunoprecipitation and by immunoperoxidase staining. Cell lines from most tumor types (e. g. lymphomas, neuroblastomas, melanomas) were negative; however, 3/6 pancreatic cell lines over-expressed c-erbB-2. Based on this finding, we examined slides from archival specimens of human pancreatic adenocarcinomas; 3/10 specimens examined to date show increased staining of tumor cells, implying an increased expression of c-erbB-2 protein. This finding suggests the identification of a unique subset of human pancreatic tumors. In work in progress we are examining further tumors and are determining clinicopathological correlations. The c-erbB-2 protein, which has a large extracellular domain, may serve as a useful target for diagnostic or immunotherapeutic procedures.

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Modification of epidermal growth factor receptor expression during the butyrate-induced differentiation of human HCT-116 colon adenocarcinoma cells Nathan, D.F., Burkhart, S.R., and Morin, M.J. Department of Pharmacology and The Cancer Center, Northwestern University, Chicago, IL 60611

Several clonal sublines of HCT-116 human colon adenocarcinoma cells were isolated and characterized on the basis of their growth characteristics, intrinsic enterocyte-like differentiation (as assessed by alkaline phosphatase and lactase activities), and responses to butyrate (an inducer of colon tumor cell maturation). There was significant heterogeneity among the clones but, in general, those sublines which exhibited the greatest degree of intrinsic differentiation responded more completely to butyrate. Among the butyrate-responsive sublines, two groups emerged: those which exhibited increases (3-6 fold) in alkaline phosphatase activity, and those in which lactase activity was increased (2-3 fold) after butyrate treatment. The epidermal growth factor (EGF) binding capacity was also found to correlate with intrinsic differentiation. Well-differentiated HCT-116 clones expressed approximately 3.5×10^4 high-affinity receptors/cell (k_D = 0.6 nM) and poorly differentiated sublines exhibited virtually no specific binding capacity for EGF. The EGF binding capacities of several moderately-differentiated clones was increased significantly (from undetectable levels to approximately 1.2×10^5 receptors/cell) after treatment with butyrate. The butyrate-induced receptors displayed lower affinities (k_D = 3.2 nM) for [^{125}I] EGF than did the receptors expressed on the more differentiated cells, and they were expressed concomitantly with the induction of lactase activity (maximal increases after 3-4 days). It was possible that proteins which regulate EGF receptor expression, including the transforming growth factors alpha (TGF-alpha) and beta (TGF-beta) played a role in the differentiating effects of butyrate. The results of northern and western blot analysis of the expression of the EGF receptor, TGF-alpha and TGF-beta, both before and after exposure to butyrate, suggested that the increased expression of the EGF receptor and altered expression of the transforming growth factors were major determinants of the differentiation responses of human HCT-116 colon tumor cell clones.

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Characterization of a tumor cell-derived collagenase stimulatory factor. Nabeshima K., and Biswas, C. Dept Anatomy and Cellular Biology, Tufts Univ Health Sciences Center, Boston, MA 02111

The isolation and partial characterization of a tumor cell derived collagenase stimulatory factor (TCSF) from the membranes of human lung carcinoma cells (LX-1) have been reported (Ellis, Nabeshima and Biswas: Cancer Research, June, 1989). Purified TCSF has an approximate mol.wt. of 58kD, and stimulates collagenase production and the level of collagenase mRNA in fibroblasts (Prescott, Troccoli and Biswas: Biochem. Int. May, 1989). Polyclonal antibody raised against the excised 58kD band from SDS-PAGE of purified TCSF inhibits stimulation of fibroblast collagenase production, indicating that the biological activity is associated with the 58kD protein band and not with a minor contaminant present in the preparation. Amino acid analysis of TCSF reveals that approximately 40% of the amino acids are hydrophobic. The sequence of the N-terminus is A-A-G-T-V-F-T-T-V-E---, the N-terminal amino acid being alanine. Electrophoresis of purified TCSF often shows doublet bands (TCSF A and B). However N-glycanase treatment of purified TCSF did not alter the mobility of either band, indicating the absence of N-asparagine linked oligosaccharide residues in TCSF-A or B. Separate analysis of each of these two bands showed close similarity in their total amino acid composition and an identical sequence for the 10 amino acids at the N-terminus. Comparison of our partial amino acid sequence data with other proteins (Bionet, NIH) showed no significant homology. Whether TCSF-A and B are products of the same gene or are derived from two separate mRNA species is being investigated. We have also compared the secreted form of TCSF (TCSF-S) with the membrane form (TCSF-M) by peptide mapping after V-8 protease digestion. Significant homology was obtained between these two forms, indicating that TCSF-S may be derived from TCSF-M. (Supported by NIH grant CA-38817)

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Bradykinin stimulation of human fibroblasts activates multiple phospholipases; Differential effects of phorbol esters.

Van Blitterswijk, W.J., Hilkmann, H., van der Bend, R.L., and De Widt, J. Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

We have stimulated human foreskin fibroblasts (HF cells) with bradykinin (BK), which results in a rapid phosphoinositide breakdown and Ca^{2+} signal, and arachidonic acid release by phospholipase A_2. The generation of the second messengers 1,2-diacylglycerol (DG) and phosphatidic acid (PA) was measured after prelabeling the cells with(^{3}H)arachidonic acid and (^{14}C)palmitic acid, which results in a ten-fold difference in the $^3H/^{14}C$ ratio between phosphatidylinositol(PI; arachidonate-enriched) and phosphatidylcholine (PC; palmitate-enriched). This allows us to evaluate the molecular source of the DG and PA generated, e.g. PI (denoted as DG_i and PA_i, respectively). Stimulation of HF cells with BK gave a biphasic DG response: an early peak at 10-15 sec, mainly of DG_i, and a second peak at 10-30 min in which DG_c predominates. DG_i appeared to be the preferred substrate for the enzyme DG kinase to yield (arachidonate-enriched) PA_i. The phorbol ester PMA completely abolished the BK-induced rapid breakdown of phosphoinositides (DG_i formation), whereas the second response (DG_c) seemed unaffected. However, treatment with PMA alone gave rise to the formation of DG_i, DG_c, PA_i, and PA_c, presumably by activation of phospholipases C and D. The latter enzyme activity was further proven by the release of free (^{3}H)choline from metabolically radiolabeled PC. Interestingly, in this way BK was also shown to evoke a rapid (15 sec) phospholipase D activity. The generation of the lipidic counterpart PA_c in this case could best be demonstrated by prelabeling cellular PC for a relatively short time (4 hrs) with (^{3}H)myristic acid. The significance of phospholipase D activation (direct formation of PA from PC) in transmembrane signalling is under further investigation.

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Two classes of antagonist interact with receptors for the mitogenic neuropeptides bombesin, bradykinin and vasopressin: studies in murine fibroblasts and human small cell lung cancer.

Woll, P.J. and Rozengurt, E., Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

Neuropeptides are increasingly recognized as growth factors, so their antagonists could be useful antiproliferative agents. Several neuropeptides have been characterized as mitogens in the Swiss 3T3 (murine fibroblast) cell line, and their signal transduction pathways described. Bombesin/gastrin-releasing peptide (GRP) is also a putative autocrine growth factor for human small cell lung cancer (SCLC). We have shown that bombesin/GRP, bradykinin and vasopressin are mitogenic for Swiss 3T3 cells at nanomolar concentrations. They act through distinct receptors as shown by (a) specific cell surface binding of [^{125}I]GRP and [^{3}H]vasopressin which is not inhibited by the other mitogens and (b) the existence of ligand-specific antagonists which are inactive against structurally unrelated mitogens. In contrast, the substance P analogues [$DArg^1$,$DPro^2$,$DTrp^{7,9}$,Leu^{11}] substance P and [$DArg^1$,$DPhe^5$,$DTrp^{7,9}$,Leu^{11}]substance P reversibly inhibit ligand binding, early intracellular signals and mitogenesis induced by bombesin/GRP, bradykinin and vasopressin. We suggest a model in which the receptors for these mitogens exhibit two binding domains. One is ligand specific, and is blocked by the ligand-specific antagonists. The second domain is common to the three receptors and is blocked by the substance P analogues. SCLC typically secretes a variety of peptides and hormones including bombesin and vasopressin. Bombesin/GRP has been suggested as an autocrine growth factor for these tumours. [$DArg^1$,$DPro^2$,$DTrp^{7,9}$,Leu^{11}]substance P and [$DArg^1$,$DPhe^5$,$DTrp^{7,9}$,Leu^{11}]substance P inhibit the growth of SCLC in vitro. GRP-stimulated Ca^{2+} fluxes in SCLC cell lines are abolished by both ligand-specific antagonists and the substance P analogues, suggesting that the receptor model derived in Swiss 3T3 cells has relevance to SCLC.

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Activation of inositol phospholipid signalling in human breast cancer cells by bombesin.

Patel, K.V. and Schrey, M.P. Dept. of Chemical Pathology, St. Mary's Hospital Medical School, London W1 1PG. U.K.

Members of the bombesin related family of peptides (BRPs) have been previously shown to act as both secretagogues and potent mitogens for a variety of cell types, however a role for these peptides has not been previously described in human breast cancer. In our studies we have demonstrated that bombesin and BRPs stimulate the hydrolysis of inositol phospholipids in at least two human breast cancer cell lines (MCF-7 and T47D). In studies with [3H]inositol prelabelled MCF-7 cells, bombesin and its mammalian homologue gastrin releasing peptide (GRP)induced a dose- dependent accumulation of inositol mono (IP1) and bisphoshates (IP2), over a 60 min period. The half maximal and maximal effective doses for both bombesin and GRP were found to be similar, 1nM and 100nM respectivily. Kinetic studies with MCF-7 cells revealed that bombesin (100nM) induces a transient increase in inositol trisphosphate at 2min, while IP1 and IP2 demonstrated an increase over basel levels at 2 and 5min respectivily, which was sustained for 30min. We were also able to completely abolish the bombesin (10nM) induced inositol phosphate accumulation by using the bombesin receptor antagonist [Leu13- -CH2NH-Leu14] bombesin at 1uM. 45Ca++ prelabelled MCF-7 cells responded to bombesin (100nM) stimulation with a rapid 3-fold increase in 45Ca++ efflux in the presence of extracellular Ca++ (Ca.ext) (1.8mM). However, in the absence of the Ca.ext, the bombesin response was greatly attenuated. Also, in the presence of a Ca++ channel blocker Ni++ (2mM), the bombesin-stimulated 45Ca++ efflux was abolished. This result suggests that Ca.ext plays a prominent or a supportive role in bombesin-stimulated 45Ca++ efflux. Although we have demonstrated that bombesin and BRPs induce intracellular signals in MCF-7 cells, we have as yet been unable to correlate these signals with a proliferative response.

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Ha-ras induced desensitization of the Ca^{2+}-mobilizing system to bombesin.

H.Oberhuber, K.Maly, J.Zaknun and H.Grunicke
Institut für Medizinische Chemie und Biochemie,
Universität Innsbruck, A-6020 Innsbruck, Austria

Addition of bombesin to quiescent 3T3-cells results in a rapid increase in inositol trisphosphate (IP_3) and a mobilization of intracellular Ca^{2+}. In cells transfected with the Ha-ras oncogene - not, however, in cells containing the corresponding protooncogene construct - expression of $p21^{ras}$ leads to a strong depression of the Ca^{2+}-response to bombesin. This desensitization of the Ca^{2+}-mobilizing system to bombesin is detectable 2 hours after expression of the oncogene with a maximum after 6 hours. Determination of the Ca^{2+}-load of intracellular non-mitochondrial stores yielded no evidence for a ras-mediated depletion. A desensitization of the phosphoinositide specific phospholipase C to growth factors in ras-transformed cells has been reported by other authors and has recently been shown to correspond to receptor down-regulation [1]. Studies in our system revealed a depression of the bombesin-induced IP_3 formation in cells expressing the transforming Ha-ras oncogene. This effect, however, is seen only after prolonged expression of ras and is not detectable up to 6 hrs after expression of Ha-ras. The desensitization of the Ca^{2+} mobilizing system, however, is clearly expressed as early as 2 hours following induction of the oncogene. These results demonstrate that the desensitization is not caused by decrease in IP_3 formation due to a reduction of bombesin receptors or receptor inactivation, but probably due to a Ha-ras mediated blockage at Ca^{2+} channels of the endoplasmic reticulum.

1) J.Downward, J.de Gunzburg, R.Riehl and R.A.Weinberg, Proc.Natl.Acad.Sci. 85, 5774, 1988.

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bFGF-stimulation of human tumor cell proliferation in vitro. Czech, J., and Sedlacek, H.H. Behringwerke AG, Dept. of Exp. Medicine, P.O. Box 1140, D-3550 Marburg, FRG

The discovery of int-2 oncogen products homology with FGFs, recent observations of bFGF stimulation of the proliferation of mammary carcinoma cells (MCF7) in vitro and observation of FGFs in urine of kidney and bladder cancer patients (1) has stimulated our search for bFGF growth stimulation of carcinoma cells. We have tested proliferation of cells in the soft agar cloning assay originating from human tumor xenotransplants (nude mice) and found stimulation in 3/5 cases of ovarian and 5/8 cases of bronchogenic carcinoma. In addition we routinely assayed for EGF stimulation and frequently found response of a tumor either to both growth factors (6/13), or to EGF alone (3/13). This shows the possible importance of bFGF, EGF/TGFα in tumor cell proliferation. Since the corresponding receptors are tyrosine protein kinases, these tumors may be targets for chemotherapy with tyrosine protein kinase inhibitors.

(1) Chodak, G.W. et al., Cancer Res. 48: 2983-2988, 1988

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Point mutations at codons 301 and 969 in the human c-fms gene (CSF-1 receptor) in myelodysplasia and acute myeloid leukaemia. Ridge S.[1], Worwood M.[1,2], Jacobs A.[2], Padua R.A.[2]. Department of Haematology, [2]Leukaemia Research Fund Preleukaemia Unit, University of Wales College of Medicine, Heath Park, Cardiff, UK.

The c-fms proto-oncogene encodes the functional receptor for CSF-1, the macrophage and monocyte specific growth factor. Binding of the ligand to the receptor induces tyrosine kinase activity, which is believed to trigger a series of cellular changes culminating in mitogenesis. Previous studies have shown that mutations at codon 301 mimic a ligand induced conformational change, resulting in constitutive tyrosine kinase activity and subsequent transformation (1). Mutations at codon 969 will further enhance the transforming activity of 301 mutants, but alone are insufficient to transform. The tyrosine residue at codon 969 is believed to serve a negative regulatory function (2), loss of which releases the receptor from its regulation.

We have addressed ourselves to the relevance of mutations at these specific codons in patients with haematological malignancies, in particular those involving myeloid lineages. DNA has been prepared from peripheral blood or bone marrow from 64 patients with myelodysplasia (including 30 patients with chronic myelomonocytic leukaemia (CMML)), and 48 with acute myeloid leukaemia. Sequences containing codons 301 and 969 have been amplified using polymerase chain reaction (PCR). We cloned and sequenced part of the intron 5' to 301 to design suitable primers. The amplified material was screened by hybridisation to mutant specific oligonucleotide probes. To date, 3 mutations at codon 969 have been detected, all Tyr -> Cys, and all in patients with CMML. Data will be presented on the prevalence of mutations at both codons 969 and 301.

(1) Roussel, M.F. et al. Cell (1988), 55, 979.
(2) Roussel, M.F. et al. Nature (1987), 325, 549.

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Liver nodules as a model system for studies of cell membrane and cell signals in cancer development. Helena Nilsson and Lennart C Eriksson. Department of Pathology, Huddinge Hospital, S-141 86 HUDDINGE, SWEDEN.

During the process of chemical hepatocarcinogenesis drug resistant preneoplastic liver cells develop and proliferate to form liver nodules. These cells are iron deficient and express low levels of eg iron containing drug metabolizing enzymes. As many fully developed tumors, they exhibit an increased expression of transferrin receptors. Liver nodules and hepatocellular carcinomas also show a higher than normal rate of spontaneous cell proliferation.

In this work we present data addressing the mechanisms of drug resistance, cell surface receptor alterations, the growth promoting effect of diferric transferrin and other extracellular redox agents, and alterations in receptormediated transmembrane and intracellular signal transduction i rat liver nodules.

We have shown that the resistance phenotype of liver nodules consists of a low phase I and a high phase II drug metabolizing activity. Diferric transferrin, as well as ferricyanide (a nonpermeable, stable ferric compond) stimulates cell proliferation in vitro in a dose dependent manner. This effect could be explained by the action of a transplasma membrane redox system (NADH diferritransferrin reductase) which increases the intracellular pH and the NAD/NADH ratio.

Another signal system affecting the intracellular pH, and also the calcium levels in the cell, is the production of inositol phosphates and diacylglycerol after hormone or growth factor stimulation. We have compared normal and nodular cells regarding this system, and found what might be explained as isomeric differences in the signal transducing compounds.

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Characterization of muscarinic cholinergic receptors in membrane preparations from rat prostatic adenocarcinoma. Batra S., Christensson P., and Hartley-Asp B. Dept Cancer Pharmacology, Pharmacia LEO Therapeutics AB, R&D, Box 941, S-251 09 Helsingborg, and The Dept Obstetrics & Gynecology, University of Lund, Sweden.

Muscarinic cholinergic receptors (MR) have been shown to be present in the glandular epithelium of human prostate. Using ^{3}H-quinuclidinyl benzilate (^{3}H-QNB) as a radioligand for the receptor, specific binding in R-3327 (Dunning) prostatic carcinoma sublines H (hormone-dependent) and AT (hormone-independent) as well in prostate and urinary bladder was examined. Whereas a significant number of high affinity binding sites for QNB were present in H tumours as in the ventral prostate and urinary bladder, they were completely absent in AT tumours. The concentration of MR (560 fmol/mg protein) in H tumours was higher than that in the ventral prostate but lower than that in the urinary bladder. Dorso-lateral prostate contained no or very low concentration of MR. The K_D value for QNB binding was between 200-300 nM in the various preparations examined. Using a number of muscarinic antagonists and agonists, MR in H-tumours were further characterized. Antagonists (atropine and scopolamine) had a much higher affinity for MR than agonists (carbacholine, pilocarpine). Verapamil and tamoxifen also competed for QNB binding sites in the membrane with IC_{50} values similar to those of the agonists. Pirenzepine had a relatively low affinity for MR in tumour indicating that the receptor was of M2 type. Similar specificity was observed in MR in prostate and in urinary bladder. The absence of MR in AT tumours may be a result of anaplasia or/and hormone independence.

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Differential response elicited from noninvasive and metastatic mouse mammary adenocarcinoma cell lines by growth factors. Bozena Korczak, J.W. Dennis, R. S. Kerbel, Div. of Cancer and Cell Biology, Mount Sinai Hospital Research Institute, Toronto, Canada.

It has been postulated that metastatic phenotype is associated with the alterations in cell signaling pathways that control expression of genes involved in metastasis. We have examined this hypothesis by analyzing the effects of different growth factors on the secretion of metalloproteases and the expression of certain genes in noninvasive (SP1) and metastatic (A1, A3, T1, M2, M3) cell lines. We found significant differences in the gelatinolytic and casenolytic activities secreted by SP1 and A3 cells as well as in the expression of stromelysin and tissue specific inhibitor of metalloproteases (TIMP). Certain growth factors (EGF, bFGF, TGFβ1) caused differential effects on the expression of stromelysin and plasminogen activator and secretion of proteases elicited from noninvasive (SP1) and metastatic cell lines (A3). For example, the secretion of 55-60 kD and 45 kD proteases was inhibited in noninvasive SP1 cells when exposed to growth factors. In contrast, the secretion of the proteases by metastatic cells was enhanced or unchanged under similar conditions. Since some of these changes might reflect alterations at the gene expression level, we tested this possibility by Northern gel analysis. We found that EGF enhanced stromelysin mRNA (2 fold) in metastatic cells but not in non-metastatic ones (SP1). The implications of these results on the dynamics of different cell populations during tumor progression will be discussed.

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Early intracellular events induced by erythropoietin in erythroid progenitor cells. Mason-Garcia, M. and Beckman, B.S. Dept. of Pharmacology, Tulane University Medical School, New Orleans, LA 70112 USA

Erythropoietin (Ep) is a glycoprotein hormone which acts on its target erythroid precursor cell (CFU-E) to induce both proliferation and differentiation. Although much work has been done to elucidate the intracellular messengers activated by Ep, no definite pathway has yet been identified. Our previous studies with chromatographic methods strongly implicated the lipoxygenase metabolites of arachidonic acid (AA). In the present study we have used specific radioimmunoassays and determined that within 5 min of the addition of Ep (0.2U) to a suspension of murine fetal liver cells (E-14;80% CFU-E), there is a 3-5 fold rise in leukotriene B4 (LTB4) and a 2 fold rise in 12-hydroxyeicosatetraenoic acid (12-HETE). Also, in the colony-forming bioassay for Ep, exogenous LTB4 or 12-HPETE, in physiological doses and in the absence of EP, causes formation of erythroid colonies in a dose-dependent manner: 10^{-10}M LTB4 is 75% as potent as Ep and 10^{-8}M 12-HPETE, 90%. We have also attempted to elucidate the source of substrate AA in these cells. Since their endogenous levels of diacylglycerol (DAG) are high, we assessed the effects of a DAG lipase inhibitor (RHC 80267) in our system. At $5X10^{-6}$M, the compound completely inhibited the Ep-induced rise in 12-HETE abd significantly inhibited the rise in LTB4, while also inhibiting Ep-induced colony formation by 80%. TMB-8, an inhibitor of the inositol trisphosphate-mediated rise in intracellular calcium, had no effect on AA metabolites, suggesting that the phosphatidylinositol pathway is not involved. Recent studies by Russell have suggested that the activation of nuclear protein kinase C (nPKC) may be a common motif in the actions of growth factors; as the liganded Ep receptor is endocytosed, we tested this hypothesis in isolated nuclei from our cells. Preliminary results show a time- and dose-dependent activation of nPKC in response to either Ep or LTB4. Our data thus suggest that the actions of Ep may be mediated by a novel intracellular pathway.

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A study on hCG binding sites of plasm membrane and hCG concentration of cytoplasm from gastric carcinoma. Li Fuhai Yang Kaiyu, Chen Manling*, Li Xinhai*, Zhang Min*, Lei Youdao*, Lan Tianhe*. Dept. of Radiology, Institute of Biochemistry and Molecular Biology, Hormone Receptor Research Laboratory*, Isotope Laboratory*, West China University of Medical Sciences, Chendu, China

The previous study of immunohistochemistry demonstrated that human chorionic gonadotropin(hCG) exists in the normal gastric mucosae and gastric cancer cells. We collected 21 specimens of gastric carcinoma which were diagnosed by the double-contrast examination, surgery and pathology and studied the hCG binding sites of plasm membrane and hCG content of cytoplasm. Pathologically normal mucosae were used as controls. The carcinomous and normal mucosae were homogenized and sonicated respectively. The homogenates were centrifuged(1000xg, 15min.). A little portion of the supernatants was obtained as cytoplasm for hCG radioimmunoassay. The rest supernates were subjected to sucrose density gradient ultracentrifugation(78000xg, 2hr.). The plasm membrane was collected from layer between 33% and 37%. The binding activity was determined with 200ug of membrane protein, 5-8ng of ^{125}I-hCG and unlabeled hCG in a final volume of 0.5ml of 0.05M/L Tris-HCL buffer and incubated at 30°c for 2 hours. The results were shown as followings:

	hCG binding sites			hCG content
	Bound(%)	Kd($\times10^{-9}$M)	No. of binding sites ($\times10^{-9}$M/mg protein)	of cytoplasm (ng/mg pro.)
Tissue				
Normal	3.51±1.92	3.78±1.87	2.77±1.76	3.01±2.58
Cancer	4.52±3.54	3.09±1.64	3.68±1.99	2.15±1.60

This study showed that: 1)hCG binding sites of normal mucosa of stomach were demonstrated. 2)Compared with the number of binding sites from normal gastric mucosa, that from gastric cancer was increased statistically(paired design t test 0.05>p>0.02). 3)hCG was found in the cytoplasm of normal gastric mucosa. 4)hCG content of gastric carcinoma was decreased significantly.

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ras proteins and peptide factors enhance the phosphorylation of a 38 kDa membrane protein (p38) whose expression is negatively correlated with cell division. Ashok N. Hegde, Ch. V. B. Swamy, B. H. Muralikrishna and M. R. Das, Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

We had previously shown that ras proteins and factors stimulating inositol lipid metabolism in rat liver cells enhance the phosphorylation of a 38 kDa membrane protein (p38). We had shown also that the phosphorylation is mediated via G-protein(s) and obtained indirect evidence to indicate that the phosphorylation occurred down stream to phospholipase C activation. We have now raised polyclonal antibodies against the 38 kDa protein. Using these antibodies which are monospecific we have studied the expression of the 38 kDa protein in various tissues of rat. The tissue distribution of p38 is highly restricted in that it is expressed only in liver, kidney and small intestine. The highest level of expression was found in the liver of adult rat. Expression of p38 in regenerating liver was less than that of adult liver and still less in fetal liver. The same pattern of expression was found in human adult and fetal liver as well. In addition, we have tested the expression of p38 in four different rat liver tumors. It was found that the expression of p38 was quite low compared to that in normal rat liver. Expression of p38 was observed in amphibian, murine, bovine, and human livers suggesting that the gene coding for p38 is evolutionarily conserved.

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A *RAS*-Related Protein is Phosphorylated and Translocated by Agonists That Increase Cyclic AMP Levels in Human Platelets

Eduardo G. Lapetina, Juan Carlos Lacal, Bryan R. Reep and Luis Molina y Vedia
Division of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC 27709.

The antigenicity of platelet proteins was assayed against various monoclonal antibodies that recognize specific epitopes of the *ras* p21 protein (J.C. Lacal and S.A. Aaronson, Proc. Natl. Acad. Sci. USA, 83, 5400-5404, 1986). Antibody M90, which detects the region of p21 within amino acids 107-130 and inhibits its GTP-binding activity, strongly reacted with a 22 kDa protein that is present in the particulate fraction of human platelets. Other monoclonal antibodies against *ras* proteins, including Y13-259, which efficiently detects *ras* proteins from a variety of organisms, did not recognize the platelet 22 kDa protein. Transfer of the platelet 22 kDa protein to nitrocellulose paper showed that it binds [α-^{32}P]GTP. Moreover, preincubation of the transfered protein with antibody M90 drastically reduced its GTP-binding activity. Treatment of platelets with iloprost, a prostacyclin analog, caused (a) a time-dependent increase of a 24 kDa protein that it is recognized by antibody M90 in particulate and cytosolic fractions and (b) the gradual decrease of the 22 kDa protein from the particulate fraction. When platelets were labeled with ^{32}P, and then treated with iloprost the 24 kDa protein was found to be phosphorylated. The [^{32}P]24 kDa protein was specifically immunoprecipitated by antibody M90. These results suggest that appearance of the 24 kDa protein results from phosphorylation of the 22 kDa protein, which causes a shift of its mobility to a higher molecular weight area. The phosphorylated *ras*-related protein is translocated from the membrane to the cytosolic fraction.

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Multiple sites of G protein involvement in the regulation of phosphoinositide metabolism. Smith C. D. Division of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC 27709

For several years, the involvement of G proteins in hormone signal transduction has been the focus of considerable investigation. In addition to regulating adenylate cyclase, G proteins have been found to regualte phosphoinositide metabolism. Hormone-stimulation of phosphoinositide metabolism can be divided into three phases: 1.) receptor-mediated stimulation of phospholipase C activity toward polyphosphoinositides; 2.) termination or desensitization of this hydrolysis; and 3.) resynthesis of polyphosphoinositides allowing repletion of PIP_2 levels. Evidence for the involvement of G proteins in all three of these phases has been established. Specifically, 1.) activated G protein(s) interact with phospholipase C allowing PIP_2 hydrolysis at physiological Ca^{2+} levels; 2.) activation of protein kinase C feeds back to uncouple the G protein and phospholipase C, thus terminating the hydrolytic phase; and 3.) G protein(s) stimulate PIP kinase activity promoting the resynthesis of PIP_2, allowing the cell to return polyphosphoinositide levels to their resting state. Therefore, G proteins appear to be involved at multiple sites in hormone-stimulated phosphoinositide metabolism.

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Purification and characterization of plasma membrane Tumor Hemolytic Protein from ras oncogene transformed fibroblasts. Zucker S., Lysik R.M., DiMassimo E. I., Bonanomi E., and Schwedes J.W. Veterans Admin. Med. Center, Northport, N.Y. 11768 and State Univ. of N.Y. at Stony Brook.

Most cancer cells have the capacity to lyse normal RBCs in vitro by a cell contact requiring phenomenon (Cancer Res. 45: 6168, 1985). We previously isolated a secreted Tumor Hemolytic Protein (s-THP; Mr=66,000) from ras oncogene transformed fibroblasts (Biochem. Biophys. Res. Comm. 140: 365, 1986). We now report the isolation of a hemolytic protein (mTHP) from the plasma membranes of ras oncogene transformed NIH-3T3 fibroblasts which is 50 fold more potent than s-THP m-THP was extracted from cells isolated from fibrosarcomas initiated by tumor transplantation in nude mice. Nitrogen cavitation and differential centrifugation were used to isolate tumor cell plasma membranes. After washing with chaotropic agents to remove extrinsic membrane proteins, 1% n-Octylglucoside was used to extract intrinsic membrane proteins. The extracted proteins partitioned by 60 -100% ammonium sulfate contained all of the hemolytic activity as measured by incubation of tumor membrane proteins with ^{59}Fe-labeled rat RBCs. Two active m-THP species (Mr=50,000 and 45,000 on SDS-PAGE) were purified by DEAE anion exchange and gel filtration chromatography. On sucrose density gradient centrifugation, m-THP behaved as a protein of 120,000 daltons suggesting a dimer association. Purified mTHP produced 50% lysis of 5 million RBCs per ug protein. mTHP is also cytotoxic for L-929 fibroblasts, but not the parent 3T3 cell line. m-THP is heat stable, requires calcium for hemolytic activity, and is destroyed by chymotrypsin treatment. m-THP is highly lipophylic as shown by a requirement for high concentrations of detergent to extract membrane -bound proteins. mTHP retained hemolytic activity when incorporated into small unilamellar vesicles prepared with egg lecithin. m-THP resembles s-THP by its inhibition by EDTA or serum, but differs by its membrane localization, lower molecular weight, heat stability, and resistance to trypsin inactivation. An important biologic role for m-THP in damaging host cells during cancer invasion is proposed.

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RAS mutations in haematologically normal patients. Padua R.A.[1], Carter G.[1], Hughes D.[1], Clark R.E.[1], McCormick F.[2], and Jacobs A.[1] Leukaemia Research Fund Preleukaemia Unit, University of Wales College of Medicine, Heath Park, Cardiff U.K.[1]. Cetus Corporation, California U.S.A.[2]

RAS mutations are frequently observed in patients with preleukaemia (Padua et al. Leukemia 1988: 2: 503). To determine whether these lesions are found as early markers of disease we investigated the incidence of RAS mutations in DNA from peripheral blood of 70 lymphoma patients at risk of secondary leukaemia following cytotoxic therapy. We have also examined DNA of peripheral blood or bone marrow from 20 haematologically normal individuals. Using the polymerase chain reaction (PCR) and oligonucleotide hybridisation we investigated codons 12/13 and 61 of N,K and HRAS.

Amongst 70 post-chemotherapy patients, 3 had RAS mutations but no sign of haematological disease. DNA from one of these patients with an N12 val substitution registered in a nude mouse tumourigenicity (NMT) assay. This mutation was also present in a bone marrow sample but not in paraffin block sections of the original tumour tissue or unaffected nodules and spleen. Another patient had concurrent N12cys and N13ala mutations. The third patients had an H61arg substitution.

Amongst 20 haematologically normal surgical patients or normal volunteer individuals,3 DNA samples showed evidence of RAS mutations. Two of them registered in a NMT assay. One had concurrent K and HRAS activations and the other had an H12val mutations. DNA from the third patient had an H12val substitution detected by PCR; there was insufficient material for a NMT assay. These patients were aged 48 - 67 years. We cannot exclude the possibility that these are constitutional mutations, but the presence of a double mutant in one case makes this unlikely.

These results raise questions about the role of RAS mutations in neoplasms and whether such lesions can be very early markers, identifying patients at risk of developing malignancies.

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RAS mutation in human melanoma; RAS activation is a frequent and early event in melanoma development. Padua R.A.[1], Hughes D.[1], Shukla V.K.[2], McCormick F.[3], Hughes L.E.[2], Leukaemia Research Fund, Preleukaemia Unit[1], Department of Surgery[2], University of Wales College of Medicine, Heath Park, Cardiff, U.K. Cetus Corporation[3], Emeryville, California, U.S.A.

Activation of members of the RAS gene family is the most common abnormality observed in human malignancies. Because of the functional and sequence homology with G-proteins, it has been suggested that they are involved in the process of signal transduction. RAS genes are known to acquire transformation inducing properties by point mutations that lead to amino acid substitutions at Codons 12, 13 and 61. We have used biological and biochemical assays to assess the frequency of HRAS activation in DNA from 12 human malignant melanoma cell lines and 45 fresh tumours. HRAS 12 mutations were observed in 50% (6/12) of the cell lines and 13% (6/45) of the tumours. Five of these mutant fresh samples had normal HRAS allele loss.

We have analysed 40 of these fresh specimens of melanotic lesions from benign naevi, primary and secondary melanomas for RAS mutations by using the polymerase chain reaction (PCR) and oligonucleotide hybridisation. In 28 cases adjacent normal skin or peripheral blood was also analysed. Apart from a single sample no mutations were observed in normal tissue. RAS mutations were detected in 2/4 benign naevi (2xK12), 7/22 primary (5xK12, 3xH12, 1xN61) and 3/14 secondary (2xK12, 1xH12, 1xN61) tumours. Three patients, two with primary and one with secondary melanoma had two concurrent RAS mutations making a total of 12/40 (30%) patients with RAS mutations. The presence of RAS mutations in naevi, which are reportedly melanoma precursors, suggests that RAS activation is an early event in melanoma development.

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Second messenger generating enzymes in human leukemic cells.
Losa G.A. and Graber R.,Laboratory of Cellular Pathology, Locarno, Switzerland.

Phosphatidylinositol 4,5-bisphosphate phospholipase C (PIP_2-PLC) and Inositol 1,4,5-triphosphate monophosphatase (IP_3-MP) are key enzymes which perform signal transduction by generating second messengers from membrane phosphoinositides.Analytical fractionation data indicate that both enzymes are mostly cytosolic while only a minor fraction (30%) is associated with plasma membrane either in normal peripheral blood lymphocytes or leukemic cells.Both PIP_2-PLC and IP_3-MP activities were measured in total cell homogenate with the aim to characterize enzymatically cells of human leukemia defined by a distinct surface marker phenotype. A suitable comparison with an activated but not leukemic state was obtained by determining these activities in lectin-stimulated peripheral blood mononuclear cells.PIP_2-PLC and IP_3-MP were found generally diminished in leukemia but increased in lectin stimulated lymphocytes with respect to resting lymphocytes. However, both activities were found to increase from very low level toward activity level expressed in control cells along with the stage of cell differentiation for both lymphoid and mieloid leukemias. These preliminary findings suggest that phosphoinositide enzymes might be altered as a consequence of an impaired inositol lipid turnover or alternatively affected by an abnormal composition and organization of plasma membrane lipids, associated with the neoplastic proliferation.

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Interactions of adriamycin with the plasma membrane. Thompson, M. G. and Hickman J.A. Cancer Research Campaign Experimental Chemotherapy Group, Aston University, Birmingham B4 7ET, U.K.

The aminoglycoside antibiotic Adriamycin is a potent antitumour drug in humans and there is evidence to suggest that it may exert its effects via the plasma membrane. As a model of this activity, we have used the enucleate human erythrocyte. Under conditions of either ATP-depletion or Ca^{2+}-loading, the cell undergoes a morphological transition from the discocyte to echinocyte form and recent findings suggest that the maintenance of phosphatidylinositol 4,5-bisphosphate (PIP_2) levels in the inner leaflet of the membrane may play a pivotal role in the maintenance of a discocyte morphology. We found adriamycin to be a potent inhibitor of the morphological transition of erythrocytes from the discocyte to echinocyte form and to inhibit PIP_2 breakdown under conditions of either ATP-depletion or Ca^{2+}-loading. However, at very low concentrations (10^{-9}M) Adriamycin appeared to increase echinocyte formation suggesting that it may stimulate PIP_2 breakdown. In an attempt to explain these results and our findings that the protein kinase C-activating phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), partially prevented echinocytosis induced by calcium loading, we have focused our attention on the regulation of phospholipase C activity. We have recently found that the addition of GTP or GTPγS (1-10uM) produced a concentration-dependent increase in inositol-phosphate production in red cell membranes. However, if phospholipase C was stimulated by Ca^{2+} ($2.5x10^{-4}$M) and then treated with GTP or GTPγS (>100uM) there was a concentration-dependent inhibition of inositol-phosphate production suggesting the presence of both a G_s and G_i linked to phospholipase C. We are currently examining the effects of Adriamycin and TPA on this system and results of these experiments will be presented.

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Effect of andriamycin on inositol phospholipid hydrolysis in human breast cancer cells. Seal, L.J., Patel, K.V. and Schrey, M.P. Dept. of Chemical Pathology, St. Mary's Hospital Medical School, London W2 1PG. U.K .

Andriamycin (ADR) is a potent antitumor drug which interacts with the plasma membrane of cells. Recent studies indicate that ADR binds to inositol phospholipid thereby preventing hydrolysis by phospholipase C. Since inositol phospholipid hydrolysis has been implicated as a mitogenic signalling pathway it has been suggested that this action of ADR may partly mediate the anti-proliferative effects of the drug. In the present study we have investigated the effects of ADR on inositol phospholipid hydrolysis in the human breast cancer cell line MCF-7. Treatment of MCF-7 cells with ADR for 80 min resulted in a dose-dependent decrease in the basal accumulation of inositol phosphate hydrolysis products, which was maximal at 10uM. Maximal growth inibition was apparant at 1uM ADR, following a 80min pretreatment and a 5 day growth period. Bombesin and the calcium ionophore A23187 both stimulated dose-dependent increases in total inositol phosphate production with maximal effective doses of 0.1uM and 5uM respectively. ADR pretreatment caused a small but significant attenuation of bombesin- but not A23187-induced phospholipid hydroloysis. Whether an impairment of inositol phospholipid metabolism in MCF-7 cells contributes to the cytotoxic action of ADR on proliferation in these cells remains to be determined.

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Inhibition of phosphatidyl inositol metabolism and c-myc gene expression in human kidney cancer cells by TNF and IFN-γ.

Y. Kubota[1], M. Yao[1], T. Shuin[1], K. Koizumi[2], and T. Yoshioka[2], 1) Dept. Urology, Yokohama City Univ., Yokohama, Japan, 2) Dept. Human Science, Waseda Univ., Tokyo, Japan.

We have demonstrated the abnormal phosphatidyl inositol (PI) metabolism (exceptional incorporation of 32p into CDP-DG) in human kidney cancer cells with extremly high level of c-myc gene expression.

In order to understand the correlation of the PI metabolism and the enhanced expression of c-myc gene, we have studied the PI metabolism and c-myc gene expression in the cells after the treatment of cytokines (TNF+IFN-γ) which might suppress the expression of c-myc gene.

The combined treatment of TNF (10ng/ml) and IFN-γ (100 unit/ml) introduced the inhibition of growth of c-myc gene overexpressed YCR-1 human kidney cancer cells at the point of 48 hours after the treatment. In the same condition, c-myc expression of YCR-1 cells was dramatically suppressed within one hour after treatment. About PI metabolism, only 32p-labelling of CDP-DG of the cells was decreased rapidly within 20min. after the cytokines treatment.

These results suggest that PI metabolism and c-myc gene expression is deeply associated with each other in human kidney cancer cells. And there is a possibility to develop a new cancer therapy which affects cell membrane functions.

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Phospholipase D activation is not sequential to phospholipase C stimulation. Jin-Keon Pai and Emily Dobek, Molecular Pharmacology Section, Schering-Plough, Bloomfield, NJ 07003, USA

Activation of HL-60 granulocytes by N-formyl-Met-Leu-Phe (fMLP) induces the hydrolysis of inositol phospholipids by phospholipase C (PLC) yielding two second messengers; inositol triphosphate and diacylglycerol (DG). The recent demonstration of phospholipase D (PLD) activation raises the possibility of a second source of DG: PLD action on phosphatidylcholine (PC) yields phosphatidic acid (PA) which can be hydrolyzed to DG by PA phosphohydrolase (PAP).

To determine the contribution of the PLC and PLD pathways to DG formation we employed the β-blocking drug propranolol (Pro), a known inhibitor of PAP. Using exogeneous [^{3}H]alkyl-lysoPC to label the cellular PC pools, Pro (50-200 μM) inhibits formation of [^{3}H]DG and increases the formation of [^{3}H]PA from PC in fMLP-stimulated cells while it does not interfere with PLD action (as shown by lack of any effect on formation of [^{3}H]phosphatidylethanol). To measure total DG mass, we utilized DG kinase to measure total DG mass. Interestingly, DG mass parallels fMLP-stimulated [^{3}H]DG formation from PC. Moreover, accumulation of DG mass is almost completely inhibited by 200 μM Pro. These results suggest that DG formed in response to fMLP is derived mainly from PLD action on PC.

To further differentiate the PLC and PLD pathways we double-labeled cells with [^{32}P]orthophosphate and [^{3}H]alkyl-lysoPC. Following fMLP-stimulation we analyzed [^{32}P]PA as a marker for PLC activation and [^{3}H]PA as a marker for PLD activation. Pro alone stimulates the formation of [^{32}P]PA via PLC and augments fMLP-stimulation of PLC tremendously, but does not activate PLD. Cytochalasin B is required for PLD activation but inhibits [^{32}P]PA formation via PLC. Preincubation with phorbol ester inhibits fMLP-stimulated PLC activation but does not inhibit PLD. These results suggest that PLD activation is not sequential to PLC activation. It is likely that both phospholipases are directly coupled to fMLP receptors by a G protein(s).

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Inhibitory effect of dolichol-containing liposomes on growth of dexamethasone-sensitive T-cell leukemia line CEM-C7 in culture. Bansal, N., and Melnykovych, G. University of Kansas Medical Center, Kansas City, KS 66103 and V.A. Medical Center, Kansas City, MO 64128.

Dolichols are naturally occurring polyisoprenoid compounds widely distributed in animal and plant tissues. Although dolichol phosphates are well recognized as intermediates in N-linked protein glycosylation, the physiological role of free dolichols and their acyl esters have not been explored until recently when several studies provided evidence for the ability of dolichols to destabilize model membranes. In our studies of the effects of glucocorticoids on a variety of cells we have observed increases in dolichol synthesis even under conditions when synthesis of cholesterol was reduced by dexamethasone. More recently, we have shown that the content of cellular dolichols was increased in the dexamethasone-sensitive CEM-C7 line but did not become elevated in the resistant CEM-C1 line even in the presence of high (1μM) concentration of dexamethasone. In order to relate dolichols to their possible cytolytic effects, we have prepared unilamellar liposomes composed of phosphatidyl choline and phosphatidyl ethanolamine, with or without inclusion of dolichols. Although both types of liposomes caused some reduction in growth, probably through stimulation of cholesterol efflux, such effects were much greater with dolichol-containing liposomes. Some growth inhibition was evident in both cell lines, sensitive and resistant, providing evidence that it is the lack of endogenous dolichol synthesis which might, in the CEM-C1 cells, contribute to their resistance to dexamethasone.

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Targeting of drug action to the plasma membrane. Tökès, Z.A., Ross, K.L., Smith, I.M., Jardillier, J.C., and Jeanneson, P. Dept. Biochem. Molec. Biol., Comprehensive Cancer Center, Univ. Southern Cal., Los Angeles, California, USA, and Institut Jean-Godinot, Reims, France.

Covalent attachment of doxorubicin (Dox) to nanospheres (Sph) forms a conjugate compound which focuses and restricts the drug action to the cell surface. These doxorubicin-nanosphere complexes (Dox-Sph) bind to the plasma membrane, perturb several membrane constituents, induce the formation of surface blebs and cause cell death. The complexes are cytotoxic to a variety of human and rodent cell lines (including bladder and ovarian carcinomas) and against human surgical tumor explants (ovarian, lung and breast carcinomas) grown in soft agar. Dox-Sph has an increased capacity to induce cellular membrane damage in comparison to free Dox as measured by chromium release assays. Affinity binding studies demonstrate that several membrane proteins bind strongly to the drug-nanosphere complexes. Negatively charged phospholipid molecules can inhibit this membrane interaction. Five different cell lines resistant to Dox are sensitive to Dox-Sph, indicating that drug resistance due to intracellular or membrane mechanisms can be overcome by this mode of drug targeting. Furthermore, when L1210 cells were exposed for 8 to 12 months to these drug complexes, no resistance developed toward either free Dox or to Dox-Sph. These studies establish that the drug is not released from the chemical conjugates, for even low concentrations of Dox resulted in significant resistance. In addition, it supports the hypothesis that multiple cell surface targets of the conjugates are not easily modulated by the cells to result in resistance. Since inactive epimers of Dox can be converted to active cytotoxic conjugates which are even cytotoxic to Dox-resistant leukemia cells, a novel mechanism of drug action has been created. This concept is further supported by experiments performed with human K562 erythro-leukemia cells. Both Dox-Sph and Dox induce differentiation in these cells, but the two forms of drug differ in the subtypes of hemoglobin induced suggesting that the induction occurs by different mechanisms. Membrane targeted delivery of anthracyclines thus offer a new and promising approach to overcome drug resistance and to increase drug efficacy. Their application for intravesicular treatment of bladder and ovarian carcinoma may be particularly feasible.

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Cellular trafficking of A-chain immunotoxin in human tumor cell. Timar J., McIntosh D., Davies A.J.S. Ist Inst.Path.Exptl.Canc.Res. Semmelweis Med.Sch. Budapest, Hungary, Dept.Med.University of Manchester, UK. Inst.Canc.Res., Sutton, UK.

The A-chain of Ricin was coupled to Fib75 anti-human monoclonal antibody to produce an immunotoxin that displays an immunospecific but moderate toxicity to target cells. This immunotoxin was two orders of magnitude less toxic than the parent toxin. The immunoelectronmicroscopic studies of gold labeled and the native A-chain immunotoxin demonstrated the internalisation of the ligand into acidic compartment, bypassing the Golgi system and ending up in the lysosomes. The B-chain posttreatment after immunolocalisation of the immunotoxin altered the intracellular traffic of the ligand resulting in the Golgi zone-involvement and recycling to the cell surface. This type of traffic is analogous with the holotoxin.
We concluded that the Ricin A-chain of immunotoxin have to reach the Golgi system during cellular traffic to achieve full power of toxicity, even in the case of sensitive target cells.

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Producing antigenic sites on tumor cells. Huber K.R. Children's Cancer Research Laboratory, Dept. of Pediatrics, School of Medicine, Univ. of South Carolina, Columbia, SC, 29208.

Present day chemotherapy is still associated with severe side effects due to the lack of unambiguous differences between normal and tumor tissues. Biological characteristics like tumor growth rates, metabolic pathways, antigenic properties, susceptibilities to drugs, etc. do not vary enough from the features of normal cells to allow selective treatment. In contrary to normal tissues, tumor growth kinetics leads to an accumulation of tumor cells over time. I intend to exploit this physical difference between normal and tumor tissue - total increase in mass - for novel treatment regimens: Any compound that is stably integrated by growing cells will eventually accumulate in tumor tissue. If the compound differs from physiological cell components it might be a target for delivery of antibodies against this compound after sufficient time for cell renewal to occur in normal tissues.

For first experiments, growing and quiescent normal human lung fibroblast (IMR-90) cells were incubated overnight with radiolabeled choline. About twice as much choline was incorporated into the membranes of subconfluent, growing, fibroblasts than into those of confluent, quiescent, cells. Most of the incorporated choline was associated with the cells for at least one week. These results prompted to synthesize a radiolabed choline analogue, Amino-N,N-dimethylaminoethanol (N-Deanol), which might vary enough from choline to be used as a specific target for antibodies. Again, subconfluent fibroblasts incorporated about twice the amount of N-Deanol than confluent cells, and most of the accumulated N-Deanol was still associated with the cells after one week. After the demonstration that growing cells stably incorporate higher amounts of the choline analogue than quiescent cells, the first in vivo experiments were performed. Tumor bearing mice were injected i.p. with 1 μCi of radiolabeled N-Deanol per day for five days. After one week, the mice were sacrificed and tumors were analyzed for remaining radioactivity. Approximately 0.1μCi of radiolabeled N-Deanol was still associated with the tumors after this time. I am now raising antibodies against N-Deanol. These antibodies will be delivered at an appropriate period after administration of N-Deanol so that most of the normal proliferating cells that have also taken up the N-Deanol are shed from the organism. Thus, after establishing the proper interval between administrations, the antibodies will hopefully bind preferentially to the tumor cells. Eventually, these antibodies could be radiolabeled for tumor imaging, or could be attached to toxins.

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Liposome-mediated delivery of Adriamycin to human tumor cells: Effect of liposome composition on in vitro cytotoxicity. Gabizon A. and Horowitz A.T. Dept Oncology, Hadassah University Hospital, 91120 Jerusalem, Israel

We have investigated the in vitro cytotoxicity of free Adriamycin (ADM) and liposome-entrapped ADM (L-ADM), using a human ovarian carcinoma cell line (OV-1063) obtained from the ascitic fluid of a patient previously treated with ADM-containing chemotherapy. Two ADM-containing liposome formulations were tested. The phospholipid components were: 1) egg phosphatidyl-choline and egg-derived phosphatidyl-glycerol (PC-PG), which are in fluid phase at 37°C; 2) fully hydrogenated soybean phosphatidyl-choline and phosphatidyl-inositol (HPC-HPI, which are in gel phase at 37°C. Twenty-four hours after plating, OV-1063 cells were exposed to a range of drug concentrations in free or liposome-entrapped form for 72 hours. Growth inhibition curves were based on colorimetric readings obtained from methylene blue stained wells. The IC_{50} values were: free ADM, $2.8\pm1.5 \cdot 10^{-7}$ M (n=6); L-ADM using PC-PG liposomes, $2.4\pm1.1 \cdot 10^{-7}$ M (n=3); L-ADM using HPC-HPI liposomes, $1.6\pm0.6 \cdot 10^{-6}$ M (n=3). The cytotoxicity observed with L-ADM may be due to either drug leakage from liposomes in the extracellular culture medium, or intracellular drug release from endocytosed liposomes. To distinguish between these two possibilities tumor cells were co-cultured with resin beads (75-180 μm diameter) which adsorb free and protein-bound ADM, without interacting significantly with liposome-entrapped ADM. Preliminary results suggest that the cytotoxic activity is significantly inhibited by the ADM-adsorbing beads, whether free ADM or ADM entrapped in any of the two formulations tested are used. Therefore, it appears that the in vitro cytotoxicity of L-ADM is mainly mediated by drug release from liposomes into the extracellular medium and that the use of gel-phase phospholipids significantly diminishes the cytotoxic activity, possibly by retarding the rate of drug efflux from the liposomes.

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P-glycoprotein fused to the membrane of sensitive cells imparts transient resistance to Adriamycin. Belli, J.A. and Zhang, Y. Department of Radiation Therapy, The University of Texas Medical Branch, Galveston, TX 77550.

A glycoprotein of 170,000 daltons (p-glycoprotein) is found in the cell membrane of multi-drug-resistant mammalian cells and is thought to be the important gene product in the expression of the multi-drug-resistance (MDR) phenotype. LZ cells, a highly Adriamycin-resistant Chinese hamster cell derived from cells of intermediate resistance, are cross-resistant to actinomycin-D, vincristine, and colchicine, have high levels of double minute chromosomes, contain an amplified gene of approximately 120 kilobases (Gros, Croup, and Houseman, Cell, 47:371-380, 1986), and have high levels of p-glycoprotein in their cell membrane. P-glycoprotein isolated and partially purified from LZ cell membrane was fused to sensitive Chinese hamster cells (V79) using polyethylene glycol (PEG) and phytohemagglutinin (PHA). The resultant fusion products were found to be transiently resistant to Adriamycin. Transfer of p-glycoprotein was successful as demonstrated by western blots and immuno-peroxidase staining. In addition, fusion products showed changes in uptake and efflux of Adriamycin consistent with the degree of resistance observed. Therefore, these results suggest that the p-glycoprotein found in the plasma membrane of multi-drug-resistant cells is the important gene product for the MDR phenotype and its transfer to the cell membrane of sensitive cells does not impair its proposed function as a transport protein. Supported by HHS grant CA34269 from the National Cancer Institute.

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Radiation inactivation studies of membranes from multidrug resistant cells. Boscoboinik, D., Debanne, M. T., Stafford, A. R., Jung, C. Y., Gupta, R. S., and Epand, R. M., Departments of Biochemistry and Pathology, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada, L8N 3Z5.

Plasma membranes from a CHO cell line, CH^RC5, which exhibits multidrug resistance was studied using target size analysis. Immunoblots after SDS/PAGE allowed specific detection of the P-glycoprotein content of the membranes. Irradiation resulted in the disappearance of the P-glycoprotein band. The dependence of the disappearance on radiation dose allowed us to calculate a target size of 250 kDa, corresponding to a dimer of the P-glycoprotein.

Vinblastine binds to the plasma membranes of CH^RC5 cells in an ATP-dependent fashion. This binding is lost upon irradiation of the membranes. The target size for vinblastine binding is 136 kDa. This does not correspond to a P-glycoprotein monomer but rather to a portion of each of the two monomers of the dimer.

This is the first evidence to indicate that the P-glycoprotein self associates in the membrane.

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Increased rate of transbilayer diffusion of amphiphiles in multidrug resistant cell lines. Boscoboinik, D. and Epand, R. M., Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada, L8N 3Z5

Rates of transbilayer diffusion were measured using radio-labelled forms of the amphiphiles lysolecithin and palmitoyl carnitine. These amphiphiles readily partition into cell membranes. They can be rapidly extracted with bovine serum albumin as long as they remain on the extracellular side of the plasma membrane. However, once these amphiphiles have undergone transbilayer diffusion they are no longer extractable with albumin. Using this method, which was developed by Haest and his coworkers, we compared the transbilayer diffusion of these amphiphiles in multidrug resistant cell lines vs the parental cell line from which they were derived. The multidrug resistant cell line, CH^RC5, had greater transbilayer diffusion than the parental cell line AB_1 (a CHO cell line). The greater transbilayer diffusion was also observed for the DC3F/ADX line in comparison with its parental line. These results are consistent with multidrug resistance being accompanied by a destabilization of the plasma membrane resulting in more facile movement of polar headgroups across the plasma membrane of the resistant cells. In addition, verapamil, quinacrine and cyclosporin A, agents known to reverse multidrug resistance, decrease the rate of transbilayer diffusion of these amphiphiles in multidrug resistant cells back to the rates observed in parental cell lines. The concomitant reversal of drug resistance and transbilayer diffusion rates suggests that these two phenomena may be associated.

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Mdr 1 expression in chronic lymphocytic leukaemia. Holmes J., Carter G., Jacobs A., Padua R.A. LRF Preleukaemia Unit, University of Wales College of Medicine, Heath Park, Cardiff, U.K.

Chronic lymphocytic leukaemia (CLL) is generally a disease of slow progression and is inherently resistant to cytotoxic treatment. Chemotherapy may cause temporary suppression of lymphocytes but cure is not usually possible. Drug resistance mechanisms and the multi drug resistant (MDR) phenotype would therefore appear to be potentially relevant in CLL. The mdr 1 gene codes for the transmembranous p glycoprotein which effectively acts as an efflux pump leading to a decreased intracellular concentration of cytotoxic drugs. We have studied 34 patients with CLL (7 untreated, 27 treated) screening both DNA and RNA with the mdr 1 gene probe to assess the relevance of this particular mechanism. In addition, pure lymphocyte populations were prepared from ten normal controls and the level of mdr 1 RNA expression in normal lymphocytes found to be low. It has therefore been possible to establish that 18 patients (4 untreated, 14 treated) have levels of mdr 1 RNA expression above the normal range. No evidence of mdr 1 gene amplification in these patients could be found. Sequential estimation of RNA levels in 3 patients has suggested that malignant lymphocytes in CLL can switch on mdr 1 transcription in response to chemotherapy which on withdrawal of the cytotoxic insult returns to basal levels. Such data raises important questions about the type and timing of cytotoxic usage in CLL.

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The role of the membrane nucleoside transporter in natural and acquired drug resistance. Thomas C. K. Chan and Marsha Janota, Department of Physiology and Pharmacology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907

The ability of tumor cells to salvage preformed metabolites from their environment has been demonstrated as one mechanism of tumor resistance against cytotoxic antimetabolites. Inhibitors of nucleoside transport such as dipyridamole and nitrobenzylthioinosine can augment the activity of antimetabolites by blocking salvage. The role of salvage metabolism in other forms of drug resistance has not been explored. Our laboratory has used a specific pyrimidine antimetabolite, N-phosphonacetyl-L-aspartate (PALA), to select for resistant human ovarian cancer cells by single exposure and cloning (model of natural resistance), and by chronic sublethal exposure (model of acquired resistance). Studies were performed on the most resistant lines isolated from each method (20-fold resistance to PALA). The naturally-resistant ovarian cancer cells demonstrated cross resistance of 2-fold to methotrexate, 5-fold to doxorubicin and 2-fold to cisplatin. The acquired-resistant cells demonstrated a 4-fold resistance to methotrexate, 20-fold resistance to doxorubicin and 6-fold resistance to cisplatin. Membrane nucleoside transport studies (zero-trans) indicate that the acquired-resistant cells have a uridine transport rate that is 8 times higher than the parent cells, while the naturally-resistant cells only have a 1.5-fold increase. Dipyridamole (1 μM) is effective in reversing the resistance in both types of resistant cells to PALA, methotrexate, doxorubicin but not cisplatin. Dipyridamole inhibited salvage of all nucleosides and some nucleobases in the resistant cells during PALA or methotrexate exposure. More interestingly, a second effect of dipyridamole exposure was a 3-fold increase in the retention of doxorubicin in both resistant cell types. Whether the membrane nucleoside transporter is the effector/target for this second effect of dipyridamole on doxorubicin is unclear at the moment and additional experiments are in progress to delineate this interaction. (Supported by the Elsa U. Pardee Foundation)

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The effect of hypoxia and other stresses on conferred drug resistance in cultured cell lines.
Jones A-M.L., Stratford I.J., and Adams G.E.
MRC Radiobiology Unit, Chilton, Didcot, Oxon OX11 ORD.
Hypoxia can be a limiting factor in the treatment of cancer by chemotherapy. Hypoxic cells in situ are located in regions of the tumour that are poorly vascularized. In addition to oxygen deprivation, these regions may also be under stress from nutrient deprivation. Such stresses are known to induce sets of cellular proteins including the oxygen regulated proteins (ORPs) and the glucose regulated proteins (GRPs). Many of these are membrane associated. These stress proteins can also control cellular resistance to certain drugs such as adriamycin (ADR). We have carried out studies to examine the effects of stress on cell survival and acquired resistance to ADR. Cells were stressed by hypoxia and/or glucose deprivation in vitro. Survival curves for cells grown under oxic and chronically hypoxic conditions show that hypoxic cells are substantially more resistant to ADR when compared to the response of oxic cells. Kinetic studies confirm that this resistance to ADR develops over several hours after the onset of hypoxic treatment. Glucose deprivation was obtained by growing cells in glucose-free media. Glucose deprivation results in cells becoming resistant to ADR. Resistance develops over several days after the onset of glucose deprivation. With both hypoxia and glucose deprivation, release from the stress results in a return to ADR sensitivity. When glucose deprived cells are also rendered hypoxic before exposure to ADR, there is no change in the rate at which ADR resistance develops or decays. Since ADR may work in part by binding to cell membranes, it is possible that the ORP/GRP system enables cells to become resistant to ADR through membrane alterations. These observations have been made with V79 cells and the ADR resistant human tumour cell line HT29. Currently we are extending this work to include a panel of human tumour cell lines with a view to characterizing their response to stress and the molecular basis for the acquired resistance to cytotoxic drugs.

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Modulation of activity of cytotoxic nucleosides against hematopoietic cells by inhibitors of nucleoside transport. Cass, C.E. McEachern Laboratory and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.

Nitrobenzylthioinosine (NBMPR), dilazep (DIL), and dipyridamole (DIP) are tight-binding inhibitors of the equilibrative nucleoside transport (NT) system of erythrocytes. Low concentrations (< 0.1 μM) of NT inhibitors will protect normal human bone marrow cells (CFU-GEMM, CFU-GM, BFU-E) from otherwise lethal *in vitro* exposures to 7-deazaadenosine (tubercidin), suggesting that permeation of nucleosides into normal hematopoietic progenitor cells is via the equilibrative NBMPR-sensitive NT system. A number of cultured human leukemia cell lines exhibit NT activity of both high and low sensitivity to NBMPR, indicating co-existence of multiple NT systems that differ in their sensitivity to NT inhibitors. We have compared the relative ability of NBMPR, DIL and DIP to protect leukemic cell lines from tubercidin cytotoxicity under conditions similar to those used in our earlier studies with normal hematopoietic progenitor cells and have related these effects to cellular uptake of ^{3}H-tubercidin. Complete protection of CCRF-CEM cells was achieved under conditions that provided only partial protection to HL-60 cells (DIL > NBMPR > DIP). Greater inhibition of uptake of ^{3}H-tubercidin was seen with CEM cells (NBMPR > DIL > DIP) than with HL-60 cells (NBMPR > DIL = DIP). These differences were due to effects on transport since dose-response relationships for inhibition of initial rates of ^{3}H-tubercidin transport at 37^o by NBMPR were similar to those obtained in protection and uptake studies. Thus, it may be possible to selectively kill neoplastic cells of low NT inhibitor sensitivity by combination therapy with cytotoxic nucleosides and NT inhibitors.
Supported by the National Cancer Institute of Canada.

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Deoxycytidine kinase "silencing" confers drug-resistance to ara-C in leukemic cells in vivo and in vitro. *Avramis V.I., and Jackson R. Department of Pediatrics, Division of Hematology/Oncology, USC & Childrens Hospital of Los Angeles, Los Angeles, CA 90027*

DNA hypomethylation has been associated with gene expression, whereas DNA hypermethylation is associated with gene silencing. Human lymphoid cells (CEM/0) were treated with 1, 20, 100μM & 1 mM ara-C for up to 48 hours. CEM/0 cells accumulated ~1 mM intracellular [ara-CTP] by 2 hrs after incubations with 0.02, 0.1 & 1 mM ara-C. Cells incubated with 1 μM ara-C accumulated an average 181 μM cellular [ara-CTP] at 24 hrs. The amount of ara-C anabolite incorporated into DNA was 15-fold higher after the 20 to 1000 μM than after the 1 μM [ara-C] reaching at least 0.015 nmoles/10^7 cell DNA. DNA methylation levels increased linearly with time and concentration of ara-C exceeding 210% after the 1 & 20μM concentrations after treatment for 24 or 48 hrs. Similar levels of DNA hypermethylation were obtained after treatment with 0.1 & 1.0 mM [ara-C] for 4 hrs. A 50% decrease in dCk expression was observed after the 1 μM ara-C treatment. This increase in DNA methylation levels may be responsible for the development of tumor drug resistance to ara-C due to "silencing" of dCk. Treatment of the CEM/dCk(-) cells with the DNA hypomethylating agents 5-Aza-C and dihydro-5-Aza-C (DHAC) resulted in reexpression of dCk gene and the establishment partial sensitivity to ara-C. Clinically, 17 pediatric patients with refractory ALL received a continuous infusion of 5-azacytidine (5-Aza-C) 150 mg/m^2/day x5 days, after not responding to (13/17) or relapsing from (4/17) high dose ara-C regimen (3 g/m^2 over 3 hr, every 12 hr, x 8 doses). Three days after the end of the 5-Aza-C infusion, the HDara-C regimen was administered again. The average cellular [ara-CTP] was 314 ± 390 μM or 2.3-fold higher after than before 5-Aza-C treatment. In 12 patients in whom the DNA methylation studies were completed, the average DNA hypomethylation level was 55.6% +15.8% of pre-treatment. DNA synthesis had a profound decline in 2/9 evaluable patients who achieved a complete response after this regimen. The data suggests that treatment with a cytostatic but DNA modulatory regimen of 5-Aza-C causes DNA hypomethylation in vivo, which is associated with dCk reexpression in the patients' leukemic blasts leading to partial reversal of drug resistance to ara-C. We conclude that DNA methylation may control the development of drug resistance to ara-C in tumor cells through dCk silencing.

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Cell-surface carbohydrate and phospholipid analogs as potential chemotherapeutic agents. Bernacki, R.J., Sharma, M., Hong, C. and Matta, K. Roswell Park Memorial Institute, Elm and Carlton Streets, Buffalo, NY, USA, 14263.

Tumor cell surface constituents play a major role in host-tumor interactions including immune recognition, cellular adhesion, invasion, angiogenesis and growth control. Therefore, plasma membrane may serve as a unique target for chemotherapeutic exploitation. A number of amino-sugar, nucleotide-sugar and phospholipid analogs have been synthesized by our group and evaluated for biologic and antitumor activity. 2-Acetamido-1,3,6-tri-O-acetyl-2,4-dideody-4-fluoro D-galactopyranose (flugal) inhibited the growth of various murine and human tumor cell lines in vitro at 10^{-6} to 10^{-4}M; with human WiDr colon carcinoma being most sensitive (IC_{50} = 1.5 X 10^{-6}M). Flugal administration to DBA/2 mice with L1210 leukemia (50 mg/kg/day X 5) increased lifespan by 68%. In culture, flugal (3 X 10^{-4}M) caused decreased incorporation of mannose (62% control) and glucosamine (72% control) and it itself appeared to be incorporated into membrane glycoconjugate. Two nucleoside-sugar analogs: uridine 5'-(2-acetamido-2,4-dideoxy-4-fluoro-α-D-galactopyranosyl) diphosphate and uridine 5'-(2-acetamido-2,6-dideoxy-6-fluoro-α-D-glucopyranosyl) diphosphate have recently been synthesized and are currently being evaluated as inhibitors of glycosyltransferases. Finally, ara-C conjugates of biologically active thioether phospholipids (e.g. ara-CDP-ß-palmitoyl-DL-thiobatyl alcohol or ara-CDP-DL-PTBA) have demonstrated significant antitumor activity against both L1210 leukemia, solid tumors and their metastases (M5076) in mice. Overall, these membrane directed agents appear to have potential for modifying tumor cell surface constitutents resulting in decreased tumor growth and/or progression.

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Role of Membrane Cholesterol in Ether Lipid Membrane Mediated Cytotoxicity. Diomede L., Bizzi A., Magistrelli A., Modest E.J., Salmona M. and Noseda A. Mario Negri Institute for Pharmacological Research, Milan 20157, Italy, and Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103, USA.

Ether linked glycerophospholipids (ether lipids, EL) have been described as a novel class of antineoplastic agents. Their mechanism of action is mediated through an interaction with the plasma membrane.
We have extensively investigated EL biophysical interactions with the plasma membrane. Preliminary observations by our group and others (Malewitz and Baumann J. Am. Oil Chem. Soc. 65: 532, 1988) suggest a possible role of cholesterol in modulating EL uptake, membrane effects and toxicity. We have observed that EL uptake by model membranes varies significantly with the cholesterol content of the vesicle preparation. ^{3}H ET-16S-OEt (1-thiohexadecyl-2-ethyl-glycero-3-phosphocholine) uptake was studied with phosphatidylcholine: ^{14}C cholesterol 1:1 and 1:0.1 (molar ratio) liposome preparations. These experiment showed a slower uptake by vesicles richer in cholesterol during incubation for 0-24 hr.

We are currently transposing this observation to biological systems. HL60 cells when incubated in cholesterol rich medium are less sensitive to the toxic action of ET-18-OMe (1-octadecyl-2-methyl-glycero-3-phosphocholine) revealing an ID_{50} significantly higher than that obtained in normal medium using trypan blue dye exclusion. Expansion of these studies is focused on the relationship between cholesterol content and EL uptake, fluidizing properties and toxicity using tumor cell lines differently sensitive to EL toxicity (HL 60 and K562, the former being more sensitive) and normal blood cells (monocytes and lymphocytes). These studies are aimed at further characterizing EL mechanism of action and identifying differences which might explain the reported EL selectivity for cancer cells.
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The Antitumoral Action of Alkylphosphonocholines in Vitro and in Vivo

U. Ries[1], A. Breiser[2], J. Stekar[3], E.A.M. Fleer[1], C. Unger[2] and H. Eibl[1].
1) M.P.I. für Biophys. Chemie,Abt. 140,D-3400 Göttingen, F.R.G.
2) Zentrum Innere Medizin, Abt. Hämatologie/Onkologie, Robert-Koch-Strasse 40, D-3400, Göttingen, F.R.G.
3) Asta Pharma AG. D-4800, Bielefeld, F.R.G.

Alkylphosphocholines as antineoplastic agents have been developed in our laboratory as a consequence of structure-relationship studies. From the data of these studies and investigations on the metabolism of alkyl-lysophosphocholines (ALP) and alkylphosphocholines (APC), it was suggested that substrate properties for phospholipase C are required for antineoplastic activity of these compounds.
In this presentation we introduce three different hexadecylphosphonocholines, that can not be degraded by either phospholipase C or D, and compare their cytotoxic properties in cell culture experiments and their antineoplastic activity in a chemically induced autochtoneous rat mammary carcinoma modell.
The results of these investigations lead to the conclusion that absence of phospholipase C susceptibility is a negative factor for cell culture cytotoxicity, but has no influence on antitumoral activity in the in vivo modell.
Part of this study was supported by the Bundes Ministerium für Forschung und Technologie (BMFT), Bonn, F.R.G.

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Metabolic Studies on 1-O-Octadeyl-2-O-methyl-rac-glycero-3-phosphocholine and Analogues in Human Leukemic Raji Cells. Fate of the Apolar Chain.

E.A.M. Fleer[1], C. Unger[2] and H. Eibl[1].

1) M.P.I. für Biophys. Chemie,Abt. 140,D-3400 Göttingen, F.R.G.
2) Zentrum Innere Medizin, Abt. Hämatologie/Onkologie, Robert-Koch-Strasse 40, D-3400, Göttingen, F.R.G.

In earlier studies we reported on the fate of the phosphocholine moiety of alkyl lysophosphocholines (ALP) and alkylphosphocholines (APC). In the present studies we report on the metabolic fate of the alkyl chain of 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (OcMeG3PC) and its analogue with elongated phosphorous-nitrogen distance (OcMeG3PC6). Furthermore, the metabolic fate of OcMeG, the product of OcMeG3PC after PC-transfer, is reported.
At high dosages, it was found that OcMeG shows cytotoxicity in cell cultures. Incorporation studies under these conditions showed that then OcmeG was taken up to a level similar to that of OcMeG3PC when it shows toxicity. The analogue OcMeG3PC6 was not toxic, even at higher dosages, despite of a similar incorporation rate as for OcMeG3PC under the same conditions. No correlation between the generation of metabolic products and cell death was detected.
Part of this study was supported by the Bundes Ministerium für Forschung und Technologie (BMFT), Bonn, F.R.G.

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Further characterisation of hexadecylphosphocholine (INN: miltefosine), an alkylphosphocholine with antitumor activity. Hilgard, P.*, Stekar, J.*, Hofmann, J.**, Grunicke, H.**, Unger, C.***. ASTA Pharma AG, D-4800 Bielefeld 14 (*), Dept. Medical Chemistry, University A-6010 Innsbruck (**), University Hospital D-3400 Göttingen (***).

Hexadecylphosphocholine (HPC) probably represents the minimal chemical structure required for the antitumor activity of alkyllysophospholipids. The compound exerts substantial activity against a broad spectrum of established rodent and human tumor cell lines in vitro, but it is inactive against granulocyte/macrophage colony forming cells in soft agar culture. As previously shown, the antiproliferative effects of ether lipids were correlated with the inhibition of the phospholipid-calcium-dependent protein kinase C (PKC). In contrast, HPC inhibited PKC only far beyond cytotoxic concentrations, and unlike standard ether lipids it had no affinity for the diacyl-glycerol binding site of PKC. The autochthonous DMBA-induced mammary carcinoma of the rat and the human KB-squamous cell carcinoma in nu/nu mice - both unresponsive to conventional chemotherapy - were the in vivo tumor models most sensitive to HPC. Neither in vitro cultivation of tumor cells in the presence of HPC nor prolonged and repeated in vivo therapies resulted in the development of drug resistance. The tolerance of HPC in rats and mice was good and without overt toxicity at therapeutic dose levels. In conclusion, the pharmacology of HPC is distinctly different from that of the known anticancer drugs and possibly also from that of the alkyllysophospholipids. Therefore, HPC may open new perspectives in the treatment of malignant disease.

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Antitumoral effect of short chain 1-O-Alkylglycerols. Unger, C[1], Wagner, A[1], Berkovic, D[1], Hilgard, P[2], Berger,MR[3], Nagel, GA[1], Eibl, H[4]. [1]University Hospital, 34 Göttingen; [2]ASTA-Pharma, 48 Bielefeld; [3]German Cancer Institute, 69 Heidelberg; [4]Max-Planck-Institute of Biophysical Chemistry, 43 Göttingen, FRG.

Depending on the length of the alkyl chain, alkylglycerols display such different biological effects as activation of protein kinase C, improvement of drug penetration through the cell membrane and of cell recovery during cryopreservation of mononuclear cells. Here we report on the antitumoral effect of short chain 1-O-alkylglycerols of different chain length (propyl to nonyl). Cell counts were determined in a Neubauer chamber, cell vitality was tested by trypan blue dye exclusion. The ID50 and LD50 values after 48 hrs were (µM): C3 3540/15400; C4 810/3820; C5 1.2/1008; C6 0.9/9; C7 0.9/30; C8 0.9/35; C9 1.8/40. Among the compounds tested, 1-O-hexyl-rac-glycerol (HG) surprisingly showed the most pronounced effect. The activity was similar to that of such established cytotoxic etherlipids as He-PC, ET-18-OCH_3 and the thioether BM 41.440 which, however, showed markedly stronger hemolytic effects than HG. HG has an LD50 on Raji cells (9 µg/ml), which is 360 times lower than the HD50 value (50% hemolysis after 48 hrs) obtained on human erythrocytes. Any surface active effects could be ruled out in our assessment of the antitumoral activity of HG. More recently we were able to show that 2 other leukemia cell lines had marked sensitivity against HG, whereas 2 others (bladder cancer) were resistant. To date, the molecular mode of action of this compound is completely unknown. Short chain alkylglycerols exert distinct antineoplastic activity against human cancer cells.

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Differential in vivo/in vitro sensitivity of methylnitrosourea-induced mammary carcinoma against hexadecylphosphocholine. Berger, M. R.[1], Yanapirut, P.[1], Unger, C.[2], Schmähl, D.F.[1] 1 German Cancer Research Center, D-6900 Heidelberg; 2 Medical Clinic, D-3400 Göttingen, FRG

Hexadecylphosphocholine (HePC) belongs to a new group of membrane-active antineoplastic agents. It exerts distinct activity against methylnitrosourea(MNU)-induced primary mammary carcinoma in the rat. HePC dosages in the range from 15 to 77 µmol/kg, administered daily for five weeks, effectively inhibited tumor growth. At optimal dosages, complete suppression of new tumors was observed and, in 30% of the animals, complete remission of existing tumors. Serum concentrations obtained from rats treated with effective HePC dosages ranged from 150 to 200 nmol/ml. In contrast to the in vivo results, exposure to HePC of tumor cells, freshly isolated from MNU-induced mammary carcinoma, for one hour or continuously and subsequent growth of these cells under clonogenic assay conditions revealed differing sensitivity. Concentrations of 2.5 to 245 nmol/ml were ineffective in reducing the growth of colonies with regard to size and number when compared with untreated controls. Only concentrations of 735 nmol/ml and above reduced the growth of colonies by 50% or more. This effect, however, is likely to arise from unspecific mechanisms as, for example, the detergent-surface activity inherent in this class of compounds. The observed striking difference between in vivo and in vitro results raises the question as to whether the activity of HePC against MNU-induced mammary carcinoma is related to direct cytotoxic or indirect growth-regulatory effects. The lack of activity following in vitro exposure of tumors which are sensitive to HePC in vivo points to the latter possibility.

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The ether lipid analogue Et-18-OCH_3 inhibits Ca^{2+} uptake and inositol 1,4,5-trisphosphate mediated Ca^{2+} release in permeabilized cells. [1]Seewald, M.J., [1]Olsen, R., [1]Powis, G. and [2]Modest, E.J. [1]Department of Pharmacology, Mayo Clinic and Foundation, Rochester, MN 55905, U.S.A; [2]Department of Biochemistry, Boston University School of Medicine, Boston, MA, 02118, U.S.A.

The platelet activating factor ether lipid analogue 1-octadecyl-2-methylglycero-3-phosphocholine (Et-18-OCH_3) is the prototype for a new group of DNA-noninteractive antitumor agents. Et-18-OCH_3 interacts with the plasma membrane of tumor cells to alter physical properties and cause morphological damage. It can also inhibit protein kinase C and interfere with phosphatidylcholine biosynthesis. We have examined the effects of Et-18-OCH_3 on $^{45}Ca^{2+}$ uptake by the endoplasmic reticulum and the subsequent release of $^{45}Ca^{2+}$ by intracellular second messengers. The system used was saponin-permeabilized Swiss 3T3 fibroblasts. ATP-dependent $^{45}Ca^{2+}$ uptake was measured at a free $^{45}Ca^{2+}$ concentration of 0.1 µM in an EGTA buffer. The mitochondrial inhibitors 2,4-dinitrophenol, antimycin A and oligomycin were used to block mitochondrial Ca^{2+} uptake. $^{45}Ca^{2+}$ uptake measured over 6 min was inhibited by Et-18-OCH_3 with an IC_{50} of 55 µM. Et-18-OCH_3 also blocked $^{45}Ca^{2+}$ release caused by inositol 1,4,5-trisphosphate (IP_3) with an IC_{50} of 350 µM. Incubating Swiss 3T3 cells with 10 µM Et-18-OCH_3 for 18 hr before permeabilization had no effect on $^{45}Ca^{2+}$ uptake (± S.D., n = 5): 141 ± 27 pmol/10^6 cells/6 min in control and 148 ± 26 pmol/10^6 cells/6 min in Et-18-OCH_3 treated ($p > 0.05$). There was, however, a significant decrease in $^{45}Ca^{2+}$ release caused by 10 µM IP_3 from 47.5 ± 6% of the total $^{45}Ca^{2+}$ store in non-treated cells to 21.1 ± 4.8% in Et-18-OCH_3 treated cells. $^{45}Ca^{2+}$ release by 10 µM GTP was not significantly affected by growing cells with Et-18-OCH_3, with a value of 38.0 ± 7.5% compared to 44.5 ± 6.2% in non-treated cells. $^{45}Ca^{2+}$ release by 100 µM arachidonic acid was also not affected by growing cells with Et-18-OCH_3 with a value of 31.3 ± 6.7% compared to 35.4 ± 9.1% in non-treated cells. Exposure of intact Swiss 3T3 fibroblasts to 5 µM Et-18-OCH_3 for 18 hr did not affect resting levels of intracellular free Ca^{2+} measured with the photoprotein aequorin but decreased the $[Ca^{2+}]_i$ responses to PDGF and bradykinin. Supported by CA 42286.

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The effect of novel ether phospholipids on thymidine uptake and clonogenicity in HL60 cells. Vogler W.R., Olson A.C., Liotta D. Emory University, Atlanta, Ga. 30322.

Ether linked analogs of lysophosphatidylcholine have been shown to be cytotoxic to a variety of neoplastic cell lines and animal tumor systems. They are unique in that the site of action appears to be cell membranes and there is relative sparing of normal marrow progenitor cells. In order to increase the selective cytotoxicity, 3 new compounds were synthesized in our laboratories and tested for cytotoxicity in HL60 cells: trans-2-octadecyloxymethyl-3-(2'-N,N,N-trimethylamoniume thyl phosphoryl) tetrahydofuran, inner salt (I), 3-O-(2',2'-dimethyloctadecyl)-2-O-methyl-1-O-(2"-N,N,N-trimethylamoniumethyl phosphoryl) glycerol, inner salt (II), 3-O-ocatadec-7-enyl-2-O-methyl-1-O-(2"-N,N,N-trimethylamoniumethyl phosphoryl) glycerol, inner salt (III). HL60 cells were incubated 4 hours at 37° in RPMI 1640 medium, 10% fetal bovine serum, 1% antibiotic mixture, 1% glutamine and varying concentrations of compounds. After washing, cells ere tested for viability (trypan blue), DNA synthesis (tritiated thymidine (^{3}HTdr) incorporation) and cloning efficiency in methyl cellulose (colonies formed from single cell suspension after incubation in supplemental medium for 7-14 days). The concentration of compound resulting in a 50% reduction (IC_{50}) in the assay was determined. In all instances, viability was greater than 90%.

	IC_{50}(µM)		
COMPOUND	I	II	III
Thymidine Incorporation	30	33	58
Colony formation	10	18	105

These data indicate compounds I and II are active and worthy of further investigation.

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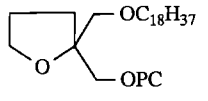
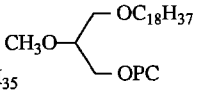
Antitumor activity of SDZ 62-406, a lactone phospholipid related to SRI 62-834. Houlihan W.J., Munder P.G.*, Lee M.L., Mason R., Handley D.A., Nemecek G.M., and Winslow C.M. Medicinal Chemistry Dept., Sandoz Research Institute, East Hanover, NJ 07936 and *Max-Planck-Institut für Immunbiologie, Freiburg, FRG.

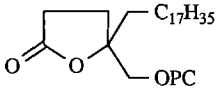
Our laboratories have reported[1] that SRI 62-834, a tetrahydrofuran phospholipid, possesses *in vivo* antitumor activity in the same range as the open-chain ether phospholipid ET-18-OCH_3. As a continuation of our program to evaluate cyclic analogs of ET-18-OCH_3 as potential antitumor agents we have prepared the butyrolactone phospholipid SDZ 62-406. SDZ 62-406 shows good *in vitro* and *in vivo* activity both p.o. and i.v. against a variety of murine tumors. In addition, the compound demonstrated *in vitro* macrophage activation, inhibition of platelet-activating factor (PAF) aggregation, and the mitogenic effect of platelet derived growth factor (PDGF). In a human foreskin fibroblast culture assay, SDZ 62-406 had weak effect on uptake of ^{3}H-thymidine and ^{3}H-uridine.



SRI 62-834



ET-18-OCH_3



SDZ 62-406

PC = $PO_3^-CH_2CH_2N^+(CH_3)_3$

[1]Houlihan, W.J., *et al.*, Lipids, *22*, 884-890 (1987).

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Simultaneous flow cytometric analysis of ether lipid SRI 62-834 modulation of membrane permeability and intracellular calcium homeostasis: Evidence of a specific biochemical perturbation. Dive C.[1], Thompson M.[1], Hickman J.[1], Watson J.[2], Workman P.[2]. 1. Pharmaceutical Sciences Institute, Aston University, Birmingham, U.K.
2. MRC Clinical Oncology Unit, Cambridge, U.K.

We used multiparameter flow cytometry (FCM) and fluorochromes Quin-2 and propidium (P) to investigate the relationship between changes in cellular calcium ($Ca^{2+}i$) and membrane permeability, respectively, induced by SRI 62-834 in EMT6 mouse mammary tumour cells. Using UV laser excitation, blue (460-510 nm, Quin-2) and red fluorescence (>630 nm, P) and light scatter were analysed. FCM has the unique advantage of subpopulation identification. After 8 min the % of cells permeable to P increased from 2% at 1 μM to 80% at 80 μM SRI 62-834. Blue fluorescence in this subpopulation was minimal. For the P-impermeable subpopulation a time- and concentration-dependent rise in the blue fluorescent calcium signal was seen for one cell subset, while no change was observed in a second intermediate subset and a third exhibited minimal blue fluorescence. At 1 μM SRI 62-834, a non-toxic dose causing no change in P-membrane permeability, $Ca^{2+}i$ was elevated by 20% at 20 min before falling to control levels at 35 min. At higher doses slight changes in light scatter indicative of cell size and/or shape changes were seen. At 15 μM SRI 62-834 the rise in $Ca^{2+}i$ was partially inhibited by preincubation of cells with the PKC-activating phorbol ester TPA (100 nm for 10 min). These results suggest that SRI 62-834 evokes specific biochemical membrane changes at low doses, but at higher doses biophysical membrane disruption may also occur.

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The relationship between cytotoxicity and elevation of intracellular calcium in ether lipid-treated leukemia cells. Charles M. Lazenby, Michael G. Thompson and John A Hickman. CRC Experimental Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, U.K.

The ether lipids represent a new class of membrane-active antitumour agents. One of these, SRI 62-834, was reported by us to bring about a concentration-dependent increase in the intracellular Ca^{2+} concentration of HL-60 human myelomonocytic leukemia cells which was inhibited by pretreatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Thompson & Hickman, Biochem. Soc. Trans. 16, 278, 1988). This led us to suggest that its effects on the membrane were not those of a detergent-like activity. Continuous incubation (72 hrs) of either $1x10^5$/ml HL-60 myelomonocytic or K562 erythroblastic leukemia cells inhibited growth by 50% at 6 μM and 90 μM respectively. Treatment of $7.5x10^6$/ml K562 human erythroblastic leukemia cells with 60 μM SRI 62-834 for 10 mins resulted in a smooth rise in intracellular Ca^{2+} from 74±4.5 nMol to 167.5±17.6 nMol (n=3), as monitored by the fluorescent calcium indicator Quin-2. Similar treatment of HL-60 cells with 30 μM SRI 62-834 brought about a rise in intracellular calcium from 101.5±18.2 nMol to 453.9±75.6 nMol (n=3). There was therefore a relationship between the toxicity of the agent to the cells and its ability to elevate intracellular Ca^{2+}. The rise in Ca^{2+} in both cell lines was inhibited by pretreatment with TPA (<100 nM) but not by the voltage-dependent calcium channel blockers verapamil or prenylamine. The nature of the Ca^{2+} rise and its relationship to toxicity is under investigation.

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Minimal resistance to the membrane active ether lipid SRI 62-834 in multidrug resistant mouse mammary tumour cells. Workman P. and Dive C. MRC Clinical Oncology Unit, MRC Centre, Cambridge CB2 2QH, UK.

A variety of evidence supports a role for a membrane-targeted mode of action for ether lipid cytotoxicity towards tumour cells in vitro. We have previously demonstrated effects on cell membrane permeability and calcium signalling in EMT6 mouse mammary tumour cells. Multidrug resistant cells frequently exhibit changes in cell membrane structure and function, including hyperexpression of the P-170 membrane glycoprotein. Using an MTT dye reduction assay, we have now investigated the comparative in vitro effects of the developmental ether lipid antitumour agent SRI 62-834 on the in vitro response of the EMT6 parent line and its MDR counterpart EMT6/AR 1.0. This MDR cell line hyperexpresses P-170 glycoprotein and exhibits a 30-fold resistance to adriamycin, an MDR drug which includes a membrane component in its mechanism of action. Inhibitory concentrations were derived from dose-response curves. The IC50 and IC90 values for parent cells were 70 and 140 μM. Very similar results were obtained from cell growth curve analysis. Significant effects were seen as low as 2 μM. EMT6/AR 1.0 cells were slightly more resistant, with IC50 and IC90 values of 100 and 200 μM respectively. These results suggest that membrane changes associated with multidrug resistance may lead to only a small though significant reduction in sensitivity to ether lipids. The universality of this effect and the mechanisms involved are currently under investigation.

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ETYA as a membrane-active agent: Studies with U937 and PC-3 cells. Harris J.E., Anderson K.M., Ondrey F., Brown M. Sections of Medical Oncology and Gastroenterology, Department of Medicine and Department of Biochemistry, Rush Medical College, Chicago, IL 60612.

ETYA (5,8,11,14-eicosatetraynoic acid), an arachidonic acid analogue, inhibits DNA synthesis in human transformed U937 monoblastoid and PC-3 prostate carcinoma cells, and partially differentiates U937 cells. The agent is not cytotoxic at the concentrations used as judged by exclusion of trypan blue, continued attachment of PC-3 cells, unchanged ^{51}Cr release and reversibility of thymidine corporation. Neither indomethacin nor acetylsalicylic acid altered DNA synthesis in either cell line. Leukotriene C4 partially reversed ETYA-induced suppression of U937 cellular DNA synthesis, suggesting a role for this eicosanoid in modulating DNA synthesis. ETYA increased whole cell and microsomal membrane fluidity, increased intracellular Ca^{2+} in PC-3 cells, altered the distribution and activity of protein kinase C in U937 cells, and rapidly down-regulated transcription of U937 c-myc. Evidence of oxidative stress including lipofuscin and myelin bodies, and disordered mitochondrial inner membranes in PC-3 cells cells was observed by transmission electron microscopy. These concatenated events are believed to represent components of the "signal" pathways responsible for reversible inhibition of DNA synthesis and the induction of phenotypic differentiation in competent cells. Arachidonic acid analogues may exert selective effects on physical and functional properties of cell membranes, and represent an additional class of membrane-active agents distinct from alkyl ether phospholipids.

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Tris as membrane active agent against Ehrlich Ascites carcinoma (EAC). Sur Pratima, Basu (Sharma) Indrani and Sur Bimanesh. Guha Inst. of Biochem. Calcutta-700029 and Jadavpur Univ. Calcutta - 700032, India.

Tris-HCl buffer augments the lytic action of surface active agents on bacterial and tumour cells. Based on this we have shown spectrophoto -metrically the effect of different conc's of Tris-HCl buffer pH 7.2 as surface active agent alone on EAC. 16 mM Tris-HCl is found to have a maximum effect with 48% lysis of the cells, indicated by decrease of O.D. at 680 nm in comparison with control cell suspension in saline, after 30 mins incubation at 35°C. On the treated cells swelling and formation of surface blebs are observed under polarising microscope, which indicate loss of surface integreity. In vitro treated EAC cells with 32 mM and 16mM Tris-HCl when transplanted ip in Swiss mice indicate significant loss of transplantability w-r. to control. MST are found to be 36 and 58 days with 32 and 16 mM Tris respectively while it is 19 days for the control group. In vivo treatment with 16 mM Tris buffer (0.7ml/mouse/day) on EAC bearing mice (10^5 cells/mouse on day 0) results in an increase in life spen over the control group by 150%. Minimum adverse hematological effect is observed in normal mice treated with 16 mM Tris buffer. There is a minor depresion of hemoglobin, WBC and platelet count 24 hrs. after treatment and are found to be recovered within ten days. However monocyte count increases after treatment. This opens up the idea of using Tris buffer as a surface active agent alone or probably in combination against EAC. Thanks are due to I.C.M.R., New Delhi and C.N.C.R.C., Calcutta-26 for financial support & experimental facilities.

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The kinetics of calcium uptake and the effect of calcium antagonists on ^{45}Ca influx and cellular growth in cancer cell lines. Popper L., and Batra S. Dept of Obstetrics and Gynecology and of Pharmacology, University of Lund, S-221 85 Lund, Sweden.

Cell growth in the various types of cancer cell lines was highly dependent on extracellular calcium. The optimum Ca^{2+}_{45} concentration was in the range of 0.6 - 2 mM. Using ^{45}Ca, the kinetics of Ca uptake in cancer cell lines (MCF-7, DU 145, HeLa) and in human fibroblasts (IBR) were studied. There was a considerable difference in both the rate and the maximum capacity of Ca uptake by the various cell lines. The resting (unstimulated) ^{45}CA uptake in fibroblasts was 4-5 times higher than that in any of the cancer cell lines. Among the cancer cell lines, DU 145 cells had the lowest uptake. Whereas K^+-depolarization of the cellmembrane with high extracellular K^+ (80 mM) caused 2-3 fold increase in ^{45}Ca influx in fibroblasts, it had no effect on ^{45}Ca influx in HeLa cells. In DU 145 cells, K^+-depolarization increased uptake by 40-50%, but only when cells had reached the plateau phase of growth. Verapamil (VPM) and trifluoperazin (TFZ) caused a significant inhibition of cell growth in cancer cells lines but had no effect on ^{45}Ca-influx. The IC_{50} concentration for VPM and TFZ were in the range of 10-20 μM and 2-5 μM respectively. Lanthanum (1-10 mM) caused a very substancial inhibition of both cell growth and Ca influx. VPM inhibited K^+-stimulated ^{45}Ca uptake in fibroblasts by 30-40% but had no effect on unstimulated cells. These results indicate that cancer cells have a considerably lower turnover of Ca and that they in contrast to fibroblasts lack potential-dependent Ca channels normally found in excitable cells.

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Pharmocological interference with intracellular signal systems results in altered sensitivity to vincristine in the human kidney tumor cell line ACHN

PETER NYGREN[#] and ROLF LARSSON[§*]. Departments of Oncology[#], Medical Cell Biology[§] and Surgery[*], University of Uppsala, Uppsala, Sweden.

The modulatory effect of substances affecting intracellular signal systems on cell growth and sensitivity to vincristine in the human kidney tumor cell line ACHN was investigated and correlated to changes in cytoplasmic free Ca^{2+} concentration ($Ca^{2+}i$) and cytoplasmic pH (pHi). Presence of the protein kinase C (pkC) activator 12-O-tetradecanoyl phorbol 13-acetate (TPA) during culture had no effect on cell growth but significantly increased the EC_{50} concentration for vincristine inhibited cell growth. However, there was no indication for endogenous pkC activity being responsible for basal vincristine insensitivity in the ACHN cells since it was not affected by the pkC inhibitor H-7. The Ca^{2+} ionophore ionomycin tended to increase cell growth and induced vincristine resistance wheras the calmodulin inhibitor W-7 had opposite effects. Presence of the adenylcyclase activator forskolin during culture dose dependently made the cells more sensitive to vincristine without affecting basal cell growth. The modulators of vincristine sensitivity did not affect pHi whereas $Ca^{2+}i$ was decreased by addition of TPA and increased by ionomycin and forskolin. There was consequently no clear cut relationship between $Ca^{2+}i$ changes and vincristine sensitivity. It is concluded that perturbation of components in the intracellular signal system may affect both cell growth and cytotoxic drug sensitivity. Increased knowledge in this field may be of great importance for the development of drugs making tumor chemotherapy more efficient.

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Modulation of hormone-induced cytosolic Ca^{2+} oscillations by depolarization and uncouplers and inhibitors of oxidation phosphorylation. Blank, L.M.[1], Kawanishi, T.[2], Tsien, R.Y.[2], and Smith, M.T.[1] School of Public Health[1] and Dept. of Physiology and Anatomy[2], University of California, Berkeley, CA 94720

The induction of cytosolic Ca^{2+} oscillations by physiological and non-physiological stimuli has been reported in various cell types. The mechanism and function of these oscillations are as yet unknown, but reports that oscillation frequency is agonist dependent and that the Ca^{2+} rise is spatially organized have led to the suggestion that oscillations may transmit information associated with inositol lipid signaling (Phil. Trans. R. Soc. Lond. B 320:325 1988); alternately, one may conjecture oscillations to be a cyclic regulatory phenomenon which functions to control intracellular Ca^{2+} levels. We have reported, (Kawanishi et al., submitted) that vasopressin and phenylephrine regularly induce periodic Ca^{2+} spikes in a large proportion of fura-2 loaded, freshly isolated hepatocytes. The frequency but not amplitude of oscillations is reduced or inhibited by depolarization. We have also demonstrated that initial oscillations require only intracellular Ca^{2+} stores, additional evidence that oscillations may be associated with phosphoinositide signaling. Here we report that valinomycin and carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) stop the oscillations immediately, while oligomycin does so gradually. The drop in ATP elicited by these agents does not appear to occur swiftly enough to explain the abrupt inhibition of oscillations, however, an immediate decrease in cytosolic pH, as measured by BCECF, follows administration of each compound. The relative contributions of pH, ATP, and transmembrane potential alterations have been investigated with regard to their role in the mechanism of phenylephrine induced Ca^{2+} oscillations and their inhibition. These results implicate intracellular pH and the transmembrane potential as potential targets for agents which block hormone-induced Ca^{2+} oscillations.

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Voltage-dependent calcium channels in MCF-7 human breast cancer cells and inhibition of cell growth by calcium channel antagonists. Worley, J.F. and Strobl, J.S. Department of Pharmacology and Toxicology, West Virginia University, Health Sciences Center, Morgantown, WV, U.S.A., 26506

The MCF-7 cell line has been used extensively as a model for hormone-dependent human breast cancer. Using the patch clamp technique, we have identified and characterized single calcium channels in MCF-7 cells. Single channel currents demonstrated unitary conductances of 9 and 26.5 pS that were selective for divalent cations over monovalent cations and anions. The open state probability of both conductance levels was voltage-dependent and increased with membrane depolarization. Thus, the properties of these unitary currents appear to be similar to those found in nerve and muscle. In a separate series of experiments, diphenylalkylamine- and dihydropyridine-type calcium channel antagonist drugs were found to inhibit MCF-7 cell growth in tissue culture. Cells grown for 4 days following passage were incubated for 24 hours in the presence of each drug, then pulse labeled with ^{3}H-thymidine for one hour. The inhibition of ^{3}H-thymidine incorporation was concentration-dependent. The apparent inhibition constant for each calcium channel antagonist was determined to be: verapamil, 2 uM; nimodipine, 0.25 uM; azidopine, 1 uM; PN200-110, 2 uM; nisoldipine, 3.5 uM; nifedipine, > 10 uM. These data indicate that voltage-dependent calcium channels exist in MCF-7 cancer cells and suggest that calcium channel antagonists may be used to inhibit cell proliferation. Supported by a WVU Biomedical Grant, the Charleston WV Area Medical Center Foundation and the Pharmaceutical Manufacturer's Association Foundation, Inc..

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The Acute Effect of Platinum Anti-Tumor Drugs on Intracellular Calcium Pools in Mouse Thymocytes

H. Simpkins, M. Rosen, M. Figliomeni
Departments of Pathology, Staten Island Hospital and the State University of New York, Health Sciences Center, NYC 11203

cis-Dichlorodiammine platinum (II) (cis-DDP) and its second generation derivative, cis-dichloro-trans-dihydroxybis (isopropylammine) platinum (IV) (CHIP) had marked effects on the binding of the fluorescent lanthanide (Tb^{3+}) to mouse thymocytes. The Tb^{3+} ion has a similar hydration radius to Ca^{2+} and is often used as a fluorescent probe of Ca^{2+} binding sites. The effect of cis-DDP was markedly ionic strength dependent in contra-distinction to CHIP. cis-Diammine 1,1 cyclobutanedicarboxylate platinum (II) (CBDCA), a lipophylic second generation derivative had no effect.
When calcium uptake into thymocytes was studied with the fluorescent dye, Indo -1 AM, all the platinum drugs affected Ca^{2+} uptake with the effect of CBDCA = CHIP > cis-DDP. The effect of the drugs on mitogen (concanavalin A) stimulated Ca^{2+} uptake paralleled their effects on cellular Ca^{2+} uptake.
From these results, it can be concluded that the lipophylic second generation drug CBDCA does not affect the ionic Ca^{2+} channel (site 1) but presumably does affect the lipophylic channel (site 2) since this drug produces marked changes in intracellular Ca^{2+} levels. cis-DDP affects the ionic Ca^{2+} site (site 1 - lanthanide site) and produces changes in calcium influx but on a smaller scale to that produced by the lipophylic drug CBDCA. CHIP, a Pt(IV) derivative acts at both sites and produces effects quantitatively similar to CBDCA. These results are important in identifying the acute cytotoxic effects of these drugs and are intriguing in light of reports showing calcium efflux changes which occur when corticosteroids kill immature thymocytes.

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Dextran sulfate inhibits growth factor dependent Ca^{2+} signalling in Swiss 3T3 fibroblasts. Seewald, M.J., Olsen, R.A., Melder, D.C., and Powis, G. Department of Pharmacology, Mayo Clinic & Foundation, Rochester, MN 55905.

Dextran sulfate (DS), a compound with heparin anticoagulant like properties, has been reported to inhibit the growth of some cell types. Changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by dextran sulfate MW 500,000 (**DS500K**) were measured in aequorin loaded Swiss 3T3 cells as previously described (Olsen et al, J Biol Chem 263:18030, 1988). **DS500K**, 100 ng/ml, decreased the $[Ca^{2+}]_i$ response to platelet derived growth factor, 100 ng/ml, ($\pm$ S.E., n = 5) from 714 $\pm$ 53 nM to 350 $\pm$ 26 nM ($P < 0.01$), but had no effect on the $[Ca^{2+}]_i$ response to 10^{-7} M vasopressin or 10^{-7} M bradykinin. Native heparin, 100 ng/ml, decreased the $[Ca^{2+}]_i$ response to platelet derived growth factor by 45% but had no effect on the $[Ca^{2+}]_i$ response to vasopressin or bradykinin. Release of Ca^{2+} from endoplasmic reticulum (er) of saponin-permeabilized Swiss 3T3 cells was measured by the method of Gill and Cheub (J Biol Chem 260:9289, 1985). **DS500K** was a potent inhibitor of Ca^{2+} release by inositol trisphosphate (1,4,5) (IP_3) with an EC_{50} of 40 nM. Ultrafiltration studies showed that **DS500K** did not bind IP_3 or Ca^{2+}. Native heparin blocked Ca^{2+} release by IP_3 with an EC_{50} of 4 μM. **DS500K** also blocked Ca^{2+} release by 100 μM arachidonic acid by 70%, and by 25 μM Ca^{2+} by 100%. **DS**, MW 5,000, was only 8% as effective on a mass basis as **DS500K** at blocking er Ca^{2+} release. Dextran, MW 500,000, did not block er Ca^{2+} release. The results suggest that the inhibition of cell growth by **DS500K** might be related to a block of growth factor-dependent Ca^{2+} signalling. This could be due to a block of the release of Ca^{2+} from er stores, although a large molecule such as **DS500K** would, presumably, have difficulty entering the cell. It is also possible that dextran sulfate could affect growth factor signalling at the cell surface membrane. Supported by CA 42286.

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Role of calcium in the anti-cancer actions of the cytokine leukoregulin. Evans, C.H. and Barnett, S.C. Laboratory of Biology, National Cancer Institute, Bethesda, MD 20892

The molecular events underlying leukoregulin (LR) induced increased tumor cell plasma membrane permeability and sensitivity to NK killing were examined in human K562 erythroleukemia cells. LR a 50 kD glycoprotein with a pI of 5.1 was purified from PHA stimulated human peripheral blood lymphocytes by sequential ultrafiltration, DEAE ion exchange chromatography, pH 4-6 ampholine IEF, and silica size exclusion HPLC. Treatment of K562 cells with 1-10 u LR/ml causes a sharp transient elevation in intracellular ionic calcium within one minute as shown by increased fluorescence of the calcium chelator indo-1. During the next several minutes a transient burst of rapidly opening and closing cation plasma membrane channel activity occurs in patch clamped cells. Cation ion channel activity is accompanied at 5-10 minutes by increasing plasma membrane permeability measurable by influx of propidium iodide and efflux of intracellular fluorescein. Increased membrane permeability reaches a maximum by 1 hour and like the accompanying inhibition of cell proliferation is reversible. Flow cytometric analysis reveals that LR permeabilizing action is partially dependent upon extracellular calcium but is effected through a mechanism other than calcium ionophore transport. Membrane protein kinase C activity increases simultaneously with membrane permeability and enhanced sensitivity to NK cytotoxicity. Flow cytometric analysis shows enhanced uptake of doxorubicin and other metabolic inhibitors concomitant with the increase in membrane permeability. FITC-dextran uptake indicates that the LR membrane channel facilitates intracellular uptake of molecules as large as 40 kD. This may permit entry of cytolytic molecules such as cytolysin, lymphotoxin or tumor necrosis factor leading to target cell destruction, the final stage of NK lymphocyte killing. Permeabilization of the tumor cell membrane and concomitant enhancement of anti-tumor drug uptake with cytokines such as leukoregulin also provides a new approach to drug targeting.

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L 651,582, A novel Antiproliferative and Antimetastasis Agent which interferes with calcium signal transduction. D. J. Hupe, J. DiSalvo, M.T. Schaeffer. Merck Institute for Therapeutic Research, Box 2000, Rahway, N.J.

The title compound, a novel carboxamido amino triazole that is an antiparasitic agent active *in vivo*, was demonstrated to act as an inhibitor of intracellular parasitic protozoal development in mammalian cells by virtue of its ability to inhibit calcium signal tranduction in the mammalian host cell. It is different from other calcium channel antagonists because it inhibits both agonist mediated and voltage dependent calcium channels and because it demonstrates surface dilution properties consistent with the cellular activity depending upon the dose per cell rather than upon the concentration of drug in solution. The drug also inhibits nucleotide metabolism by regulating PRPP synthesis in some cell types, and this results in non-toxic anti-proliferative activity. Because high plasma levels at non-toxic oral doses predicted the ability to dose single cells in plasma, a study of antimetastatic activity was carried out in the mouse PMT-6 model developed by Liotta. Little effect on the primary flank tumor was found at oral doses of up to 500 mg/kg/day, however, a dramatic reduction in the number of pulmonary metastases in mice surviving 21 days was found in both the number and size of metastases at oral doses of 100 mg/kg/day.

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Altered ion transport in human colorectal cancer.
Davies R.J. and Thompson S.M., Dept. Surgery, University of California, San Diego, CA 92103

Studies of crypt cell kinetics in colorectal cancer suggest that a "field-defect" may exist wherein the entire colonic mucosa exhibits increased proliferation even in patients who develop only a single tumor. Mitogenic signals responsible for this increased proliferation are unknown, but may involve altered ion transport. The short-circuit current (I_{SC}) is a measure of net rheogenic transepithelial ion transport. We have compared effects of ion substitution on the I_{SC} in samples of distal colonic mucosa derived from patients undergoing surgery for colorectal cancer with those from patients with benign disease. Specimens tested were normal appearing mucosa located at least 10 cm from a histologically confirmed distal colonic or rectal adenocarcinoma (n=12) or from similar sites in non-cancer bearing colon (n=9). The mucosa was stripped from underlying tissue and mounted in a modified Ussing chamber. Values of I_{SC} ($\mu A \cdot cm^{-2}$) for colonic mucosa from cancer patients (CA) versus benign controls (B) were not different in NaCl Ringer, but became significantly lower upon switching to Cl-free Na-gluconate Ringer's (CA: 48 ± 5* and B: 81 ± 15 ($p<0.05^{\#}$). Mucosal amiloride ($2x10^{-5}$ M) promptly reduced the I_{SC} in both groups to similar values (CA: 35 ± 5 and B: 40 ± 6 ($p<0.005$)) so that the change in I_{SC} was greater in colons from benign patients. Upon washout of amiloride and return to NaCl Ringer, the I_{SC} of controls returned to their initial values (81 ± 17) whereas CA tissues remained depressed (49 ± 8). Subsequent serosal addition of theophylline (1 mM) to stimulate Cl secretion increased I_{SC} in both groups by similar amounts (CA: 23 ± 4.5 and B: 30 ± 8, $p>0.5$). Differences in the I_{SC} responses to Cl-free Ringer and amiloride, but similar responses to theophylline in colonic mucosa from cancer versus benign patients are consistent with a field-change in ion transport. Whether these differences are related to altered proliferative signals in the mucosa or are epiphenomenon is not established; however these differences are provocative in view of reports of altered Na transport that have been observed during growth stimulation or transformation in cell culture and animal models of colonic carcinogenesis.

* (mean ± sem); # Paired or unpaired Student t-test as appropriate.

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Therapeutic potential of compounds that inhibit membrane-based regulation of intracellular pH. Tannock IF, Newell K, and Rotin D, Ontario Cancer Institute, Toronto, Ontario, Canada. M4X 1K9

Solid tumours are known to develop regions of acidity, and the survival of cells under acidic conditions is dependent on membrane-based ion-exchange mechanisms which regulate intracellular pH (pH_i). These mechanisms include the Na^+/H^+ antiport, the Na^+-dependent HCO_3^-/Cl^- exchanger and an H^+/lactate symport. We have shown recently that the Na^+/H^+ antiport may be required for growth of some tumours, since variant MGH-U1 human bladder cancer cells which lack Na^+/H^+ exchange activity had reduced ability to generate xenografts. Moreover, the few tumours which grew contained revertant cells (Rotin et al, 1989). Further experiments have demonstrated that the K^+/H^+ ionophore nigericin, and the protonophore CCCP cause rapid acidification of cells under acidic conditions in culture, and kill cells at pH_e 6.5. Cytotoxicity was enhanced when these agents were used in combination with inhibitors of Na^+/H^+ exchange (e.g. amiloride), or Na^+-dependent HCO_3^-/Cl^- exchange (e.g. DIDS), and permissive values of pH_e for cell killing were increased to pH_e 7.0. Since pH_e is often below 7.0 in tumours but usually above 7.0 in normal tissues, these results suggest a mechanism for the selective killing of tumour cells. This approach is currently being tested using experimental tumours in mice, with or without the use of vasoactive drugs such as hydralazine which may lower pH in tumours.

Rotin et al. Requirement of the Na^+/H^+ exchanger for tumour growth. Cancer Res 49:205-211, 1989.

Supported by the Medical Research Council of Canada.

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pH effects on tumor growth and an $H^+$and Na^+/H^+ antiport system related approach to the treatment of at least ras p21 and v-mos dependent tumors.

Harguindey S., Cragoe E. J. Jr., and García Cañero R. Clínica la Esperanza, Vitoria 01002, Spain.

From basic research to different situations where tumors arise there is enough evidence to propose that a local and chronic pH elevation may play a crucial role in carcinogenesis. Also, a pH rise is thought to be a terminal and mediating mechanism of action of ras p21 and v-mos as well as of many other growth factors. Our results will show the cause-effects relationships among pH abnormalities and the onset of cancer at different biological levels.

From the therapeutic point of view, Amiloride and derivatives have been shown to suppress ras p21 activity, cell proliferation, etc, through a pH effect. Growth and dissemination of ras p21 dependent breast cancer is related to the activity of this oncogen. Therefore, the use of Amiloride derivatives and other specific intracellular acidifiers represent a rational and promising avenue in the adjuvant treatment of certain human tumors, an appraisal which is related to the role of the Na^+/H^+ antiport system.

Our data and this approach may also contribute to understand some most important aspects of tumor growth and dissemination as a process mediated by a mechanism of an intimate and non-specific acid-base nature. Preliminary protocols in these lines will be discussed.

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Na^+/H^+Antiport blockers as possible inhibitors of in vivo tumour growth. Hedley, David W. Ludwig Institute for Cancer Research, University of Sydney, NSW2006, Australia. Reduction in cytoplasmic pH([pH]i) causes cell death in vitro. Solid tumours often contain acidotic regions, caused by anaerobic glycolysis. Cells producing excess H^+ions can extrude them via a transmembrane Na^+/H^+antiport, which is blocked by drugs of the amiloride series. We used flow cytometry to measure [pH]i in B16 melanomas growing in C57Bl mice. (Hedley&Jorgensen Exp. Cell. Res., 1989, 180:106). Because any acidotic cells would be expected to recover via the Na^+/H^+antiport during sample preparation, we investigated the use of amiloride to prevent this in cells grown in vivo. Cells were acid-loaded using 10mM NH_4Cl, and rate of [pH]i recovery monitored at room temperature. Controls regained basal [pH]i in approx 10min, while the highest non-toxic concentration of amiloride(0.5mM)halved this rate. Treatment at 4°C+amiloride "froze" cell acidosis for at least 1hr, and was used to prepare the B16 tumours. Mean [pH]i of 5 samples was 7.32±0.05SD, with a narrow range within individual tumours. Pre-treatment of 5 animals with glucose, 0.25g i.p., 1hr, prior to sacrifice gave a mean [pH]i of 7.17±0.17SD, suggesting that hyperglycaemia may reduce [pH]i in solid tumours, in addition to [pH]e, but this degree of acidosis is unlikely to produce cell death. Trapping of the excess H^+ions by coadministration of a Na^+/H^+antiport blocker might achieve the required reduction in [pH]i. Amiloride itself is toxic at the concentrations needed. A series of analogues with 10^3-fold greater affinity for the antiport have been synthesised, however, and could be a novel form of anti-cancer agent, selective for hypoxic cells.

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Altered intracellular pH and Na^+/H^+ antiport activity in multidrug resistant cell lines. Boscoboinik, D., Gupta, R. S., and Epand, R. M., Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada, L8N 3Z5

The intracellular pH of a number of multidrug resistant cell lines was compared with the intracellular pH of the parental line from which they were selected. Intracellular pH was measured using the fluorescent probe bis-carboxyethylcarboxyfluorescein. In four different cases, cells having 5-fold resistance or more, exhibited an intracellular pH which was 0.10 to 0.17 units higher than that of the parental cell line. A CHO cell line, AB_1, and its 180-fold resistant counterpart, CH^RC5, were further investigated. The intracellular pH could be perturbed by changes in the extracellular pH but the intracellular pH was always higher for the CH^RC5 cells than for the AB_1 cells. The Na^+/H^+ antiport activity was greater at lower intracellular pH and at any intracellular pH was greater for the CH^RC5 cells than the AB_1 cells. This antiport activity could thus be responsible for the higher pH observed in multidrug resistant cells. Drug accumulation in these cell lines is also pH dependent. Both the AB_1 and CH^RC5 cell lines exhibit a similar gradual increase in intracellular accumulation of vinblastine between pH 7.4 and 6.9. Thus, the higher intracellular pH of resistant cells can contribute to the lower uptake of drug by these cells.

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A protein kinase that resembles casein kinase type II is regulated by cations. *Reynolds, C.H., †McDonald, O.B. and †Sahyoun, N. The Wellcome Research Laboratories, *Beckenham, BR3 3BS, UK and †Research Triangle Park, NC 27709, USA.

Cytosol from several tissues was examined for protein kinase activity using an alkali- and heat-treated extract of rat brain homogenate as the source of substrate proteins. A polypeptide (Mr42,000) was readily phosphorylated on serine in a highly cation-dependent manner. K^+ was the most effective, followed by NH_4^+ and Na^+; Li^+ and $Tris^+$ were inactive. Activation was co-operative with activity being highly dependent on $[K^+]$ or $[K^+ + Na^+]$ at physiological intracellular concentrations. Polyamines and (at supra-physiological concentrations) Mg^{2+} also activated partially, although Mg^{2+} at millimolar concentrations was essential. The enzyme phosphorylated phosvitin and casein at higher concentrations but was inactive on histones, could use GTP or ATP, and was inhibited by heparin, thus resembling casein kinase type II (CKII). The enzyme was purified from rat brain using columns of DEAE-cellulose, octyl-Sepharose, casein-Sepharose and heparin-Sepharose, yielding subunits of 38 and 26 kDa and only the latter could be autophosphorylated. On gel-filtration it behaved as a polydisperse system with complexes of Mr > 300,000 at low salt concentrations, but salt induced disaggregation. Thus its physical properties also resemble CKII and suggest that the aggregation state may affect the catalytic activity. CKII phosphorylates topoisomerases, RNA polymerase II and initiation factors of protein synthesis, and can be activated by insulin or IGF-1 treatment of cells. CKII levels appear to be under hormonal control, and are elevated in some transformed cells. Intracellular $[K^+]$ is reportedly elevated in actively growing cells. These results may aid understanding of the regulation of tumour growth.

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Flavones and quinazolinones as potential inhibitors of tyrosine kinases. Dale, I.L., Farnell, K., Groundwater P.W., Hickman, J.A. and Solomons, K. CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET.

Quercetin (a flavone) and genistein (an isoflavone) are known inhibitors of tyrosine kinase activity and kinetic analyses have shown that this inhibition was competitive with ATP. This competitiveness with ATP suggests that these compounds should inhibit a number of protein kinases, including protein kinase C and c-AMP dependent protein kinase, however, genistein is selective for tyrosine kinases and quercetin shows a degree of selectivity over other kinases. These compounds bear no structural relationship to ATP but molecular orbital calculations by Ferrel et al. suggest that the charge distribution of the pyrone ring is similar to that of the pyrimidine ring of ATP. As part of our programme involving the synthesis of potential tyrosine kinase inhibitors we have prepared a number of 2-aryl-1,2,3,4-tetrahydroquinazolin-4-ones in the hope that by making the charge distribution of the heterocyclic ring more like that of the pyrone ring we could prepare more potent inhibitors. The compounds prepared were tested in two screens; (i) comparison of inhibition of cell growth between a "normal" cell line (3T3 murine fibroblasts) and a transformed cell line (ANN-1; infected with Abelson murine leukaemia virus), and (ii) inhibition of the kinase domain of the cloned Abelson virus protein ptab150. Both the 2-(3'-aminophenyl)- and 2-(4'-aminophenyl)-derivatives were more toxic than quercetin and brought about cell death in the same manner but neither of these compounds were inhibitors of the enzyme and none of the compounds tested showed selective toxicity against the transformed cell line. Further studies on these and other compounds are being carried out.

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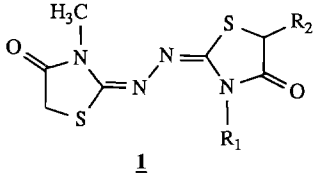
THIAZOLIDINE-DIONES - A NOVEL TYPE OF SELECTIVE INHIBITORS OF PROTEIN TYROSINE KINASE

Traxler P.*, Lydon N.*, Geissler J., Meyer Th., Regenass U., Roesel J. and Storni A.
Research Department, Pharmaceuticals Division, CIBA-GEIGY Ltd., Basle, Switzerland.

A series of >100 thiazolidine-dione derivatives of general formula **1** have been tested against the EGF-R and v-abl tyrosine kinases. The derivatives CGP 520 (R_2: $=CH_2$), CGP 645 (R_2: $=CH_2$) and CGP 4303 (R_2: $-CH_2N(CH_3)_2$ have been found to selectively inhibit the EGF-R tyrosine kinase (IC_{50}: 1.3 - 5.7 µM) relative to PKC (IC_{50}: >100 µM). No inhibition of v-abl tyrosine kinase was observed with these 3 compounds. In a cellular test system using an EGF-dependent mouse epidermal keratinocyte cell-line (MK-line), CGP 520, CGP 645 and CGP 4303 showed potent antiproliferative effects (IC_{50}: 1 - 4.2 µM).
Structure-activity relationships within this class of compounds and mechanistic studies with respect to enzymatic and cellular activity will be discussed.



1

	R_1	R_2
CGP 645	$CH_2CH=CH_2$	$=CH_2$
CGP 520	$CH_2C(CH_3)=CH_2$	$=CH_2$
CGP 4303	$CH_2C(CH_3)=CH_2$	$CH_2 \cdot N(CH_3)_2$

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Mechanisms of action of genistein, an inhibitor of EGF receptor tyrosine kinase activity, on NIH-3T3 cells.

Linassier C., Pierre M.*, Le Pecq J-B.+, Jacquemin-Sablon A., and Pierre J. URA158 CNRS, U140 INSERM, Institut Gustave Roussy, rue Camille Desmoulins 94805 Villejuif Cedex France. *U96 INSERM, Hôpital de Bicêtre, 94240 Kremlin Bicêtre, France. +Institut de Biotechnologie Rhône-Poulenc, Quai J. Guesde, 94401 Vitry/Seine, France.

Genistein has been shown to inhibit specifically in vitro EGF-receptor-tyrosine kinase activity (Akyama et al. J.Biol.Chem. 262, 5592-5597, 1987). Genistein has a cytostatic effect at concentrations below 40 µM, whereas at higher concentrations it elicit a cytotoxic effect on NIH-3T3 cells. Genistein prevents the mitogenic effect mediated by EGF or by insulin on NIH-3T3 cells. Genistein was also able to block the mitogenic effect mediated by thrombin, although the thrombin receptor does not involve a protein tyrosine kinase activity. Genistein at cytostatic concentrations did not prevent the induction of c-myc m-RNA synthesis mediated by EGF receptor activation. The stimulation of S6 kinase activity in quiescent NIH-3T3 cells stimulated by EGF was prevented by genistein. In addition, S6 kinase extracted from cells treated by EGF was also inhibited, both effects occuring at similar drug concentrations. This effect of genistein on the cytosolic S6 kinase activity might account for its ability to inhibit EGF induced DNA synthesis.

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A rapid and sensitive technique for the assay of protein kinase C: application to screening large numbers of inhibitors.

Aftab, D.T., and Hait, W.N. Depts. of Pharmacology and Medicine, Yale University School of Medicine, New Haven, CT 06510.

Protein kinase C (PKC) is involved in many cellular processes including carcinogenesis and drug resistance. Since several isozymes of PKC are expressed in mammalian tissues, a distinct form may be involved in tumor-specific or resistance-related phenomena. Therefore, specific isozymes of PKC may constitute novel targets for cancer chemotherapy. In order to determine the structural characteristics of a large number of phenothiazines and related compounds required for inhibition of PKC, we have developed a semi-automated 96 well plate assay for the enzyme. PKC (purified from rat brain by DEAE-cellulose chromatography and TSK-phenyl HPLC) and reaction buffer (giving a final concentration of 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 µg/ml diacylglycerol, 200 µg/ml histone H1, phosphatidylserine, and either 500 µM $CaCl_2$ or EGTA) are loaded into the wells using multichannel or repetitive pipettors. The reactions (100 µl final volume) are initiated one column at a time by the addition of 25 µl of 100 µM [γ^{32}P]ATP using an 8 channel pipettor, and terminated in the same fashion by the addition of 100 µl of 25% TCA. The samples (12 at a time) are then filtered over receptor binding glass fiber filtermats (1.0 µm pore size) and washed with 5% TCA using a Skatron® semiautomatic cell harvester. The samples are then transferred to scintillation vials using the Skatron® filterdisk transfer system. Direct comparison with traditional filtering techniques (Millipore 0.45 µm cellulose acetate) and with the phosphocellulose paper binding assay yield identical results for sensitivity and accuracy, but an increase in efficiency of over 500%. Using this modified assay system, we have screened over 60 phenothiazines and related compounds, and the structure-activity data will be presented.

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Teleocidine inhibits cell proliferation and increases cellular and nuclear proteins of human hepatoma cells probably by c-kinase-independent pathways. Kaneko Y., Nakayama T., Tsukamoto A., Toda G., and Kurokawa K. First Department of Medicine, University of Tokyo, Hongo, Tokyo 113.

A tumor promoter teleocidin which had phorbol ester-like activity inhibited the proliferation of PLC/PRF/5 human hepatoma cells. The hepatoma cells treated with teleocidin transformed morphologically into polygonal cells of large cytoplasm which was occupied by numerous pinocytotic vesicle-like subcellular structures. Since teleocidin inhibited cell proliferation without suppressing new protein synthesis, the amounts of cytoplasmic and nuclear proteins per cell increased remarkably. In addition, [^{14}C]leucine incorporation into proteins immuno-precipitable with anti-actin and anti-albumin antibodies was increased, while [^{14}C]leucine incorporation into β2 microglobulin was decreased. These data indicate that the teleocidin effects on cell proliferation and cell morphology were associated with altered gene expression and differentiation state. Teleocidin inhibited [^{125}I]EGF binding to receptors only transiently, and did not interfere the internalization and nuclear accumulation of [^{125}I]EGF, suggesting that the teleocidin effects were not mediated by the initial stage of signal transduction of growth stimulators. The effects of teleocidin were not negated by protein kinase C inhibitor H7 nor mimicked by a diacyl glycerol agonist, OAG. In addition, the levels of c-fos and c-myb protein as measured by Western blot analysis using specific antibodies were not reduced remarkably by teleocidin treatment. Therefore, the inhibition of cell proliferation, morphological transformation and increase in cellular proteins of PLC/PRF/5 human hepatoma cells induced by teleocidin appeared to be mediated by C- kinase-independent pathways. The present data also suggest that teleocidin may be one of the differentiation-inducing agents available for the chemotherapy of human hepatoma.

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Comparison of the growth-inhibitory properties of phorbol esters in A549 human lung carcinoma cells grown in the presence or absence of serum. Bradshaw, T.D. and Gescher, A. CRC Experimental Chemotherapy Group, Aston University, Birmingham B4 7ET, U.K.

The growth of A549 cells maintained in serum supplemented medium is potently arrested by activators of protein kinase C (PKC) such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (at nM concentrations). Growth inhibition lasts for 6 days, after which time cells become desensitised towards the inhibitory effect of TPA. We wished to test the hypothesis that TPA-induced inhibition is dependent on the presence of factors contained in serum.

When cells were introduced into serum-free medium supplemented with 2% Ultroser G (US), a significant loss in sensitivity to the growth inhibitory properties of phorbol esters was observed compared to serum supplemented cultures. Following 96 hours exposure to 10nM TPA, cells supplemented with 2% US were growth inhibited by only 20 ± 9% (n=9) when compared to controls. In contrast serum supplemented cells were inhibited by 91±4% (n=6). When serum was reintroduced into US maintained cultures, cells regained much of their sensitivity towards TPA such that 69±8% (n=9) inhibition was observed following 96 hrs exposure to 10nM TPA.

Translocation of PKC activity from cytosol to membrane and enzyme down-regulation occured in a time and concentration dependent fashion in cells supplemented with serum or 2% US. However, cytosolic enzyme activity in untreated 2% US supplemented cells was only 29 ± 4% (n=3) of activity observed in serum supplemented cells. Specific phorbol ester binding to cytosolic extracts of cells from the two populations illustrated the same dramatic loss in available binding sites. The results suggest that: i) factor(s) within serum act in concert with TPA to induce arrest of A549 growth and ii) the decreased sensitivity of cells in serum free medium towards the growth inhibitory potential of TPA may be related to decreased levels of PKC activity in these cells.

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Phorbol esters inhibit phospholipase C activity in the human promyelocytic cell line HL-60 : Relationship with protein kinase C activation and differentiation. Geny B., Cost H., and Cockcroft S.*. INSERM 204, Hopital Saint Louis 75475 paris cedex 10 FRANCE, and * UCL Medical school, experimental pathology, London WC2E 6JJ U.K.

The tumor promoter, phorbol 12-myristate 13-acetate (PMA) is a potent inducer of differentiation in Hl-60 cells leading in 2-3 days to adherent macrophage-like cells. We have studied its effect on phospholipase C (PLC) activity. This molecule is shown to be able to inhibit PLC activity by 80% within 1 hour. This inhibition by PMA occurs on the basal , on the GTPγS-stimulated and on the Ca^{2+}-stimulated PLC activity indicating that the tumor promoter acts on the enzyme PLC itself.

We have tested the effect of two different PKC inhibitors on PLC activity. Cells were treated with either protein H7 or staurosporine then, PMA was added. In such conditions, PMA-induced PLC inhibition was prevented by both PKC inhibitors. Moreover, we found that PMA did not modify PLC activity in an HL-60 subline resistant to PMA.

We observed that concentrations of PMA which affect the PLC activity are those which induce differentiation of HL-60 cells. We also found a relationship between the differentiating potency of several phorbol esters and their inhibiting effect on PLC activity.

These results indicate 1) that the effect of PMA on PLC activity is likely to be secondary to PKC activation and 2) that the inhibition of PLC might be an important step in the induction of differentiation.

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UCN-01 and calphostin (UCN1028), microbial product inhibitors of protein kinase; Selective inhibition *in vitro* and antitumor activity *in vivo*.

Nakano H., Kobayashi E., Takahashi I., Takahashi K., Akinaga S., Azuma M., Morimoto M. & Tamaoki T.
Tokyo Res. Lab. & Phamaceutical Res. Lab., Kyowa Hakko Co. Ltd., Machida, Tokyo, Japan

In the course of screening of microbial product inhibitors of protein kinase C, we have found :

1. Staurosporine, an alkaloid from *streptomyces*, is a potent inhibitor of protein kinases (IC50; 2.7nM for C-kinase, 8.2 nM for A-kinase, 6.4 nM for p60 v-*src* kinase, 630 nM for tyrosine kinase of EGF receptor).
2. UCN-01 (7-hydroxy staurosporine), isolated from *streptomyces*, is a selective inhibitor of C-kinase (IC50; 4.1 nM for C-kinase, 42 nM for A-kinase, 45nM for p60 v-*src* kinase).
3. Calphostin(UCN1028), isolated from fungus *Cladosporium*, is a specific inhibitor of C-kinase. (IC50 of calphostin C; 50 nM for C-kinase. >50 μM for A-kinase and p60 v- *src* kinase).
4. The regulatory domain of C-kinase is the target of calphostin whereas the catalytic domain of protein kinases are targets of UCN-01 and staurosporine.

We have examined antitumor activity of the potent and selective inhibitor of protein kinase C with murine tumor models and human tumor xenografts. UCN-01 showed antitumor activity *in vivo* against p388 murine leukemia model and other models including transformed cells with oncogene while staurosporine had not antitumor activity against *in vivo* models so far tested. Calphostin C was also cytotoxic to transformed cells and showed antitumor activity *in vivo*.

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Effects of anthracyclines on signalling pathways related to protein kinase C activation. Lanzi C., Banfi P., Franzi A., Guazzoni L., Perego P., Gambetta R.A. Istituto Nazionale Tumori, Milano, Italy.

The antitumor anthracycline antibiotics are known to bind negative phospholipids and to affect cell membrane structure and functions. We have used different model systems to evaluate the impact of doxorubicin (DX) and daunorubicin (DN) on pathways which involve the calcium and phospholipid-dependent protein kinase (PKC). In a previous study we have shown that DX and DN have different effects on PKC activation in human platelets (Biochem. Pharmacol. 337, 3497, 1988). Here, we report that, in ^{32}P-labeled platelets, DX also induced phosphatidic acid production and the exixtence of a correlation between anthracycline-induced PKC activation, as shown by 40K protein phosphorylation, and lipid peroxidation, as measured by the TBA assay. In Swiss/3T3 fibroblasts either DX ($\geq 10^{-4}$M) and DN ($\geq 10^{-5}$M) induced phosphorylation of the PKC substrate 80K protein. As PKC has been implicated in epidermal growth factor receptor transmodulation, we assayed the effect of DX on tyrosine kinase activity of this receptor by western blotting using a polyclonal anti-phosphotyrosine antibody. In Swiss/3T3 cells DX markedly reduced EGF-induced autophosphorylation of the EGF receptor while in A431 human carcinoma cells the anthracycline inhibits either basal and EGF-induced tyrosine autophosphorylation of the receptor. These effects are evident at DX concentrations in the nanomolar range. The results suggest that signal transduction pathways could be one of the targets for the anthracycline action.

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Phosphatidylinositol 4,5-bisphosphate competes with phorbolester for binding to protein kinase C. Chauhan, V.P.S., Chauhan, A. and Brockerhoff, H. N.Y.S. Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Rd., Staten Island, New York, 10314

Recent evidence (BBRC 155, 18 (1988) that phosphatidylinositol 4,5-bisphosphate (PIP_2) may function as primary activator of calcium-phospholipid dependent protein kinase C (PKC) suggests that PIP_2 plays an important role in signal transduction other than generating two intracellular messengers i.e. inositol trisphosphate (IP_3) and diacylglycerol (DG). A further demonstration of its role as PKC effector was obtained in experiments employing phorbol ester as ligand. Scatchard analysis showed that PIP_2 competitively displaced phorbol ester from the kinase in both triton-phosphatidylserine (PS) micellar system and liposomal system. The plot of apparent dissociation constant (K_d) against PIP_2 concentration was linear over a range of 0.01-1 mol% and confirmed the competitive nature of inhibition between PDBu and PIP_2. In the presence of PS and Ca^{2+}, PIP_2 like DG and phorbol esters could also translocate PKC from cytosol to the membrane in a dose-dependent manner. Phosphatidylinositol (PI) and PI-5-phosphate (PIP) also competed with phorbol ester for binding to PKC, though less efficiently. PI and PIP are, however, incapable of activating the kinase. Thus, all phosphoinositides can bond at the effector-binding region of the regulatory moiety of PKC, but only the inositol 5-phosphate group turns the lipids into activator. These findings support a role of inositide shuttle, PI $\rightleftharpoons$ PIP $\rightleftharpoons$ PIP_2 in the control of protein phosphorylation.

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Peripheral blood lymphocytes (PBL) of steroid-dependent brain tumor patients may have defective second messenger systems. McVicar D.W., Merchant R.E. Young H.F. Depts. Anatomy and Surgery, Virginia Commonwealth University, Richmond, VA, 23298.

Brain tumor patients receive relatively high doses of dexamethasone for the control of cerebral edema. This therapy is often prolonged and the peripheral lymphocytes of these patients exhibit a significant depression in their mitogenic response to lectin or antigenic stimulation. As dexamethasone has been shown to bind to, and induce the translocation of PKC in some cells, it is feasible that this chronic translocation has depleted the lymphocytes reserve of functional PKC. This study evaluates the second messenger systems of PBL isolated from patients receiving chronic steroid therapy through differential stimulation of PKC and/or induction of high intracellular Ca^{2+} levels using phorbol 12,13-dibutyrate (PDBu) and ionomycin (Io), respectively. The PBL of 6 patients, 4 with malignant glioma and 2 with metastatic melanoma, and 5 normals were tested. PBL were suspended in serum-free X Vivo 10 and pipetted, in triplicate, into 96 well plates at a density of $1.5X10^4$ cells/well. Recombinant interleukin-2 (rIL-2, 1000U/ml, Hoffmann-La Roche) and various other stimuli were added to each well bringing the final volume to 200ul/well. After a 42 h incubation at 37°, each well was pulsed with ^{3}H-thymidine for an additional 6 hours, the cells were harvested onto glass fiber filters and ^{3}H-thymidine incorporation was evaluated. The results show that the PBL of patients had a significantly lower mitogenic response to the lectin PHA than did normal controls. In addition, the patient's PBL exhibited a slightly reduced response to stimulation with PDBu (10^{-8}M) plus Io ($5X10^{-7}$M) and were more susceptible to over stimulation when all three agents (PHA, PDBu, and Io) were added together. Neither the addition of PDBu at 10^{-9}, 10^{-8}, or 10^{-7}M, or Io at $5X10^{-8}$, $5X10^{-7}$, or $5X10^{-6}$M, could restore the patients PHA response to the level of the normals. While the response of the patients' PBL to Io alone was similar to that of the normals, PBL of normal donors demonstrated a dose dependent increase in proliferation in response to stimulation with PDBu alone and the PBL of the patients did not. Both the group of patients with primary brain tumor and those with metastatic lesions were tested with similar results. Therefore, it appears that the histological type or grade of tumor has little effect on the observed abnormalities. All the patients were receiving anticonvulsants, but no connection between PKC and these drugs has been established. These data and observations suggest that prolonged treatment of PBL with corticosteroids results in the loss of PKC function or a reduction in the availability of that PKC to be bound and activated by PDBu.

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Lipid-specific phospholipase C activity is reduced by retinoic acid in HL-60 cells. An early step in induction of differentiation. Geny B., Cost H., Barreau P., Abita J. P., and *Cockcroft S. INSERM 204, Hopital Saint Louis 75475 Paris FRANCE, and * UCL, Experimental Pathology, London. U.K.

We have shown that phospholipase C (PLC) is inhibited by PMA, after this molecule has activated protein kinase C (PKC). It is likely that PKC leads to PLC phosphorylation and hence to its inactivation.

Retinoic acid (RA), which provokes the differentiation of HL-60 cells towards granulocytes-like cells, also inhibits by 70% within 1 hour PLC activity. However, RA acts on PLC activity through a different pathway. We have found that this molecule modifies neiter PLC basal activity nor Ca^{2+}-stimulated PLC activity. RA only inhibits GTPγS- or NaF-stimulated activity, indicating that this drug would modify the coupling of the specific G-protein (Gp) to phospholipase C.

The relationship between the decrease in the GTPγS-stimulated PLC activity and the process of differentiation was studied by two ways. 1°) Another retinoid, RO 10-1670, a poor inducer of differentiation, provokes a small inhibition of PLC acitivity. 2°) In a retinoic acid resistant HL-60 subline, PLC acivity cannot be inhibited with this drug whereas PMA leads to an important decrease in PLC activity.

These results indicate that retinoic acid provokes a rapid inhibition of PLC activity through a decrease in the enzyme stimulation by the Gp and that this decrease in PLC activity is an early event related to differentiation.

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Membrane events associated with adriamycin-induced differentiation of the leukaemia cell line K562.
Hoffman R.M. & Newlands E.S. Dept of Medical Oncology, Charing Cross Hospital, Fulham Palace Road, London W6 8RF.

In some cell lines, adriamycin increases inositol lipid turnover resulting in increased protein kinase C (PKC) activity. These processes are associated with the differentiation of HL60 cells, but the role of inositol lipids in erythroid differentiation of K562 cells is unclear since K562 cells do not differentiate in response to phorbol 12-myristate 13-acetate (PMA), an activator or PKC. Adriamycin (0.1uM) was cytostatic to K562 cells and induced differentiation in 44% of cells. 1-(5-Isoquinolinylsulphonyl)-2-methylpiperazine (H7) inhibited K562 PKC (IC50=30uM) but caused less than 50% inhibition of proliferation and adriamycin-induced differentiation at concentrations up to 600uM. The PKC activator dioctanoyl-sn-glycerol did not induce K562 differentiation either alone or in combination with the calcium ionophore A23187. Modulators of inositol lipid turnover such as lithium chloride and neomycin failed to prevent adriamycin-induced differentiation. DMSO at non toxic concentrations (1%) blocked adriamycin-induced differentiation. DMSO alone did not induce differentiation. The protein kinase inhibitor quercetin (50uM) also blocked adriamycin-induced differentiation. These results suggest that PKC activity is not important for adriamycin-induced differentiation, but they do not rule out a role for other membrane events.

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Modulation of cellular differentiation associated with membrane fatty acid modification.
Burns C.P., Petersen E.S., North J.A., and Ingraham L.M. Dept. Medicine, Univ. of Iowa, Iowa City, IA 52242.

The effect of fatty acids(FA) on the linked processes of cellular differentiation and growth arrest was studied in the HL-60 human leukemia cell line using a membrane lipid modification model. HL-60 cells were grown in RPMI 1640/10% fetal bovine serum supplemented with six FA of diverse structure containing 1-6 double bonds. Gas chromatographic analysis of phospholipid extracts of HL-60 cells revealed enrichment with the supplemented FA or its 2-carbon elongation product (18:1ω9 36%, 18:3ω3 8%, 20:4ω6 19%, 20:5ω3 16%, 22:4ω6 8%, 22:6ω3 10%). Retinoic acid(RA)-induced differentiation was measured in cells enriched with each FA using phorbol ester-induced nitroblue tetrazolium reduction, superoxide generation and growth arrest. Unmodified control cells and 18:1-enriched cells showed oxidative burst activity and $G_{1/0}$-growth arrest beginning at 48h after the addition of RA. In contrast, 22:6-enriched cells exhibited increased oxidative activity and growth arrest as early as 24h which is equivalent to about one cell cycle time. The augmentation could be demonstrated after 1-120h exposure to the FA. Cells enriched with the other FA showed less or no augmentation. The addition of the antioxidant butylated hydroxytoluene(BHT) further accelerated differentiation in a concentration and time dependent manner, but other antioxidants and various metabolic inhibitors(ascorbic acid, ibuprofen, indomethacin, aspirin, 4-pentenoic acid) had variable effects. We conclude that the rate and extent of HL-60 cellular differentiation can be modulated by membrane components.

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Physiological cAMP stimulation and oncogene expression in HL-60 cells.
Aarbakke J., Moens U and Bang B.E. Institute of Medical Biology, University of Tromsø, N-9001 Tromsø, Norway.

The rate of transcription of a number of genes, expressed in tissues which are responsive to hormones or regulatory factors, is rapidly altered by cAMP (reviewed in J Biol Chem 263:9063-9066, 1988). The promoter-regulatory regions of several of these genes have been isolated and cAMP responsive elements (CRE) have been characterized with the consensus sequence TGACGTCA (Proc Natl Acad Sci 83:6682-6686, 1986). The oncogene c-fos encodes a nuclear protein that exhibits DNA-binding activity in vitro and has been proposed to act as a nuclear intermediary in the transduction and translation of cell surface signals at the level of gene expression (Science 234:192-196, 1987). c-fos belongs to a class of genes containing CRE and which transcription is altered by cAMP. Since the mRNA of c-fos has a short half-life, it is possible to detect changes in the rate of gene transcription shortly after changing cAMP levels. Expression of c-fos is associated with HL-60 growth and differentiation (Science 237:854-856, 1987; Cell 45:497-504, 1988). It is unknown, however, whether an increase in cAMP mediated via stimulation of the physiological beta-adrenergic/adenylate cyclase pathway affects oncogene transcription in the cell line. We recently demonstrated beta-2-adrenergic receptors functionally coupled to adenylate cyclase in the promyelocytic cell line HL-60 (J Leuk Biol 44:41-45, 1988). Stimulation of the cells with isoproterenol caused a rapid increase in cAMP levels. We herein report that theophylline (4mM) or theophylline (4mM) plus isoproterenol (10μM) increased basal cAMP concentrations from 0.4 pmol/10^6 cells to 0.8 and 7.3 pmol/10^6 cells, respectively. These changes were associated with a similar, marked reduction in c-fos transcription as measured by Northern blot analysis 30 to 60 min after drug addition.

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The induction of cell death by nitrogen mustard - direct or indirect damage to the cell membrane?
Nardone, A., Wilcock C. and Hickman, J.A. CRC Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET.

Nitrogen mustard (bis(2-chloroethyl)methylamine, HN2) is a prototypical cytotoxic bifunctional alkylating agent which is capable of covalently crosslinking cellular target molecules, such as DNA. Although the formation of DNA interstrand crosslinks has been demonstrated in HN2-sensitive cells, there is no clear explanation of why the cells go on to die. HN2 selectively inhibited the activity of a $Na^+K^+Cl^-$ cotransporter in L1210 leukemia cells (Wilcock et al BBA, 946, 368, 1988). This inhibition was concentration and time dependent, so that a selective inhibition of the L1210 $Na^+K^+Cl^-$cotransporter by 10 μM HN2 (>3 log cell kill) was not observed until after 3 h. At this time, K^+ influx was inhibited but intracellular [K^+] was maintained (138 mM) by a fall in cell volume. It has been reported previously that high concentrations of HN2 inhibit K^+ influx in a non-specific manner. In human erythrocytes, no inhibition of K^+ influx was observed with 100 μM HN2, even after 4 hours, and at higher concentrations (2 mM) the 50% inhibition of K^+ influx was observed to be non-specific, with equal inhibition of the ouabain-sensitive and insensitive fractions of K^+ influx. This raises the possibility that the selective inhibition of the co-transporter by HN2 (<10 μM) is via an indirect mechanism and might represent a (lethal?) response of the cells to damage.