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*Review*

# Cell-signaling targets for antitumour drug development*

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## Introduction: Cancer as a disease of all signaling

The proliferation and differentiation of normal cells is tightly regulated by a balance between the action of the growth-stimulatory proto-oncogenes and the growth-inhibitory tumour-suppressor genes. This balance is upset by the over-expression or mutation of the oncoprotein products of oncogenes or by the inactivation of tumour-suppressor genes [142]. Both kinds of genetic changes are now known to be essential for the development of the full malignant phenotype. The sequence of multiple changes has been particularly well documented in the skin [10] and colon [41]. Moreover, genes involved in angiogenesis and metastasis are now being identified [84].

In the consideration of possible new targets for the design of more innovative anticancer agents, oncogenes, tumour-suppressor genes and their products are extremely attractive. It is now clear that most, if not all, products of the various kinds of dominantly acting cancer genes are components of cellular signaling pathways (Table 1). For example, mutated *ras* genes are found in many human cancers, where the oncogene encodes an abnormal, transforming variant of a low-molecular-weight G-protein. Enhanced expression of c-*sis* is also found in many human cancers such as sarcomas and glioblastomas. The oncogene product of c-*sis* is analogous to the B chain of platelet-derived growth factor (PDGF). Increased expression or mutation of key signaling elements, including growth factors and their receptors, can result in constitutive activation of the signaling pathways employed by normal cells in autocrine and paracrine mitogenic stimulation [1]. The malignant cell thus behaves like a normal cell undergoing continuous mitogenic stimulation and is frequently independent of external mitogenic growth factors. Unrestrained growth is an inevitable consequence.

**Table 1.** Some oncogene products and their cellular functions

| Oncogene | Function |
|---|---|
| Growth factors: | |
| *fgf*-5 | FGF-like growth factor |
| *hst* | FGF-like growth factor |
| *int*-2 | FGF-like growth factor |
| *sis* | B-chain PDGF |
| Protein tyrosine kinases – receptor-associated: | |
| *erb*B | Truncated EGF receptor |
| *fms* | CSF-1 receptor |
| *kit* | Truncated stem-cell receptor |
| Protein tyrosine kinases – non-receptor-associated: | |
| *abl* | |
| *lck* | |
| *src* | |
| *yes* | |
| Protein serine/threonine kinases: | |
| *mos* | |
| *pim*-1 | |
| *raf* | |
| G-proteins: | GTP |
| *ras* | GTP-binding protein |
| Nuclear oncogenes: | |
| *fos* | Combines with c-*jun* product to form AP-1 transcription factor |
| *jun* | Sequence-specific DNA-binding protein |
| *myb* | Sequence-specific DNA-binding protein |
| *myc* | Sequence-specific DNA-binding protein |

Recent developments indicate that the recessive tumour-suppressor gene products are components of signal-transduction pathways by which the cell processes autocrine or paracrine growth-inhibitory signals [142]. The cell may respond to such negative signals by arresting growth in the G1 phase or by engaging programmes that result in differentiation, senescence or apoptosis. The loss or mutation of such genes therefore results in a growth advantage. The deleted-in-colon-cancer gene (*DCC*) is a 190-kDa transmembrane phosphoprotein that is likely to

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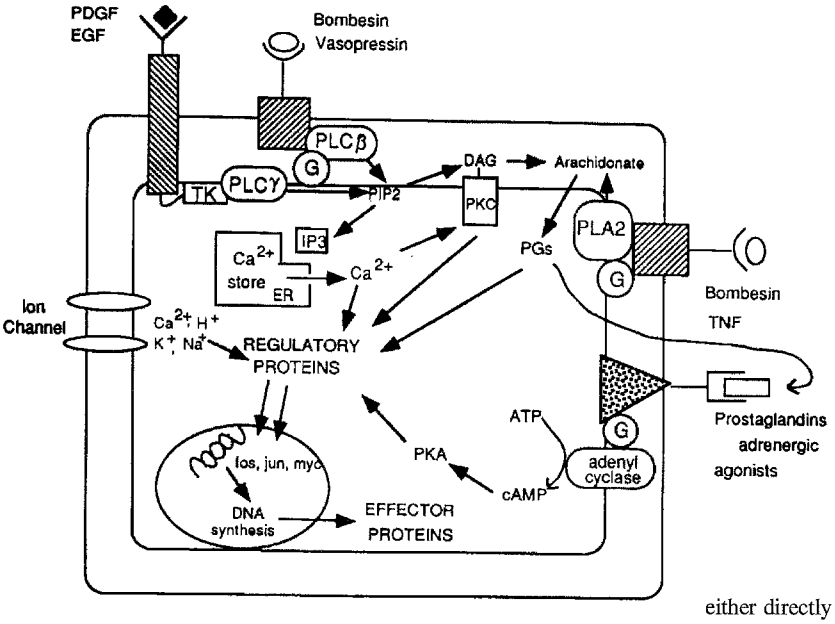


**Fig. 1.** Cellular signaling pathways. Growth factors such as PDGF and EGF bind to specific receptors with intrinsic cytosolic tyrosine kinase domains. PLCγ associates with the activated receptor, and hydrolysis of $PIP_2$ to $IP_3$ and DAG follows. Other mitogens such as bombesin also activate this hydrolysis pathway. However, the bombesin receptor is G protein-linked and probably associates with PLCβ. Bombesin stimulation is also known to induce phosphorylation of certain as yet unidentified cellular proteins, suggesting a tyrosine kinase pathway for bombesin signaling. The $IP_3$ formed moves from the membrane and binds to specific receptors on the ER, resulting in calcium release. The increase in intracellular calcium in turn modulates the activity of certain regulatory proteins. DAG activates PKC, whose activity is also regulated by calcium levels. PKC phosphorylates many proteins, leading to kinase cascade systems that have not been fully identified. PKC down-regulates receptors such as the EGF receptor. Bombesin receptors are also linked through G proteins to $PLA_2$, which releases arachidonate from membrane lipids. Arachidonate is then converted into prostaglandins, which either directly regulate cytosolic signaling events or are transported out of the cell. PGs can then bind to specific PG receptors that stimulate adenyl cyclase, so increasing cAMP levels, which results in activation of PKA. Another source of arachidonate is DAG. Further early responses following activation by mitogens include increases in ion fluxes across the plasma membrane and induction of c-*fos* and c-*myc* expression in the nucleus. Although the sequence of events outlined above leads to cell proliferation, activation of other effector proteins in a similar way also controls differentiation and apoptotic pathways. *Abbreviations used: DAG*, diacylglycerol; *ER*, endoplasmic reticulum; *EGF*, epidermal growth factor; *G*, G protein; *$IP_3$*, inositol (1,4,5)-trisphosphate; *PDGF*, platelet-derived growth factor; *PG*, prostaglandin; *$PIP_2$*, phosphatidylinositol 4,5-diphosphate; *PKA*, protein kinase A; *PKC*, protein kinase C; *PLC*, phosphoinositide-specific phospholipase C; *TK*, tyrosine kinase; *TNF*, tumour necrosis factor. Note that tyrosine kinase-linked receptors are shown as monomers for simplicity (see text). Other signals downstream of tyrosine kinase-linked receptors are shown in Fig. 2 and additional lipid pathways are illustrated in Fig. 3.

be a signal-transducing receptor [42]. The neurofibromatosis tumour-suppressor gene (*NF1*) resembles proteins such as guanosine triphosphatase (GTPase)-activating protein (GAP), which interact with the $p21^{ras}$ oncoproteins [151]. The retinoblastoma gene (*RB*) encodes a 105-kDa phosphoprotein that binds to DNA and is probably involved in transcription regulation [80] and plays an important role in cell-cycle progression [110a].

Due to the diverse nature of the signal-transduction pathways there are many possible points of pharmacological intervention, from inhibiting growth-factor binding through interference with the phosphorylation of regulatory proteins and transcription factors via specific protein kinases and phosphatases. For an introduction to this new area of cancer pharmacology the reader is referred to previous reviews [53, 101, 102, 134, 149, 149a]. Here we examine many of the sites for drug hunting in the area of cancer-cell signaling, starting with the cell membrane and moving through the cytoplasm to the nucleus (Fig. 1). Following the interaction of a ligand with its receptor there are several possible signaling pathways. The initial response is transduced either directly via a receptor protein tyrosine kinase (PTK) or through activation of G-protein-linked, membrane-bound enzymes such as phospholipase C (PLC) or adenyl cyclase. These are distinct pathways, but at the level of gene expression the different mitogenic pathways share a precocious induction of c-*fos* and c-*myc* expression, although striking kinetic differences are observed, which suggest independent controls of proto-oncogene expression [108].

Although the main focus of this review is on prospects for the pharmacological inhibition of signals for proliferation and transformation in cancer cells, there are clearly also opportunities for intervention with similar or related pathways for differentiation, apoptosis, angiogenesis and metastasis.

## Interaction with growth factors and their receptors

The first point of intervention in the signaling cascade is at the level of the receptor. Inhibition of ligand binding can be achieved by two methods: disabling the ligand or blocking the ligand-binding site on the cognate receptor. A related approach that is mentioned here is the use of receptor ligands to target cytotoxic drugs.

*Suramin*

Suramin, a polysulphonated naphthylurea, was first introduced for the treatment of African trypanosomiasis. The demonstration of its potent reverse transcriptase inhibition and anti-human immunodeficiency virus (HIV) activity in vitro led to its clinical trial in patients with acquired immune deficiency syndrome (AIDS) [28]. It was here that a response was seen in an HIV-associated lymphoma, and subsequent studies confirmed activity in Kaposi's sarcoma [127]. Because of its toxicity against the normal gland, the subsequent clinical studies were planned for adrenal carcinoma, where activity was also seen, but as experience was accumulated and the drug's mechanism of action became clearer, this was expanded to other tumour types [127].

Suramin inhibits the binding of certain growth factors, such as epidermal growth factor (EGF), transforming growth factor α (TGFα), Kaposi's sarcoma-derived fibroblast growth factor (K-FGF) and PDGF, to their respective receptors. Suramin binds directly to various growth-factor molecules. This property has been linked to its ability to inhibit the growth of certain tumour cell lines in vitro [17, 35, 66, 77, 125]. However, presumably due to the polyanionic nature of the compound, suramin can also bind non-specifically with a wide range of proteins and therefore interacts with a large number of effector systems [77, 85]. For example, inhibition of inositol 1,4,5-trisphosphate ($IP_3$)-induced $Ca^{2+}$ release by suramin has been postulated to be due to its interference with the $IP_3$ receptor on the endoplasmic reticulum [116]. Inhibition of the putative membrane phosphatidic acid receptor has been observed (Moolenar W, personal communication). Suramin also inhibits the crucial signaling enzyme protein kinase C (PKC) [85] and exhibits an unusual form of interaction with topoisomerase [21c].

Clinical trials of suramin as an anticancer agent have been promising and responses have been seen in prostatic and ovarian carcinoma [6, 127, 139]. There is, however, a high level of toxicity, which may be a reflection of the relatively non-specific nature of the binding of this compound to various proteins. This could be unrelated to its effects on growth-factor binding. On the other hand, it appears possible to minimise toxicity by careful attention to pharmacokinetics [113]. Other polysulphates such as the heparin analogue pentosan polysulphate have also shown antitumour activity, inhibiting the K-FGF-dependent growth of a human adrenal carcinoma cell line both in vitro and in vivo, whereas heparin remained inactive [143]. This was achieved at a sufficiently low dose to prevent heparin-like in vivo toxicity and is postulated to be due to the binding of pentosan polysulphate to K-FGF, thus preventing interaction with its receptor. Various analogues and related agents are under development.

An interesting feature of suramin and related molecules is their ability to antagonise the binding of growth factors to the extracellular matrix components of tumours, thereby altering the local presentation of stimulatory (and potentially inhibitory) factors to the tumour cells. Such effects may contribute to both their antiproliferative and their anti-angiogenic activity.

*Neuropeptide-receptor antagonists*

Small-cell lung-cancer (SCLC) tumours secrete multiple mitogenic neuropeptides and hormones. These include bombesin-related peptides such as gastrin-releasing peptide, adrenocorticotrophin, vasopressin and substance P [146]. Receptors for these ligands are also expressed in SCLC. Bombesin-like peptides are thought to act as autocrine growth factors in SCLC, and for this reason specific bombesin receptor antagonists were developed [148]. Some early success was achieved using these substance-P analogues to antagonise bombesin-driven growth of SCLC lines in vitro and also with an anti-bombesin antibody against human SCLC xenografts in immune-suppressed mice [34]. However, it was soon realised that these would have limited use in the treatment of SCLC, as there is increasing evidence to suggest that the growth of such tumours is regulated by multiple autocrine and paracrine interactions, a phenomenon referred to as redundancy or degeneracy in signal transduction. Redundancy in mitogenic neuropeptide signaling certainly appears to undermine attempts to inhibit SCLC growth by a single neuropeptide-receptor antagonist. On the other hand, several substance-P analogues were found to inhibit simultaneously the mitogenic effects of a number of neuropeptides in Swiss 3T3 cells and to block the growth of SCLC in vitro [147]. Such broad-spectrum antagonists would appear to be more effective and useful antiproliferative agents in SCLC than antagonists specific to a single receptor type. Two of these are being developed for phase I clinical trial in the United Kingdom.

Recently a bombesin antagonist has also been shown to inhibit the growth of human colon-cancer xenografts in nude mice [105] and to antagonise the growth of nitrosamine-induced pancreatic cancers in hamsters when used in combination with a somatostatin analogue [129]. This suggests that such agents may have a wider role than was originally envisaged.

*Somatostatin analogues*

The cyclic tetradecapeptide somatostatin, acting via its own receptors, has a broad spectrum of biological actions, exerts growth-suppressive effects on a large variety of cells and appears to be an endogenous growth inhibitor. The therapeutic use of somatostatin is limited because of its multiple actions and extremely short half-life. Several somatostatin peptide analogues with enhanced selectivity and greater stability than the parent compound have been designed. These compounds have been clinically successful in prostatic and endocrine pancreatic tumours and are thought to work through multiple mechanisms [112]. In a human pancreatic cancer cell line, somatostatin analogues inhibited EGF-stimulated growth [83]. The somatostatin analogues stimulate the tyrosine phosphatase activity of the somatostatin receptor. The increase in tyrosine phosphatase activity inhibits the EGF-induced phosphorylation of tyrosine residues on the EGF receptor, which is essential for the growth-stimulatory effects of EGF. The reduction in phosphorylation correlates with the decrease

in pancreatic tumour size induced by a somatostatin analogue in vivo [97]. It is possible that somatostatin and its analogues could similarly inhibit the action of other endogenous growth factors that are regulated by phosphorylation status (see phosphatase section).

*EGF-receptor strategies*

The discovery that the viral oncogene product of v-*erb*B is a truncated analogue of the gene encoding the EGF receptor (also known as HER1) provided the first functional mechanism for a genetic abnormality in a human cancer. Although the truncated EGF receptor, which does not require ligand binding for activation, is not found in human cancers, overexpression of the EGF receptor is observed in various types of human tumours, including breast cancer, squamous carcinomas and gliomas [22, 30, 61, 81]. A related gene known as c-*erb*B-2 (or HER2), which encodes the rat neu oncoprotein homologue, is highly homologous to the EGF receptor. Both the EGF receptor and c-erbB-2 are often amplified and over-expressed in human breast adenocarcinomas, a finding associated with a poor prognosis [124]. Ligands for the EGF receptor include EGF, TGF α and amphiregulin [133], and the likely ligand for c-erbB-2 is heregulin [63a].

Monoclonal antibodies raised against the extracellular domain of the EGF receptor have been shown to inhibit the growth of squamous cancer cell lines and to retard their growth as xenografts [2, 87]. Phase I clinical trials with anti-EGF-receptor monoclonal antibodies have been undertaken in patients with squamous-cell lung carcinoma [37]. A monoclonal antibody raised against the extracellular epitope of the c-erbB-2 protein inhibits the in vitro proliferation of human breast-tumour cell lines that overexpress c-erbB-2 [66c].

Such antibodies can also be used as delivery vehicles. A monoclonal antibody against the EGF receptor, conjugated with doxorubicin, was shown to be an efficient carrier of this cytotoxic drug to tumour sites that express high receptor levels [3]. This may allow lower doses of the cytotoxic agent to be used, thus minimising cytotoxicity. A similar strategy has been taken by conjugating *Pseudomonas* exotoxin to the extracellular ligands for the EGF receptor, EGF and TGF α. Many tumour cell lines are sensitive to the chimeric toxins, which have yielded promising results when tested on human tumour xenografts in nude mice [59]. The growth factor itself could also be used to deliver drugs or radioisotopes. Particular specificity would be anticipated for antibodies that recognise mutated epitopes.

## Protein tyrosine kinases and SH domains

PTKs phosphorylate tyrosine residues (rather than serine or threonine) in target proteins, thereby altering their activity. There are two main groups of PTKs, those linked to growth-factor receptors that have a single hydrophobic transmembrane domain and the non-receptor PTKs such as the src family. The metabolic roles played by the non-receptor PTKs are poorly understood, and research

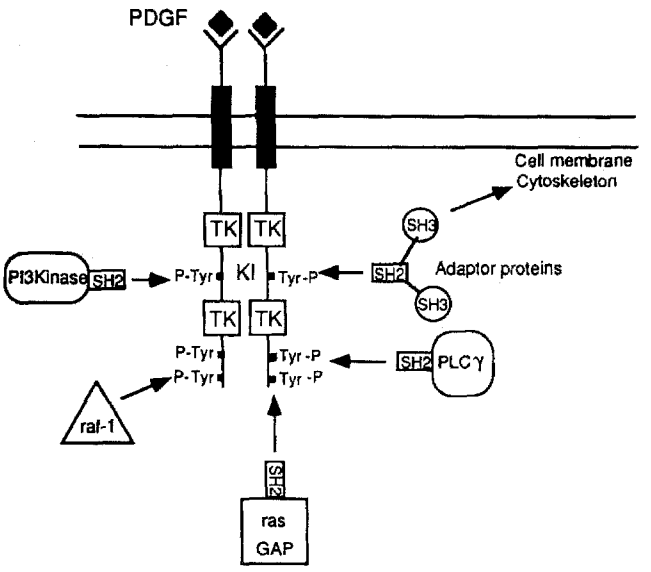


**Fig. 2.** Cellular signaling at protein tyrosine kinase receptors, using PDGF as a prototype. Ligand binding to the extracellular ligand-binding domain induces a conformational change that results in receptor dimerisation. This allows adjacent cytosolic domains within a receptor dimer to cross-phosphorylate each other on tyrosine residues and enhances the intrinsic tyrosine kinase activity of the receptor towards other cellular substrates. The multiple tyrosine-phosphorylated sites on the receptor provide specific binding sites for these cytosolic proteins, such as PLCγ, PI3kinase and ras-GAP, by virtue of their SH2 domains. In the case of raf-1, there is no SH2 domain and this enzyme is mainly phosphorylated on serine. Binding of these proteins to the activated receptor acts to increase their catalytic potential. The kinase-insert domain is unique to the PDGF receptor and it is a phosphorylated tyrosine residue in this region that is responsible for the binding of PI3kinase. Conclusive proof of the specific binding sites for the other substrates has not yet been obtained, but it is believed not to be within the kinase-insert domain. The increased activity of these cytosolic regulatory proteins then dictates the passage of events to the nucleus. Adaptor proteins that contain SH2 and SH3 domains but no catalytic domain can also associate with phosphorylated tyrosine residues. They may facilitate the association of other SH2-containing proteins to the activated receptor. *Abbreviations used: PDGF*, platelet-derived growth factor; *PLC*, phosphoinositide-specific phospholipase C; *PI3kinase*, phosphatidylinositol-3′-kinase; *KI*, kinase insert; ras GAP, ras-GTPase-activating protein; *SH2, src* homology 2 domain; *TK*, tyrosine kinase

into PTK inhibitors has therefore focused mainly on the membrane-bound growth-factor-receptor PTKs. There is increasing evidence that the PTK activity intrinsic to the growth-factor receptors is essential for the induction of mitogenic stimulation by growth factors such as EGF. For example, mutations that disable the tyrosine kinase activity of the receptor result in an inhibition of the signal-transduction pathways leading to cellular proliferation [24].

Activation of the receptor tyrosine kinase following ligand binding involves receptor dimerisation [153] and autophosphorylation of the EGF-receptor dimer. This leads to the phosphorylation of a set of cytoplasmic proteins such as phosphoinositide-specific PLC, ras GTPase-activating protein (GAP), phosphatidylinositol 3′-kinase (PI-3′-kinase) and the serine-threonine kinase $p74^{raf}$ (or raf-1), all of which may directly regulate intracellular signal-transduction pathways (Fig. 2). These proteins, apart from $p74^{raf}$, contain conserved sequences of about 100 amino

acids, called src homology region 2 (SH2) domains, as they were first identified in non-receptor PTKs like src. These SH2 domains have been shown to bind to activated growth-factor receptors such as EGF and PDGF, providing a common mechanism by which functionally diverse regulatory proteins can physically associate with the same receptor [76]. Studies using nuclear magnetic resonance spectroscopy and X-ray crystallography are now elucidating the structure of these SH2 domains, raising the possibility of designing specific inhibitors [97b]. It seems that the molecular recognition potential of the different but related SH2 domains for specific phosphorylated tyrosine residues in the cytoplasmic domains of various PTK receptors is a major mechanism by which a varied repertoire of signaling responses can be achieved from a limited number of downstream effector proteins.

Proteins with SH2 domains often have accompanying SH3 domains. These are regions of approximately 50 amino acids whose function remains unknown, although they are found in cytoskeletal proteins such as spectrin [76]. It may be that the combination of SH2 and SH3 domains on proteins such as the PDGF receptor leads not only to the activation of specific signal-transduction pathways following ligand binding but also to regulation of the cytoskeleton.

Recently, in *Caenorhabditis elegans* a new gene *sem*-5 was identified that encodes a protein consisting of two SH3 domains and one SH2 domain. However, it lacks a catalytic domain [27]. Other proteins similar to sem-5 that contain SH2 and SH3 domains but lack a catalytic domain include v-crk and nck. These adaptor proteins can bind phosphotyrosine-containing proteins and may facilitate the association of catalytic components of signaling pathways with their substrates and/or localise these components to their appropriate cellular sites of action. Such proteins may also represent interesting new therapeutic targets. However, the major current interest is focused on tyrosine kinase inhibitors [149b].

### *Adenosine triphosphate-competitive tyrosine kinase inhibitors*

The PTK activity of the first oncogene product to be discovered, the cytosolic protein $pp60^{v\text{-}src}$, was inhibited by the bioflavonoid quercetin [52]. However, quercetin and its analogues were found to be very toxic. These compounds are competitive inhibitors at the adenosine triphosphate (ATP)-binding site of the kinase [54]. As the ATP-binding site is a conserved structural region in all protein kinases, these agents would be expected to exhibit fairly minimal selectivity for particular kinases, including serine/threonine kinases in addition to tyrosine kinases. The reputed toxicity of these agents presumably is related to the inhibition of multiple kinases. Surprisingly, some degree of specificity has been reported for the isoflavone genistein. Inhibition of tyrosine kinases exceeded that of serine/threonine kinases such as cyclic adenosine monophosphate (cAMP)-dependent protein kinase and PKC [5]. More recently, flavone analogues have been developed that are competitive inhibitors of $p56^{lck}$ with respect to ATP binding, and these are highly selective over protein serine/threonine kinases [33].

### *Substrate-competitive tyrosine kinase inhibitors*

Another approach to the problem of selectivity is the development of PTK inhibitors that compete for the specific substrate-binding site of the different kinases. While increasing molecular selectivity, this approach should also limit toxicity. Umezawa and co-workers [137] identified a compound called erbstatin from the medium of *Streptomyces,* which inhibited the autophosphorylation of the EGF receptor in membranes of A431 epidermoid carcinoma cells, while being relatively ineffective at inhibiting cAMP-dependent protein kinase. Based on the structure of erbstatin, which clearly resembles that of tyrosine, a series of tyrosine kinase inhibitors termed tyrphostins have been developed. In this review we have classified all substrate-competitive tyrosine kinase inhibitors as tyrphostins, following the nomenclature of Levitzki and co-workers [47, 152]. Inhibitors have been designed that can distinguish between tyrosine kinase activity intrinsic to the EGF and PDGF receptors, c-erb-B2/neu kinase [20, 47, 48] and also the cytoplasmic PTKs such as $pp60^{c\text{-}src}$ [121]. These highly specific inhibitors might be extremely valuable where the malignant transformation is dependent on signaling through a single class of receptor. However, many of the compounds exhibit a broad spectrum of specificity. As it is becoming increasingly evident that malignant cells are under multiple growth controls, it is possible that these may prove to be the more effective antitumour agents. This may also circumvent the problem of growth-factor redundancy (see below). However, there will be concerns about effects on the insulin-receptor tyrosine kinase in the pancreas.

Many of the tyrphostins potently block cell proliferation in a way that correlates well with their potency to inhibit tyrosine kinases [152]. They are also ineffective inhibitors of other non-tyrosine protein kinases, which is reflected in their comparatively low toxicity [152]. At present these tyrosine kinase inhibitors have the disadvantage of being unstable in aqueous solution. Even when given intraperitoneally to mice bearing intraperitoneal tumours, erbstatin has shown antitumour activity only on its co-administration with foroxymithine, which chelates $Fe^{3+}$ ions [68]. Subsequent in vitro studies suggest that both serum proteins and ferric ions are required for the breakdown of erbstatin to an inactive compound. Recently a new tyrphostin was reported to inhibit the EGF-driven growth of a human squamous-cell carcinoma in vitro and to suppress the growth of the corresponding tumour in nude mice while increasing the life span of the animals [154]. This cell line expressed high levels of the EGF receptor and EGF stimulated its growth in vitro, suggesting a positive role for EGF in the control of mitogenesis in this system. The tyrphostins are also capable of inhibiting other signaling events downstream of EGF-receptor stimulation, such as phosphorylation of PLC, release of intracellular calcium [86] and phosphatidylinositol 4,5-diphosphate ($PIP_2$) breakdown [100].

## Phosphotyrosyl phosphatases

As with the PTKs, there are two classes of phosphotyrosyl phosphatases (PTPs): cytoplasmic PTPs, which are small soluble proteins, and receptor PTPs, which are large transmembrane proteins. SH2 domains have been identified on the soluble PTP1C isozyme and this was found to form a high-affinity complex with the activated EGF receptor and other phosphotyrosine-containing proteins [118]. This suggests that PTPs may selectively dephosphorylate tyrosine residues in response to changes in the cellular environment, analogous to but opposing the role of growth-factor-receptor PTKs.

Transformation of chicken fibroblasts with Rous sarcoma virus results in a 30%–50% increase in tyrosyl phosphatase activity [93], and the differentiation of HL60 leukaemic cells is accompanied by both an increase in tyrosine kinase activity and a larger increase in tyrosine phosphatase activity [44]. Although the precise details of phosphatase signaling remain to be established, the abundance of these enzymes in mammalian tissues suggests an important role that may be exploited for the development of antiproliferative agents. A likely scenario would involve the pharmacological stimulation of those phosphatases that promote growth-inhibitory signals. It seems probable that such phosphatases will function as tumour suppressors. For example, one of the receptor PTPs (PTPγ) has been proposed as a candidate tumour-suppressor gene at human chromosome region 3p21 [79]. However, it also appears likely that some phosphatases will transduce undesirable effects, in which case the development of inhibitors would be important.

We currently have such reagents as vanadate and okadaic acid, which can be used to inhibit PTPs and serine-threonine phosphatases, respectively, with varying specificity in the laboratory, and it seems very likely that potential drug candidates will emerge in this area. The antitumour agents gallium nitrate and suramin have been reported to act as phosphatase inhibitors [17a].

## G-proteins

The cascade initiated by mitogen-receptor binding may also be coupled to the generation of intracellular events by G-proteins, so called because they bind guanine nucleotides. G-proteins couple more than 70 receptors to enzymes such as adenyl cyclase and phospholipases as well as to ion channels. Mitogenic peptides such as bombesin and vasopressin fall into this category, having receptors with the classic seven transmembrane domains that pack into anti-parallel helical bundles. The high-molecular-weight G-proteins associated with these receptors are heterotrimers consisting of α,- β- and γ-subunits. Occupation of G-protein-linked receptors leads to binding of guanosine triphosphate (GTP) to the α-subunit and to its subsequent self-limiting hydrolysis. Multiple inhibitory and stimulatory G-proteins are known and some are uncoupled from the physiological response by toxin-catalysed adenosine diphosphate (ADP) ribosylation. However, there appears to have been little progress in the development of selective chemical inhibitors.

The second class of G-proteins have a lower relative molecular weight (20,000–30,000 Da) and are not associated directly with membrane receptors. There are numerous members of this large family, including the $p21^{ras}$ proteins. The cellular functions of the small-molecular-weight non-receptor G-proteins is less well understood, but it is believed that they function as molecular switches in signaling events of cell growth and differentiation [55]. The $p21^{ras}$ proteins bind the guanine nucleotides GTP and guanosine diphosphate (GDP) with high affinity and possess relatively low intrinsic GTPase activity. When GTP is bound, the ras proteins are in an 'active' state, whereas the GDP complexes comprise the 'inactive' state. The very low intrinsic GTPase activity of the normal cellular $p21^{ras}$ protein is accelerated by interaction with a specific cytosolic GTPase-activating protein, ras-GAP, which induces hydrolysis of GTP to GDP, thus acting as a negative regulator of ras function.

Single-point mutations of the *ras* genes converts them to their oncogenic form [130]. The mutated $p21^{ras}$ proteins are believed to play an important role in the development of cancer [11]. The activated *ras* genes have been identified in a wide variety of human neoplasms. For example, they occur in about 40% of human colon cancers and 95% of human pancreatic cancers. The activated *ras* oncogene product has a higher proportion of GTP-bound molecules than do normal ras proteins. Although the mutated protein binds GAP, there is no 'off' signal as the GTPase is not activated. Mutants that do not bind GAP are non-transforming and it thus appears that GAP is a downstream effector for oncogenic ras and probably also for normal ras. The NF1 protein appears to play a similar role [151]. The *ras* transformation of cells is not sufficient for the development of full malignancy, but the prolonged activation of the mutated ras proteins described above may confer a growth advantage on these cells and certainly contributes to the malignant phenotype.

Strategies for antagonists of $p21^{ras}$ transformation involve inhibition of the physical interaction between ras and its downstream effectors GAP and NF1. GAP and NF1 in turn have further downstream effectors which will be targets for therapy [51a]. Restoration of GTPase stimulation would also present a viable target and interference with guanine nucleotide exchange factors is emerging as a potential approach. The problem with the strategies aimed directly at the ras p21 protein is that the structural differences between normal and oncogenic ras are likely to be very small. Although X-ray crystallography studies have helped to elucidate the conformational changes in Ha-ras p21 protein on GTP hydrolysis [114], the structural changes in oncogenic ras do not immediately suggest a strategy for the rational design of inhibitors. A more viable strategy may be to inhibit the localisation of $p21^{ras}$ oncoproteins in the plasma membrane, which is an essential requirement for their transforming activity.

A series of post-translational modifications at the carboxy terminus must occur before binding of $p21^{ras}$ to the plasma membrane [51]. The carboxy terminus includes the CAAX motif, where C is cysteine 186, AA are two

aliphatic amino acids and X can be one of several amino acids. Cysteine 186 is modified by a cholesterol biosynthesis intermediate, farnesyl. This step is followed by the proteolytic removal of the tripeptide AAX and carboxymethylation of the farnesylated cysteine 186 [56]. The processed $p21^{ras}$ can then associate with the plasma membrane and be further modified on cysteines 181/184 by another lipid, palmitic acid. Site-directed mutagenesis demonstrates that farnesylation is a prerequisite for the membrane binding and transforming activity of $p21^{ras}$. The origin of the farnesyl groups resides in the mevalonic acid pathway of cholesterol biosynthesis, therefore providing a target for inhibitors of $p21^{ras}$ transforming potential. Lovastatin (mevinolin), an inhibitor of cholesterol biosynthesis, inhibits the growth of human H-*ras*-oncogene-transformed cells in nude mice [115]. However, the severe toxicity seen in this model may limit its use as a single agent in chemotherapy. This is probably due to the observation that lovastatin inhibits a very early stage of isoprene metabolism, namely, the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and thus cholesterol and steroid synthesis will be compromised. More selective strategies such as inhibition of the recently purified $p21^{ras}$ farnesyl:protein transferase, the enzyme responsible for transfer of the farnesyl moiety to $p21^{ras}$, are currently being explored as potential targets for inhibiting ras functions [106]. This enzyme is also involved in the farnesylation of lamin A and B, but differences in the affinity for the various substrates have been observed, suggesting the possibility of selective intervention. Moreover, membrane proteins that are modified by the related geranylgeranyl transferases I and II would remain unaffected by farnesyl transferase inhibitors. The biological significance of geranylgeranyl post-translational modification is not yet clear, but the CAAX motif contains all of the critical information for the determination as to whether a protein is preferentially farnesylated or geranylgeranylated. The anticarcinogen limonene has an interesting profile of activity. It is capable of specifically inhibiting the farnesylation of ras proteins while having no effect on several other CAAX-containing proteins, including the farnesylated lamins. It inhibits at a point in the mevalonate pathway distal to HMG-CoA reductase [31]. Although limonene is only a weak inhibitor its metabolites are more potent and clinical trials are anticipated.

Inhibitors of the potentially very specific CAAX protease are of considerable interest, and carboxymethylation and palmitoylation antagonists might also have some utility, but the latter especially may have relatively low specificity, and ras palmitoylation seems to be less important than farnesylation.

Other pharmacological approaches to the suppression of *ras* transformation also appear highly promising. The antibiotic azatyrosine can reverse the *ras*-transformed phenotype of NIH3T3 cells without inhibiting the growth of untransformed NIH3T3 cells [120]. Azatyrosine also caused the permanent reversion of NIH3T3 cells transformed by activated c-*raf* and c-*erb*B-2 but not certain other oncogenes. In addition, human tumours with activated *ras* could also be reverted. *Ras* expression is maintained in all cases. Several genes were expressed specifically in these revertants, including the *ras* recession gene (*rrg*), *rho*B and collagen type III. Prolonged treatment with interferon α/β also reverted the H-*ras*-transformed phenotype in NIH3T3 cells. As with azatyrosine, the revertants expressed pre-reversion levels of *ras* and the *rrg* gene was also switched on. A cDNA sequence search with *rrg* revealed a match with the cDNA of rat lysyl oxidase. This enzyme catalyses the oxidative deamination of peptidyl lysine in elastin and collagen, leading to covalent cross-linkages that insolubilise these matrix proteins. Such observations suggest that lysyl oxidase action on extracellular matrix proteins may be important in the maintenance of cells in the non-transformed state. A more potent azatyrosine analogue (SF 2698) has been isolated from the culture medium of a *Streptomyces*. It was 10 times more potent at reverting the *ras* transformation of NIH3T3 cells than was azatyrosine.

Interestingly, the *rrg* gene product can be post-translationally modified by geranylgeranyl but not by farnesyl (see above). This provides some degree of selectivity between rrg and ras post-translational modification that might be exploited pharmacologically. The *rho* gene products are small-molecular-weight G-proteins having around 30% homology with ras proteins. Microinjection of recombinant Rho protein into mammalian cells induces dramatic changes in cell shape [55]. This is further support for the link between signal-transduction cascades and cytoskeleton organisation in transformation. Pharmacological interference in this area is therefore a possibility to consider.

In addition to azatyrosine, another antibiotic, oxanosine, also restores the normal morphology of NRK cells transformed with v-K-*ras* [71]. This antibiotic decreases the pool of guanine nucleotides, thus reducing *ras* activation. However, the ubiquitous nature of guanine nucleotides renders this a very non-specific approach. A further fungal product, depudecin, was capable of inducing morphological reversion of K-*ras*-transformed NIH3T3 cells. This reversion was reversible, unlike the effect of oxanosine and azatyrosine.

## Adenyl cyclase

Following the interaction of certain hormones such as insulin and glucagon with their G-protein-linked receptors, adenyl cyclase is activated, resulting in increased cAMP levels. This increase leads to activation of cAMP-dependent protein serine/threonine kinases (PKAs). There are two isozymes of PKA, having different receptor sites for cAMP, and these appear to have distinct roles in certain physiological processes such as cell proliferation and differentiation. Type I PKA is the major or sole PKA in a variety of human cancer cell lines and this isoform has been related to growth stimulation and transformation, whereas an increase in type II is related to growth arrest and differentiation [26]. Site-directed cAMP analogues such as 8-chloro-cAMP that compete with cAMP for the receptor on type II PKA are capable of increasing the ratio of II/I and thereby specifically inhibit the growth of a wide range of transformed cancer cells while being ineffective

against non-transformed cells [25]. The precise mechanism of action of 8-chloro cAMP remains unclear since effects at the cell membrane and incorporation of the metabolite 8-chloroadenosine into DNA and RNA may also be important. In addition, there appear to be concerns about the potential toxicity of the drug.

cAMP phosphodiesterases (PDEs) are responsible for the hydrolysis of cAMP to 5′-AMP. Addition of the PDE inhibitor mopidamol to standard chemotherapy was shown to prolong the survival of a small group of patients with non-SCLC, which suggested that there may be clinical potential for the use of this inhibitor in that disease [156]. The initial rationale behind this study was the concept that platelets were involved in malignancy, as mopidamol is also an antiplatelet drug. It was subsequently proposed that by inhibiting the breakdown of cAMP, mopidamol may promote the transduction of growth-inhibitory signals in neoplastic cells. This was consistent with an earlier view that the cAMP levels were inversely correlated with proliferation. However, there are now several systems where it is recognised that cAMP enhances or initiates proliferation [39]. As the control of growth by cAMP appears to vary between different systems, this may explain the limited use of such treatments. Mopidamol was also shown to modify the expression of $p21^{ras}$ and to inhibit the growth-promoting effects of EGF in rat mammary adenocarcinoma cells, which may be unrelated to its inhibition of PDE [82].

Recently a new cAMP PDE inhibitor was found to inhibit the growth of B16 melanoma and MCF-7 mammary carcinoma cell in vitro [38]. Drug-induced PDE inhibition was highly specific for the type IV isoform of the enzyme, whereas type I and type V PDE inhibitors were not growth-inhibitory. This suggests that PDE IV may be a viable target for antiproliferative therapy, although further information on the functions and distribution of the isozymes is required.

## Phospholipase $A_2$

G-protein-linked phospholipase $A_2$ ($PLA_2$) acts on cellular phospholipids to release arachidonic acid, which can then be converted into a variety of biologically active metabolites such as prostaglandins and leukotrienes. It has been proposed that arachidonic acid and some of its metabolites may act as second messengers in the action of certain growth factors and mitogenic neuropeptides such as PDGF [109] and bombesin [90]. Recent studies have suggested a link between arachidonic acid, its metabolites and the proto-oncogene product $p21^{ras}$ [58], with the prostaglandins $PGE_2$ and $PGF_2\alpha$ stimulating GAP, whereas arachidonic acid inhibits the ability of GAP to stimulate the GTPase activity of $p21^{ras}$. Data for oncogenic ras are not available. Effects of tumour necrosis factor have also been linked to activation of $PLA_2$ and the arachidonic acid cascade [126]. In addition, arachidonic acid can be generated indirectly as a product of diacylglycerol (DAG) breakdown, and activation of PLC can influence arachidonic acid metabolism and release, although the precise relationships between these different pathways is not known. However, it is clear that as more information becomes available, therapeutic intervention within the $PLA_2$-arachidonic acid pathway may provide new approaches to drug development.

## Phospholipase C

PLC is the enzyme responsible for hydrolysis of the inositol lipid $PIP_2$ to $IP_3$ and DAG [88]. Generation of $IP_3$ via hydrolysis of $PIP_2$ (Fig. 1) causes release of calcium from intracellular stores, whereas DAG activates the important signaling enzyme PKC (see below). Thus, both products of the PLC reaction are important mitogenic signals triggered by growth-factor-receptor activation [18]. Furthermore, activation of PKC by the phorbol ester tumour promoters led to the belief that cell proliferation was controlled by phosphatidylinositol turnover [144]. At least 16 forms of PLC and 4 PLC genes have thus far been identified [87a, 107]. PLCγ associates with activated tyrosine kinase-containing growth-factor receptors such as PDGF and EGF via its SH2 domains, resulting in tyrosine phosphorylation and activation of the phospholipase function [128]. Elevated levels of PLCγ have been found in primary human breast carcinomas as well as phosphorylation of the enzyme [9]. The same tumours also hyperexpress the EGF receptor or c-erbB-2, consistent with elevated signaling through this pathway to PLCγ activation. It should be noted however that genetic evidence now indicates that PLCγ is not involved directly in the mitogenic signaling via the FGF receptor [97a]. Thus its activation by receptor tyrosine kinases may control related housekeeping functions rather than proliferation *per se*.

Studies on PKC regulation of PLC in mammalian cells suggest that PLCβ is coupled to membrane receptors via a G-protein [110]. Inhibitors of this phosphatidylinositol pathway should thus be potential antitumour agents.

There are several non-specific inhibitors of PLC such as mepacrine [19] and manoalide [15], but these also inhibit other phospholipases. The aminoglycoside antibiotic neomycin inhibits PLC by forming a complex with $PIP_2$ [46]. No further data have been reported on the therapeutic potential of these compounds. The only agents available that are more specific inhibitors of PLC are the ether lipids [104a]. ET-18-$OCH_3$, the thioether BM 41.440 and hexadecylphosphocholine are currently undergoing clinical trial and are showing some promising results without producing excessive toxicity [16, 96, 138]. Another analogue, SRI 62-834, may be evaluated shortly by the Cancer Research Campaign. The ether lipids appear to be highly selective cytotoxic agents towards some tumour types as compared with normal cells [14]. Structurally they are analogues of the local inflammatory mediator platelet-activating factor (PAF) and they have a wide range of biochemical effects, mainly involving the cell membrane. As well as exerting direct actions on plasma-membrane structure and permeability [36, 94], these compounds also interfere with other membrane-associated signaling systems such as phosphatidylcholine synthesis [141]. Although the antitumour ether lipids are related in structure to PAF, it is now clear that PAF receptors are not involved in their mechanism of action. Thus, PAF antagonists do not protect against the cytotoxicity of ether lipids [150]. Of potential importance is the finding that they may also inhibit PKC

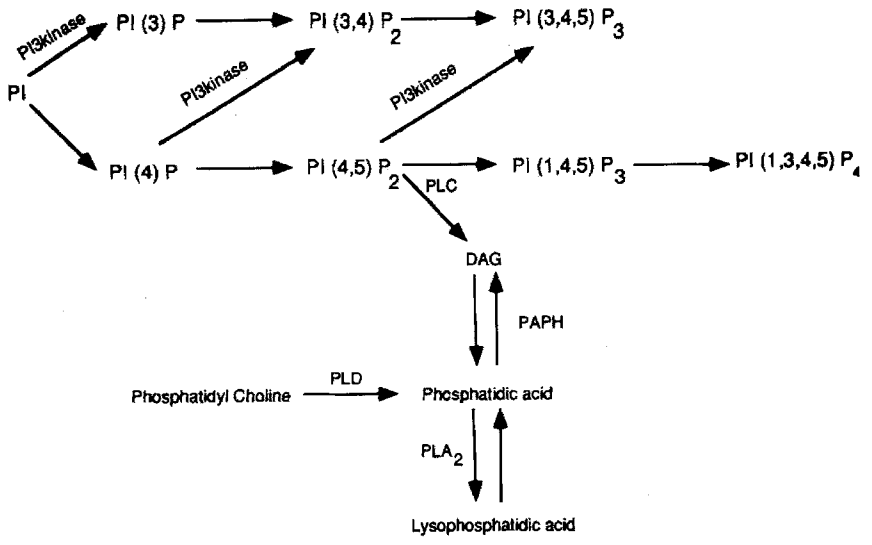


**Fig. 3.** Formation of novel phosphoinositides involved in signaling pathways. PI3kinase phosphorylates on position 3 of the inositol ring giving rise to phosphoinositides that are not substrates for PLC and whose exact role in signaling pathways has not been fully elucidated. Other possible sources of DAG formation are also shown that are thought to be important in mitogen signaling. *Abbreviations used: DAG*, diacylglycerol; *PAPH*, phosphatidic acid phosphohydrolase; *PI*, phosphatidylinositol; *PI3kinase*, phosphatidylinositol-3′-kinase; *$PLA_2$*, phospholipase $A_2$; - *PLC*, phosphoinositide-specific phospholipase C; *PLD*, phospholipase D

[60, 135]. The exact contribution of these diverse actions in the antitumour activity of the ether lipids remains to be established, but blockade of PLC signaling may well be involved, as these agents are the most potent of the known inhibitors.

Recently, phosphonate analogues of phosphatidylinositol have been synthesised that inhibit PLC [117]. U-73122 [1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino} hexyl)-1H-pyrrole-2,5-dione] has been shown to inhibit both growth-factor-induced PLC and G-protein-linked PLC in Swiss 3T3 cells with reasonable potency [104]. No information on the specificity of this compound is available.

## Phospholipase D

Emerging evidence indicates that growth factor and oncoprotein-induced mitogenesis may be less dependent on the breakdown of PI by PLC and more directly associated with hydrolysis of phosphatidylcholine (PC) by PLC, $PLA_2$ and also phospholipase D (PLD) [20a, 28a, 84a]. The latter pathway is particularly interesting from the point of view of therapeutic intervention as the action of PLD on PC liberates phosphatidic acid (itself a second messenger that interacts with membrane receptors, PKCζ, PLC, ras-GAP and phosphatidylinositol-4-phosphate kinase) which in turn provides a sustained source of DAG by the action of phosphatidic acid phosphohydrolase (PAPH) (Fig. 3). Natural products related to wortmannin inhibit PLD in neutrophils [21a] and agents like chlorpromazine, propanalol, sphingosine and spermine antagonize PAPH [72a].

## Protein kinase C

PKC is a $Ca^{2+}$- and phospholipid-dependent protein serine-threonine kinase that plays a crucial role in cell proliferation and differentiation [50]. Treatment of cells with PKC activators causes translocation of the inactive enzyme from the cytosol to the membrane where it is activated. The most common physiological activator of PKC is DAG. At least ten subspecies of PKC have been identified thus far and they are known to phosphorylate multiple cellular proteins [9a]. The main problem with PKC as a target for pharmacological intervention is the ubiquitous nature of this enzyme and its involvement in many aspects of cell signaling. However, recent evidence indicates that the same PKC isoenzyme can activate quite different phenotypic programmes, depending on the nature of the cell in which it is expressed. For example, expression of PKCβ1 enhances the growth of rat fibroblasts but inhibits growth and tumorigenicity in human colon-cancer cells. Decreased levels of PKCβ1 and DAG have also been found in human colon tumours as compared with normal colon [99]. Transformation of rat fibroblasts by an activated *ras* oncogene leads to increases in PKCα and PKCγ but to a decrease in PKCε. The expression and function of PKC is obviously very complex, but differential isoform distribution and function may allow for selective modulation of PKC. It is probably highly significant that the various isoforms of PKC are activated differentially by alternative second messengers such as DAG, calcium and phosphatidic acid [9a]. It is not yet clear whether we should be developing specific antagonists or agonists for PKC, but further information on the role of multiple isoforms should clarify this issue. It is possible that both types of agent could be useful.

The most potent inhibitor of PKC available is staurosporine, a microbial alkaloid acting on the catalytic domain of the enzyme [132]. However, this agent also inhibit PTKs and both cAMP- and cGMP-dependent protein kinases, and this probably accounts for its reputedly high level of toxicity. 7-Hydroxy-staurosporine shows more selectivity and is less toxic than staurosporine and it exhibits antitumour activity in vivo [4]. Calphostin, isolated from the fungus *Cladosporium cladosporioides*, specifically inhibits PKC without inhibiting other protein kinases but requires activation by light [131]. In addition to their effects on PLC as described earlier in this review, the ether lipids also antagonize PKC. BM 41.440 inhibits PKC and blocks phorbol ester-induced differentiation of human leukaemic cells [122], whereas hexadecylphosphocholine inhibits PKC and antagonises phorbol ester-induced proliferation of canine kidney cells [49]. Recently the dihydropyridine

derivative B859-35 was shown to inhibit PKC in cell-free extracts from NIH3T3 cells and to antagonise 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced expression of c-*fos* [136]. It is clear from the above discussion that several compounds have been identified that inhibit PKC, but the therapeutic relevance of these findings remains to be determined.

Despite this, a number of clinically useful drugs have previously been shown to exert effects on PKC. The anti-oestrogen tamoxifen inhibits PKC. This may play a significant role in the antitumour activity of this non-steroidal drug in breast cancer, as not all of the antiproliferative effects of tamoxifen can be attributed to blockade of the oestrogen receptor [95]. Doxorubicin is a weak inhibitor of PKC [157], but the relevance of this to its antitumour action is not clear. Suramin, described earlier in the present review, also blocks PKC [85].

The phorbol ester tumour promoters are an interesting group of compounds that have a diverse range of actions on a number of different cell systems. TPA, the most active of these esters, can both induce and inhibit differentiation, depending on the cell type, consistent with the ambiguous role of the target enzyme. Although it would be impossible to use these agents therapeutically, this information does raise the possibility that PKC activators may be useful antitumour agents.

Bryostatins are a highly potent group of complex macrocytic lactones isolated from a marine bryozoan sea moss [98]. They activate PKC at the same site on the regulatory domain as the phorbol esters. Both agents initially activate PKC and then promote membrane localisation and subsequent degradation of the enzyme. However, the bryostatins can antagonise certain effects of the phorbol esters, including tumour promotion in mouse skin [62] and induction of differentiation in HL60 cells [78]. In vitro and in vivo antitumour activity has been observed for bryostatin 1 in a number of different tumour types [65]. More mechanistic information on the relationship between these compounds and PKC is required before their full therapeutic potential can be assessed. Nevertheless, bryostatin 1 is currently undergoing clinical trial with the Cancer Research Campaign in the United Kingdom and a wide range of biochemical measurements are being made, including cytokine release, which should provide valuable information on this PKC partial agonist. Interestingly, the dose-limiting toxicity of bryostatin 1 in patients is myalgia, but it is not yet clear whether the drug actually inhibits PKC *in vivo*.

## Phosphatidylinositol-3′-kinase

As well as the established $IP_3$ pathway, there is now evidence that other inositol phosphate pathways are involved in cell signaling. PI-3′-kinase is a heterodimer consisting of an 85 kDa subunit containing SH2, SH3 and bcr domains together with a 110 kDa catalytic subunit [96a]. It is activated by receptor tyrosine kinases and phosphorylates the D-3 position of the *myo*-inositol ring of PI, giving rise to products that are not substrates for PLC (Fig. 3) [145]. The evidence for these inositol phosphates as signaling messengers comes from work in cells with a mutated PDGF receptor that fails to associate with PI-3′-kinase. The mitogenic response to PDGF in these cells was greatly reduced, suggesting that formation of these phosphoinositols by PI-3′-kinase plays an important role in the mitogenic response of PDGF [29]. Inhibitors of PI-3′-kinase may therefore prove to be useful as antiproliferative agents. However, little is currently known about the distribution and regulation of this enzyme in tumour or normal tissues, or about the precise function of its lipid products.

D-3-Deoxy-3-substituted *myo*-inositol analogues have recently been synthesised. These selectively inhibited the growth of v-*sis*-transformed NIH3T3 cells, a cell line containing higher constitutive levels of PI-3′-kinase than the wild-type cells [103]. The *myo*-inositol analogues are substrates for phosphatidylinositol synthetase, and D-3-deoxy-3-fluoro-Ins(1,4,5)$P_3$ is as effective as Ins(1,4,5)$P_3$ ($IP_3$) in releasing calcium from intracellular stores. It is thought that the inhibition of cell proliferation involves antagonism of the formation of 3′-phosphorylated inositol phosphates as *myo*-inositol competes with the analogues and attenuates their growth-inhibitory properties. This may be the reason for the low potency of these compounds and would certainly restrict their activity in vivo.

## Other inositol phosphate approaches

Plant phosphatidylinositol (PI) has been tested for antitumour activity and showed remarkable selectivity, being cytotoxic toward eight of nine tumour cell lines, but ineffective against four normal cell lines [73]. More recently it has been shown to kill preferentially multidrug-resistant cells [74]. It is thought that the plant PI may alter the intrinsic PI turnover in tumour cells as there are several structural differences between plant and mammalian PIs. The fatty-acid composition differs and isomers other than the *myo* form are found in plants. This opens up a potentially very interesting area for the development of other analogues that could interfere with the normal PI balance in tumour cells.

Psi-tectorigenin and inostamycin, antibiotics isolated from *Nocardiopsis* and *Streptomyces*, respectively, inhibit EGF-induced PI turnover in A431 cells. They do not inhibit PLC. Rous sarcoma virus-transfected NIH3T3 cells and activated c-*erb*B-2-transformed NIH3T3 cells convert increased levels of [$^3$H]-inositol into phospholipids, and this was inhibited by both agents, as was the proliferation of these cells [69].

Other possible targets in this area include tetrakisphosphate ($IP_4$; Fig. 3). This inositol phosphate has been reported to act synergistically with $IP_3$ by controlling calcium entry through the plasma membrane [70]. The exact role of $IP_4$ as a signaling messenger is presently under investigation.

Phosphatidic acid and lysophosphatidic acid (Fig. 3) have been shown to exert a mitogenic action [91], and interference with the pathways involved in the generation of these compounds may prove useful when more is known about the role that they play in cell signaling. A membrane receptor may be involved and receptor antagonists might be envisaged. Work in reconstituted vesicles has identified a binding protein for $IP_3$ that has been localised to the

endoplasmic reticulum. This binding protein acts as a receptor for $IP_3$, facilitating the release of calcium by this second messenger from endoplasmic reticulum stores [43]. Subsequently the $IP_3$ receptor was cloned and sequenced [45]. To date there are no known specific antagonists of the receptor that would be useful as biochemical tools or possible drugs. Their use as antiproliferative agents may be restricted due to the presumably wide distribution of the receptor and its participation in multiple signaling processes. However, as mentioned earlier, suramin may antagonize both the $IP_3$ and the phosphatidic acid receptors.

## Nuclear signaling targets

As more information becomes available as to how the signals at the cell membrane trigger nuclear events, it is possible to define downstream targets for drug therapy. Following the addition of PDGF, for example, to quiescent fibroblasts there is a rapid induction of the expression of the cellular proto-oncogenes c-*fos* and c-*myc*. Since these cellular oncogenes encode nuclear proteins, it seems likely that they play a role in the transduction of the mitogenic signal in the nucleus [108].

The 41- to 45-kDa mitogen-activated protein (MAP) kinases and the 85- to 90-kDa ribosomal S6 protein kinases (RSKs) have emerged as candidates for transfer of information from cytoplasmic signaling elements to the nucleus [21, 92]. It has recently been shown that these protein serine-threonine kinases are localised in the nucleus as well as in the cytoplasm [21]. MAP kinase substrates include microtubule-associated protein 2, which modulates microtubule polymerisation, and also the RSKs. These RSKs phosphorylate the ribosomal S6 protein, which is thought to participate in the regulation of protein synthesis. Preliminary data suggested that a MAP kinase can phosphorylate the cytoplasmic raf-1 protein serine/threonine kinase [7]. However, it now seems more likely that raf-1 acts upstream of the MAP kinase pathway [66a]. The tyrosine and serine/threonine kinase cascades are complex and probably act as branched, interconnecting pathways. However, recent evidence suggests that, at least in certain situations, membrane tyrosine kinases combine with ras p21 to activate a sequence which involves raf-1 → MAP kinase (kinase) → MAP kinase → RSK [107a]. Both MAP kinase and RSK translocate to the nucleus and may phosphorylate jun and fos (see below) [107a]. All of these kinases are potential drug targets. Interestingly MAP kinase requires concomitant phosphorylation of threonine and tyrosine and MAP kinase may catalyse both reactions by acting as a 'dual specificity kinase' [9b].

Interference with the binding of important transcription factors such as AP-1 (activator protein 1) to DNA may be possible by developing small polypeptides with the same binding domain sequence. AP-1 is a complex of several different proteins, including the c-*jun* and c-*fos* gene products [32]. There are specific AP-1 binding sites in both viral and cellular genes that are stimulated by treatment of cells with phorbol esters. Leucine zippers and the helix-loop-helix motif, found in transcription factors, are involved in their binding to DNA. It is possible that sequence-specific inhibitors to these regions could act as broad-spectrum transcription-factor blockers, but there would be concerns about their potential therapeutic selectivity as multiple genes would be affected.

There is considerable interest in structural and mechanistic aspects of protein-protein interactions between certain nuclear proteins and tumour-suppressor gene products. Identification of critical amino-acid sequences should facilitate the targeting of potential growth-inhibitory molecules. For example, small peptides have been synthesised that attach to the binding site for human papillomavirus product, E7, on the *RB* gene product. These peptides must be restricted to very short amino-acid sequences (eight or less) to allow their entry into the cells. The *RB* gene product is a nuclear phosphoprotein that binds DNA and also virus-encoded oncoproteins such as E7, large T-antigen and E1A. It has been proposed that cellular homologues of these binding proteins are involved in inhibiting *RB*-induced growth suppression [142]. RB is intimately involved in cell-cycle progression and its phosphorylation status is highly regulated throughout the cell cycle. The hypophosphorylated form is thought to suppress cell proliferation. The cdc2 kinase is an obvious candidate for the phosphorylation of RB at the $G_1$/S boundary.

In normal cells, major proliferation controls operate at the $G_1$/S interface, thereby restricting entry into the S phase. Activation of cdc2 kinase and possibly other related proteins appears to be the rate-limiting step. Activation of this kinase requires association with the important regulatory proteins known as cyclins. A series of these complexes exists. Each specific cyclin/cdc2 kinase complex may have a distinct role in controlling cell-cycle progression [40]. It seems that in malignant cells the normal control systems are altered such that their proliferation is not constrained by the usual $G_1$/S-boundary checkpoints in the same way. Further understanding of the components of these cell-cycle control pathways in normal and transformed cells is likely to provide substantial insight into normal and abnormal cell proliferation and may identify new therapeutic targets. In support of this the oncogenes PRAD 1 and *bcl*-1 have been identified as D-type cyclins [99a]. Moreover, recessive oncogenes encoding RB and p107 appear to be targets of the G1-activated kinase regulated by G1 cyclins [75b].

The cdc-2 kinase is involved in the phosphorylation of p53. Mutational alterations of the p53 gene represent the commonest genetic alteration yet found in human tumours. The gene codes for a nuclear phosphoprotein transcription factor that is now known to play a crucial role in the regulation of DNA replication at the G1/S checkpoint. Wild-type p53 allows cells to arrest in G1 so as to provide an opportunity for DNA repair prior to commencement of replicative DNA synthesis [75, 78a]. Mutant p53 proteins are unable to act in such a way and this failure is consistent with their inability to function as transcription factors [105a]. Because of that, p53 mutations are now believed to be a major cause of genetic instability in cancers and this in turn probably explains how wild-type p53 acts as a tumour suppressor. At the same time, the genetic instability (which is a hallmark of malignant cells) leads to a predisposition

for gene amplification and drug resistance [152a]. In addition to mutation, p53 function can also be subverted by binding to viral proteins or the product of the recently identified *mdm* 2 oncogene [90a] which is frequently amplified in human sarcomas [79a].

In view of the crucial role played by p53 and the frequency of abnormalities in the p53 pathway in human cancers, p53 is now seen as a major target for drug hunting. Possible therapeutic strategies include drugs which would restore p53 to its wild-type conformation or interfere with the binding of wild-type p53 to viral or cellular proteins. In addition, the *gadd* 45 gene product is now known to lie alongside p53 on the DNA damage recognition – G1 arrest pathway, providing further opportunities for drug intervention. It should be remembered however that in additon to providing a means for drug resistance to develop, aberrations in the p53 pathway will also render cancer cells initially more sensitive to chemotherapy and radiotherapy, because the defective G1/S checkpoint decreases the probability of DNA repair. Therapies directed at correcting p53 might therefore be most appropriate at a very early stage in tumour development or at a much later time when resistance to a particular agent is already established. p53 is multiply phosphorylated by at least four protein kinases (including p34 *cdc2 kinase*, DNA-activated protein kinase, casein II kinase and casein I-like kinase) and this appears to be critical for DNA binding and growth arrest [66b]. This again presents therapeutic possibilities.

When considering nuclear signaling targets in general it appears that pharmacological interference with protein-protein and protein-DNA interactions is quite complicated, and for this reason there is currently greater emphasis on agents that directly inhibit proteins involved in cell-membrane and cytoplasmic signaling rather than nuclear events. On the other hand, the involvement of various kinases and phosphatases is to some extent a unifying feature that does allow a common pharmacological approach.

## Signaling and resistance

There is growing evidence that pharmacological modulation of signal-transduction proteins may be an important means of circumventing resistance to conventional anticancer drugs. The previous section described the role of p53 in G1/S checkpoint control and drug resistance, and also emphasized the pitfalls of pharmacological intervention in this area. A similar checkpoint operates at the G2/M boundary, allowing cells to stop and inspect correct DNA fidelity prior to mitosis. The molecular basis of this checkpoint is not understood in mammalian cells but the *rad* 9 gene product is known to be a key player in yeast [141a]. Inhibition of the G2 arrest by caffeine provides encouragement to pursue cell cycle checkpoint controls as targets for resistance modulation [94a].

Signaling through membrane receptor tyrosine kinases and protein kinase C is also emerging as an important area for drug modulation. Antibodies to the EGF and c-erbB2 receptors have been shown to enhance the effect of certain antineoplastic drugs such as cisplatin and doxorubicin [3, 57, 67], although the mechanism of action is not fully understood. Elevated EGF-receptor numbers have been reported in some multidrug-resistant cell lines [89]. Simultaneous amplification of EGF-receptor and multidrug-resistance genes was also observed in a newly established human lung-cancer cell line [119]. EGF itself has also been shown to increase radiation and drug sensitivity [78b].

Both PKC and PKA modulate cisplatin toxicity. Increased levels of cAMP induced by stimulation of adenyl cyclase by forskolin has potentiated cisplatin accumulation and sensitised the cells to cisplatin cytotoxicity [8]. However, in a cisplatin-resistant cell line there was no potentiation of cisplatin cytotoxicity following forskolin treatment. PKA may phosphorylate a protein that modulates cisplatin accumulation in parental cells but is deficient in the resistant cells [8]. Inhibition or down-regulation of PKC has been shown to enhance cisplatin cytotoxicity in Walker rat carcinoma cells and HeLa cells [12, 64]. Conversely, in HeLa cells treated with the PKC activator lyngbyatoxin A, there was also sensitisation to cisplatin [13]. PKC has also been reported to phosphorylate P-glycoprotein in multidrug-resistant human KB carcinoma cells [23]. Transfection of PKCα into multidrug-resistant breast cells increased the resistance to doxorubicin, with an increase in phosphorylation of P-glycoprotein [155]. These results suggest that PKC does have a role in modulation of multidrug resistance. Recent data also show that activation of PKC increases expression of the *mdr1* gene, and this is blocked by PKC inhibition [23a].

Increases in *ras, fos* and *myc* mRNA have been reported in cisplatin-resistant cells, and NIH3T3 cells transformed with activated *ras* or hyperexpressing the normal gene show cisplatin resistance [111, 123]. There is as yet no biochemical explanation for the effects of these oncogenes on the cytotoxicity of cisplatin.

The relationship between signaling pathways and drug resistance is obviously very complex. It is, however, not inconceivable that agents that interfere with signaling pathways may be useful in modulation of drug resistance in cancer patients. To date, most of the interest has concentrated on the possible regulation of drug-transport proteins by phosphorylation. However, it is likely that other elements of cytotoxic drug resistance may be influenced by signal-transduction pathways. A case in point is topoisomerase II, which is regulated by phosphorylation. Modulation of cell-signaling pathways involving p53 and other checkpoint control proteins, EGF receptor, raf-1 and the nuclear oncoproteins is emerging as a possible means of altering both drug and radiation sensitivity. The interplay of various factors, including cell proliferation and loss kinetics, may be extremely complex, but the potential to circumvent drug and radiation resistance in a novel way is considerable.

## Apoptosis

Although this review has focused primarily on the cell signals leading to proliferation it is becoming clear that inhibition of programmed cell death or apoptosis is also very important in cancer biology [35a]. Despite the fact that apoptotic signaling is defined in only limited molecu-

lar detail, it is obvious that there are exciting possibilities for drug development in the future.

The *bcl*-2 gene product is a protein found in the inner mitochondrial membrane, but also in the nuclear envelope and the endoplasmic reticulum. It has been identified as a survival factor for some cells, although its cellular function is not fully understood. However, a link between *bcl*-2 and apoptosis or programmed cell death has been identified. Cells transfected with *bcl*-2 fail to acquire the morphological changes associated with apoptosis following treatment with agents that are known to induce programmed cell death [140]. Furthermore, expression of the *bcl*-2 gene in lymphoma cells confers resistance to thymidylate synthase-induced cell death by apoptosis [63]. Possible interventions would include the inhibition or down-regulation of *bcl*-2. Identification of extracellular signals that regulate *bcl*-2 and those that control downstream nuclear events linked to apoptosis may also provide useful points of pharmacological intervention. It is now known that, in the absence of growth factors, deregulated c-*myc* expression is a potent inducer of apoptosis and that the *bcl*-2 protein specifically abrogates c-*myc* induced apoptosis without affecting c-*myc* mitogenic function [40a]. A problem will be that of selectivity as the gene is expressed in a number of normal tissues.

Also relevant to pharmacological manipulation of programmed cell death is the identification of a human cell-surface antigen that mediates apoptosis. The Fas antigen is a polypeptide with a single transmembrane domain and an extracellular domain that shows remarkable similarity to tumour-necrosis-factor and nerve-growth-factor receptors [72], but again links to the nucleus remain undefined. Numerous other genes involved in apoptosis are now being defined, particularly in *C. elegans*. All represent potential drug targets.

## Perspectives on selectivity

As with all approaches to therapy, the pharmacological manipulation of signal transduction is not without its potential pitfalls. It is obviously important that these be anticipated so that we can be prepared to deal with the difficulties that arise. There are three major potential problems.

*Problem 1: where is the therapeutic index?*

The main issue concerns the degree of selectivity that we might expect by interfering with cancer cell signaling, given the importance of the same or similar transduction cascades in normal cells. There are several responses to this. Firstly, in many cases, elements of normal signaling processes are over-expressed or overactive, and thus a 'dampening down' of these hyperstimulated pathways might yield a cytostatic effect in tumours without unduly affecting normal tissues. There is clear evidence that transfection of normal cells with activated oncogenes produces an elevation of second messengers such as phosphoinositols and DAG. Particular signaling proteins such as EGF and the c-erbB2 receptor show increased expression in human breast cancer, and downstream signaling proteins are known to be simultaneously up-regulated and hyperphosphorylated.

A second answer concerns the participation of multiple isoforms of signaling proteins in different normal tissues as well as in tumour versus its normal counterpart. PKC is a good example of this. The multiple forms are differentially expressed in various cell types and may have different cellular locations and functions. Thus, isoenzyme-specific PKC modulators can now be envisaged for various signaling proteins.

Yet another solution to the selectivity problem is that aberrant cell signaling can result from a mutation in, rather than an altered expression of, cancer genes – including growth-factor receptors, oncogenes and tumour-suppressor genes. These mutations are often highly specific. This means that new therapeutic strategies can be conceptualised that will be targeted precisely to a mutated gene or its protein product. Hence, we might envisage an especially tumour-selective outcome. At the gene and RNA level the use of antisense oligonucleotides allows a high degree of specificity for a particular point mutation (e.g. *ras*) or chromosomal rearrangement (e.g. *bcr-abl* in leukaemia). Targeting of DNA-binding drugs lags somewhat behind in molecular recognition potential: sequences of 5–6 bases at best can be achieved, whereas runs of 15–20 would be required to hit a single mutated gene. However, there is considerable interest in this area as agents like distamycin mustard and CC-1065 analogues enter clinical trials. It can be argued that a modest increase in sequence recognition capability could generate sufficient specificity to inhibit expression of a relatively small number of cancer cell genes. Changes at the protein level can be exploited by the construction of epitope-specific antibodies (e.g. EGF receptor and c-erbB2). Some of these amino-acid alterations can also result in conformational changes in the effector or regulatory domains of the protein, which provides another avenue to the discovery of highly tumour-specific drugs.

Further encouragement comes from genetic knockout experiments in transgenic animals to indicate that the disabling of key signaling proteins is not necessarily a lethal event for the organism. Surprisingly, for example, complete removal of both copies of the p53, *src* or *fos* genes is not lethal [37a]. Moreover, we are now accruing initial experience with prototype drugs that act on cell-signaling loci and with antibodies that disable oncogene products and growth-factor receptors. It is clear that the toxicity can be quite acceptable at doses that give a readily measurable therapeutic benefit in vivo. This is the case with antibodies to the EGF receptor in experimental animals. Early clinical trials in patients have also shown a surprising lack of toxicity [37]. Drugs such as the ether lipids, which inhibit both PLC and PKC and probably other signaling elements, are surprisingly non-toxic in patients. Although suramin causes considerable toxicity (not necessarily related to its effects on growth factors), therapeutic concentrations can be achieved by careful attention to pharmacokinetics. Continuing human studies with these early agents will shed further light on the toxicological sequelae associated with the inhibition of signal-transduction pathways in cancer therapy. Such studies should continue even if we regard some of the early anti-signaling

drugs as sub-optimal. It is also worth remembering that in addition to their more conventional roles as DNA-damaging agents, existing chemotherapeutic drugs such as alkylating agents and anthracyclines have also been shown to exert various effects on a number of cell-signaling pathways.

*Problem 2: the antiproliferative trap?*

A second concern about targeting signal-transduction genes and proteins for cancer therapy is that this will simply generate a new pharmacopoeia of antiproliferative agents. The first pragmatic response to this is that new types of antiproliferative drugs would in any case be of significant value, for example in combination with traditional cytotoxic drugs or in situations where resistance to standard cytotoxics has developed. Moreover, these new agents would probably be cytostatic rather than cytotoxic. The second line of defence is that certain known anti-signaling, antiproliferative drugs are of proven clinical utility: the major breast-cancer drug tamoxifen exerts at least part of its activity by antagonising the oestrogen receptor and switching off autocrine production of TGFα and insulin-like growth factor, together with its possible effects on PKC and calmodulin. Another important response to the concern about the antiproliferative trap (and indeed to the selectivity issue) is that signal-transduction-inhibiting drugs will exhibit diverse effects on different target cells, depending on the biochemical context in which they are expressed. As mentioned earlier in the review, transfection of PKCβ1 can either promote or inhibit tumour-cell growth and tumourigenicity. Similarly, EGF can induce a mitogenic response in some target cells and an inhibition of proliferation in others. We would therefore expect drugs working on such signaling pathways also to have markedly diverse effects. Where such phenotypic response differences can be shown to exist in tumour versus normal tissues, these might be exploited by signaling drugs to produce an unusually high degree of antiproliferative selectivity. Moreover, in some cases the phenotypic response to cell-signaling changes is to induce a differentiated phenotype. This is really another, more sophisticated type of antiproliferative effect, since a terminally differentiated tumour cell is no longer of concern to the therapist. A further type of desirable response that might be created by meddling with signal transduction is apoptosis or programmed cell death. This is a process that appears to be compromised in tumour cells and for which the responsible genes (such as *bcl*-2 and *myc*) and associated signaling mechanisms are now being defined [40a]. Once again, it might be imagined that the normal tissue toxicity could be lower than that produced by conventional cytotoxic or cytostatic drugs.

*Problem 3: multistep oncogenesis means dirty drugs or combinations?*

The third and final anxiety about cell-signaling drugs in cancer is that since oncogenesis is a concerted multistep process, it may be necessary to block simultaneously several of the resulting alterations in signal-transduction pathways. It is not clear to what extent this is true, since it seems quite conceivable that each one of the various individual changes may be necessary to induce particular aspects of the malignant phenotype, such as immortalisation, uncontrolled mitogenesis, de-differentiation, angiogenesis and metastasis, for example. On the other hand, it is also possible that some of the genetic changes are not essential for the cancerous phenotype but represent epiphenomena or pathological alterations that are required for an earlier stage of transformation but no longer play a role in the advanced form of the disease as it presents clinically. In addition, many examples are known in the laboratory where a collaboration of two oncogenes or growth factors is required for tumour-cell growth. Frequently one, such as EGF, acts as a competence factor to initiate proliferation, whereas a second, such as insulin and related molecules, functions as a progression factor to sustain completion of the cell cycle [1]. Both factors are essential and removal of any one type prevents proliferation. However, the redundancy of signaling pathways can clearly provide multiple means by which cells can activate and maintain proliferation programmes. Thus, specific blockade of any one component might or might not generate a valuable therapeutic advantage [21b]. Examples are known where introduction of wild-type p53 or RB causes inhibition of cell growth or cell death even in tumour cells with multiple genetic abnormalities [21b, 110a].

If it does turn out to be necessary in some situations to inhibit two or more signaling pathways, this does not by any means negate the whole approach. The use of combinations of the appropriate specific antagonists would, however, be more appropriate. For example, a ras p21 farnesylation inhibitor might be combined with an oncoprotein tyrosine kinase inhibitor. Equally well, a single drug with the simultaneous capacity to inhibit multiple signaling mechanisms might be envisaged. Such a 'dirty' drug would be less intellectually appealing but therapeutically valuable nonetheless. An example is the substance-P analogues (mentioned earlier) which act as broad-spectrum mitogenic neuropeptide antagonists. Alternatively, one might envisage using comparatively low-specificity protein kinase or phosphatase inhibitors in a similar way. The same purpose might be fulfilled by drugs acting on a downstream signaling protein through which multiple pathways converge, such as PKC and MAP kinases. Of course, more side effects are likely to be encountered in these broader approaches to inhibit multiple oncogenic signaling targets, although again the experience with the neuropeptide antagonists indicates that this will not necessarily be the case in vivo. It is possible that the redundancy of signaling pathways in normal cells might act in our favour if the dynamic mix of parallel signaling elements were suitably different in tumour versus normal cells.

The issue of signaling redundancy also relates to another matter that we have not yet touched on: that of the development of resistance to our new anti-signaling drugs. The genetic instability of tumours coupled to the selection pressure of drug treatment means that resistance will inevitably develop, just as it does with more conventional agents. However, some of the mechanisms will be quite different. In particular, blockade of one signaling pathway

is likely to trigger the activation of another to maintain cell growth. In this situation the circumvention strategy will involve the use of a dirty drug that would inhibit both the new and the old pathways or the introduction to the chemotherapy schedule of an additional specific inhibitor to antagonise the new signal-transduction route. It also follows that research aimed at understanding the molecular basis of drug resistance is vitally important.

## Concluding remarks

In addition to the various specific arguments rehearsed above, there is the classic response of the ever optimistic chemist, pharmacologist or clinician that no new drug would ever be developed if concerns about potential or even predictable toxicities were to be taken too much to heart. We would be paralysed into inactivity. Naturally, we would like to develop agents acting on new molecular targets that would produce minimal or at least acceptable normal tissue toxicity. The high therapeutic index of current antihormonal therapies together with our rapidly accruing clinical experience with first-generation anti-signaling drugs suggests that this exciting goal may well be achievable.

It will be clear from this review that there are very many potential targets for drug hunting in the wiring diagram of cancer cells. It will also be apparent that many of our current leads were identified by the traditional approaches of cellular screening – either 'random' screening for cytotoxicity or, increasingly, mechanism-based test systems. Serendipity also continues to play a key part in drug discovery. However, although we can point to very few examples as yet, it seems clear that rational strategies will gain increasing influence. High throughput molecular screens are already in place to search for new leads with targets such as p53, ras and tyrosine kinase. Moreover, rational design based on the solution of protein structures and computational chemistry is a clear way ahead. Antibody-based drug design and the use of phage display libraries show considerable promise. Most likely, optimum benefit will be gained by using these various approaches in combination.

It is crucial that new anti-signaling drugs are developed sensibly with a 'feel' for their novel mode of action. They must be evaluated in relevant models where the appropriate molecular target is known to be important. Clinical protocols must also be in tune with the novel mechanism involved. Evidence of specific drug-target interaction must be obtained in preclinical models and in patients. It is essential that potentially valuable drugs are not lost to ignorant screens or inappropriate clinical schedules.

Signal transduction drugs are entering clinical trials in cancer patients at the same time as gene therapy approaches. Potentially these will be aimed at the same cancergene targets. It will be an exciting challenge to develop both avenues with enthusiasm, yet with our critical faculties intact.

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