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COMMENTARY

TYRPHOSTINS—POTENTIAL ANTIPROLIFERATIVE AGENTS AND NOVEL MOLECULAR TOOLS

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In recent years, there has been an explosion in the knowledge of cell growth and proliferation. This applies to normal cells, whose growth is tightly controlled, as well as to proliferative tissues whose growth is enhanced but programmed and to malignant cells whose growth is out of control. Knowledge has also been gained about the signalling events which convert a resting cell to a proliferative cell and the biochemical events involved in cell transformation.

Probably the most important development is the realization that the two groups of genes—those which control the tight schedule of normal cells and the oncogenes which are involved in the enhancement of proliferation and transformation—are one and the same. The oncogenes are actually normal genes whose function has been subverted by mutation(s), translocation(s), or amplification; in many cases, it is a combination of these events. It has become apparent that the molecular-genetic cause of cancer and other proliferative diseases is not "foreign" genes encapsulated in virus-like particles. The changes in normal genes which convert them to harmful oncogenes are small and sometimes confined to a change in their position rather than in their coding sequence. Sometimes these subverted genes are "captured" by viruses which then become oncogenic. This, however, is the exception, not the rule. This sombering realization [1] implied that, for the foreseeable future, chemotherapy is bound to remain a major tool in combating proliferative diseases.

Recent developments in the understanding of proliferative processes, however, can give the pharmaceutical chemists new directions. The identification of the biochemical activities of many oncoproteins, the protein products of oncogenes, serves to focus our attention on these as potential targets for new drugs. Rather than designing toxic molecules which either block the synthesis of DNA or its precursors, one can now focus on the "villain"—oncoproteins expressed in tumors— and try and design selective blockers which inhibit their biochemical activity. Table 1 identifies three main classes of oncoproteins. The first two classes do not easily lend themselves to specific drug design for the following reasons: Little is known about the chemistry of interaction of nuclear oncoproteins with DNA, although this knowledge is emerging quickly. Also, from the accumulating data it seems that DNA–protein complexes involved in transcription or replication are actually multiprotein complexes. Thus, it is the combination of protein–protein interactions and DNA–protein interactions which generates the specificity in the system. At this stage, it is also difficult to envisage how the detailed knowledge of DNA–protein interaction can lead to a rational design of selective drugs which compete against specific DNA sequence, which interact with a specific protein. Knowledge about the interaction of $p21^{RAS}$ proteins with their target effector systems has also been slow in coming, although the so-called "effector domains" of these proteins are well recognized. Clearly, designing GTP antagonists would result in cytotoxicity since GTP binding sites are conserved among many essential cellular proteins.

Protein tyrosine kinases, on the other hand, present themselves as potential targets for drug design. These proteins are all enzymes which catalyze a well defined chemical reaction: the phosphorylation of a tyrosine residue. It is also generally known that this unique kinase activity is essential for the protein to express its biological activity. Sequence analysis has identified the active sites of these proteins, which can be conceptually divided into two subsites: the ATP subsite and a juxtaposed subsite for the substrate to be phosphorylated. Currently, about 100 protein tyrosine kinases are known and the catalytic domains of many have been compared to each other [2, 3]. If one designs blockers for the substrate domain of the protein tyrosine kinase (PTK*), it is likely to yield an effective and selective antiproliferative agent. An ATP antagonist, on the other hand, is likely to be highly toxic to the cell.

Recognizing that the residue phosphorylated by PTK is the phenolic residue of a tyrosine, a much more distinctive chemical entity than serine or threonine, we felt this to be a promising starting point for the design of PTK blockers. Indeed the natural compound quercetin [3, 4] and its related compound genistein [5] inhibit not only a variety of PTKs but also other protein kinases such as cAMP-dependent protein kinase and protein kinase C. This undesirable feature of the two compounds and their synthetic derivatives [6] renders them highly cytotoxic, and therefore, of little or no potential as candidates for antiproliferative drugs.

We therefore began with phenolic compounds and

* Abbreviations: PTK, protein tyrosine kinase; TGFα, transforming growth factor-α; EGF, epidermal growth factor; EGFR, EGF receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; BMN, *cis*-benzene malononitril; and PLC, phospholipase C.

Table 1. Biochemical characterization of oncoproteins

Type of oncoprotein	Biochemical reaction(s) catalyzed	Biological and pathological role
(1) Nuclear oncoprotein	DNA binding, transcription	Gene expression
(2) RAS proteins	GTPases, interaction with GAP*. Mutated "oncogenic" forms have reduced GTPase activity.	Essential for cell proliferation. In their "oncogenic" form, lead to cell transformation.
(3) Protein tyrosine kinases, e.g. EGFR, erb B-2, $p210^{bcr\text{-}abl}$, PDGFR	Kinase, phosphorylation on Tyr residues of the oncoprotein and intracellular substrates	Normal cell proliferation. Enhanced kinase activity leads to transformation.

* GAP = GTPase activating protein, recently discovered and found to enhance the hydrolysis of RAS bound GTP to GDP.

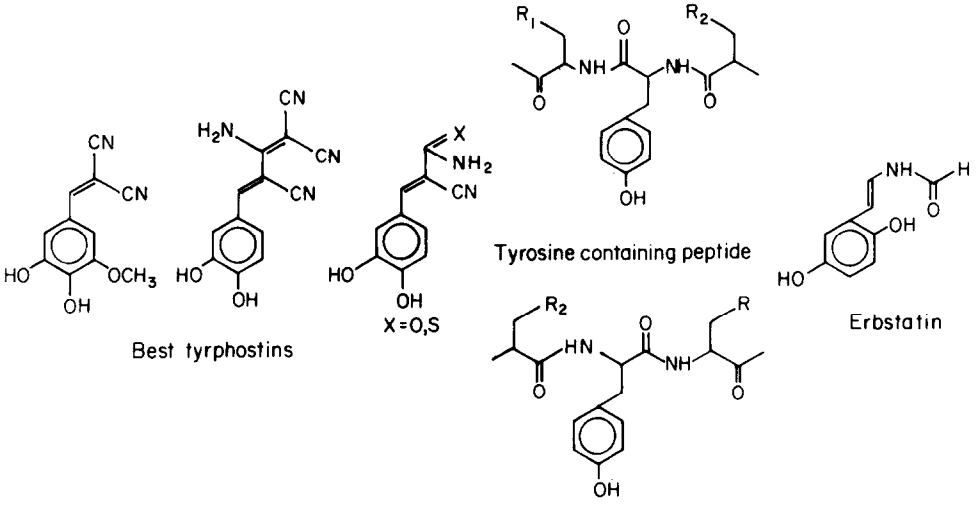


Fig. 1. Tyrphostins and erbstatin compared to a tyrosine containing peptide.

then proceeded to modify their chemistry. From the beginning, we also aimed at slightly hydrophobic compounds soluble in ethanolic solutions and dimethyl sulfoxide (DMSO) to enhance their chances to cross the hydrophobic cell membrane. Last, but not least, we concentrated our efforts on low molecular weight non-peptidic compounds to simplify synthetic procedures and enhance biological stability and cell permeability.

Protein tyrosine kinases

It has been a decade since the first PTK was discovered. In the past 10 years, about 100 proteins with this previously unknown enzymatic activity have been discovered. The biological role of protein tyrosine kinases is well defined for only a few proteins, although it is quite apparent that most, if not all, of them signal cell proliferation.

PTKs fall into three main categories: (i) hormone and growth factor receptors; (ii) products of c-oncogenes; and (iii) products of v-oncogenes (viral oncogenes). Many forms of cancer, or some claim all forms of cancer, are associated with elevated levels of activity resulting from amplification of a PTK protooncogene, translocation of a c-oncogene which results in higher expression or point mutations which enhance the PTK activity, or a combination of these events.

Many types of data support the hypothesis that the kinase activity of the PTK is essential for the protein to transmit a proliferative signal. (i) Transfection of cells with c-oncogenes coding for PTK activity results in their transformation, where the degree of transformation is proportional to the kinase activity; (ii) nullification of the oncoprotein PTK activity by site-directed mutagenesis eliminates its transforming activity; and (iii) elevation of the oncoprotein PTK activity through mutations increases its transforming activity.

The recognition that PTKs are involved in cell proliferation identifies these proteins as specific targets for chemotherapy of proliferative diseases. It must be stressed that not only malignancies need to be considered, but also benign conditions which afflict many more people than cancer. Two such conditions should be mentioned: psoriasis and atherosclerosis. Psoriasis is the hyperproliferation of keratinocytes in the psoriatic epidermis. In psoriasis, the proliferation of keratinocytes most probably results from the over-expression of the TGFα gene within the cell. This gene amplification results in the constant secretion of TGFα and, therefore, sustained

autocrine signalling of the keratinocyte through its normal EGF receptor [7]. Therefore, it is highly likely that inhibiting the activity of the EGFR kinase may alleviate symptoms of the disease. PTK blockers can, in principle, fulfill this task. Similarly, signalling of smooth muscle cells in the intima of the walls of blood vessels by PDGF released from aggregated platelets probably plays a pivotal role in the development of the atherosclerotic plaque [8]. Action of PDGF is mediated through the PDGF receptor whose tyrosine kinase activity is triggered by the binding of PDGF to its receptor. Like in psoriasis, a valid strategy to treat this disease is to develop selective PTK blockers acting at the PDGF receptor in these diseases.

Blockers of PTKs and tyrphostins

The interest in PTK inhibitors began immediately after the discovery of the PTK activity of the first oncogene product, namely $pp60^{v\text{-}src}$ [9–11]. The bioflavonoid quercetin, known to inhibit various kinases, was tested immediately as an inhibitor of PTK activity of $pp60^{v\text{-}src}$ and found to be effective (IC_{50} = 3–4 μM). However, it was soon discovered that quercetin and its analogs are rather toxic. The toxicity of these flavone compounds probably results from their non-specificity towards PTKs. These compounds inhibit other protein kinases at almost the same potency as their inhibitory activity towards $pp60^{v\text{-}src}$. The non-specificity stems from their being competitive inhibitors of the ATP binding site which is a conserved structural feature among all protein kinases. This property of quercetin [6] is also shared by another natural compound genistein which has been found to inhibit PTKs as well as other protein kinases [5].

Since the PTK reaction involves the phosphorylation by ATP of a tyrosine residue within a particular amino acid sequence, we assumed that compounds which compete for the "substrate site", and not with ATP, stand a better chance of being more selective and non-toxic blockers of protein tyrosine kinases. Since it is more than likely that different PTKs possess different substrate sites, it is reasonable to assume that one should be able to design selective PTK inhibitors for each PTK. We suggested the general term "tyrphostins" for such compounds [12]. Indeed, it was already shown by Braun *et al.* [13], as well as by other workers, that L-tyramine and tyrosine containing peptides can serve as inhibitors for insulin receptor kinase and EGF receptor kinase. These inhibitors exhibit low affinity towards the kinases tested but their very existence encouraged us to design and synthesize non-peptide PTK inhibitors. These inhibitors can serve not only as antiproliferative agents but also as molecular tools to investigate signal transduction pathways of protein tyrosine kinases (see below).

EGF-receptor kinase inhibitors—first class of tyrphostins

In the past 2 years, we have synthesized a few dozen hydroxylated benzylidene malononitrile compounds, many of which are competitive inhibitors of the EGF receptor kinase with respect to the substrate but not with respect to ATP [12, 14]. The common

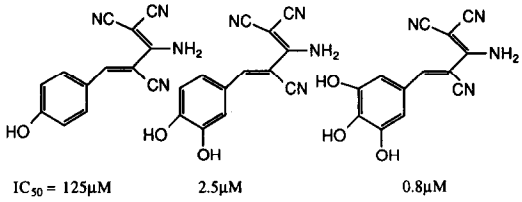


Fig. 2. Some benzenemalono nitrile tyrphostins. The increasing number of hydroxyl residues and the more elaborate structure of the side chain increase the selectivity and affinity of the compound for the EGF receptor kinase.

structural denominator which was found to be essential for effective inhibition of EGFR PTK activity was the hydroxylated *cis*-benzene malononitril (BMN) moiety conjugated to a double bond. We found that many of the EGF receptor inhibitors with the structures described in Figs. 1 and 2 are competitive with the substrate for both EGFR and insulin receptors, but are 100- to 800-fold less effective in inhibiting the kinase activity of the insulin receptor [12, 14]. The structural homology between EGF receptor and insulin receptor in the tyrosine kinase domain is limited to the ATP subdomain [15], suggesting different substrate specificity. So far, we have succeeded in synthesizing tyrophostins which inhibit EGF kinase with inhibition constants approaching $K_i = 10^{-7}$ M. Some of the many features [14] of the structure–activity relationships are summarized in Fig. 2. Our initial success in designing relatively selective tyrphostins strengthened our confidence in the basic idea that, in the long run, it will be possible to generate selective tyrphostins for each of the known PTKs (see below).

Figure 1 shows some of the best tyrphostins, the natural compound erbstatin, and the general pattern of a tyrosine containing peptide. It can be seen that tyrphostins, as well as erbstatin [16], can be aligned so that they both mimic, to some extent, the tyrosyl moiety within the polypeptide chain. Thus, one can also view tyrphostins and erbstatin as novel peptidomimetics. Tyrphostins, like erbstatin, possess the moiety of the hydroxylated phenyl ring. Tyrphostins, however, differ from erbstatin in the essential role of the nitrile residue which is *cis* to the hydroxylated benzene ring. In erbstatin, the *trans* formylamino group may play a role. Extensive substitutions around the double bond and on the ring, while preserving the *cis* nitrile position, have allowed us to reach dissociation constants in the range of 100 nM (unpublished results).

Biological activity of anti-EGFR kinase tyrphostins

The biological activities of some of the best tyrphostins (those found to inhibit the EGF receptor kinase in the low micromolar range) were tested on three different cell lines: (i) the cell line A431 clone 15 which possesses many EGF receptor molecules but, in contrast to its parent cell A431, its growth is specifically stimulated 200–250% by EGF [12, 14]; (ii) NIH3T3 cells into which the EGFR gene has been stably transfected (HER14) so that their growth is stimulated by EGF [17] and (iii) normal human keratinocytes whose growth is strongly stimulated by EGF*. In these three cell types, we found that some

* Dviz A *et al.*, manuscript in preparation.

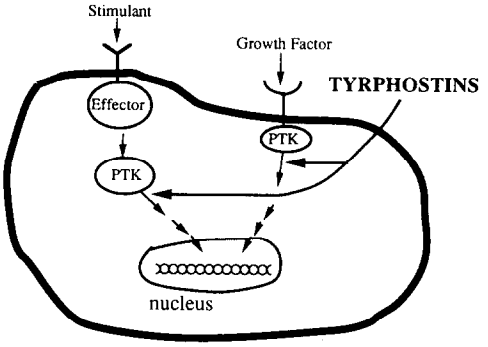


Fig. 3. Role of protein tyrosine kinases in cell proliferation. Protein tyrosine kinases function as signal transduction elements. The PTK can appear as a growth factor receptor and, therefore, as the trigger of signal transduction. It can also appear as an intermediate element of the signal transduction pathway. This may be the case of $p56^{kk}$ which may mediate signalling through the CD4 receptor on T-lymphocytes [18].

of the most effective tyrphostins inhibited *in vitro* the EGF-stimulated cell proliferation and much less effectively serum-stimulated proliferation.

Two groups of tyrphostins were identified. One group inhibits EGF-stimulated growth with IC_{50} values 5- to 10-fold lower than the IC_{50} values for the inhibition of serum-induced proliferation [14, 15]. The other group consists of compounds which discriminate only by a factor of 3 or less between EGF-dependent growth and serum-induced growth. For both groups, however, we observed that once the blocker was removed from the tissue culture medium, the cells resumed growth at the same rate and their viability remained maximal (unpublished observations). Also, the so-called "non-specific" effect was due most probably, at least in part, to the inhibition of the PTKs stimulated by serum growth factors. The behavior of tyrphostins strengthens our confidence that the compounds stand an excellent chance of becoming antiproliferative drugs with minimal toxic effects (Fig. 3).

Selectivity of tyrphostins

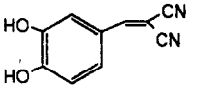
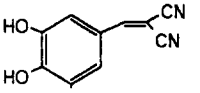
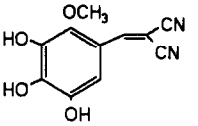
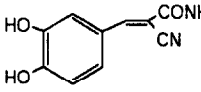
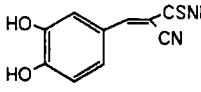
Our published and on-going studies demonstrate the feasibility of synthesizing selective anti-proliferative agents. We can now define a family of tyrphostins which potently inhibit the EGF receptor kinase and poorly inhibit the insulin receptor kinase [12, 14], and a family of tyrphostins which inhibit PDGF-dependent cell proliferation better than EGF dependent (unpublished experiments). These PDGF receptor directed tyrphostins are up to 50-fold less potent blockers of the EGF receptor kinase and EGF-dependent cell proliferation. Similar results are beginning to emerge from other systems which we are currently testing. Table 2 shows that even among the benzenemalono nitrile family of tyrphostins, selectivity is striking. These findings support the basic idea that each PTK has a specific spectrum of substrates, although they do not negate "relaxed" specificity with partial overlap, especially among related PTKs like EGFR (HER1/ErbB) [19] and neu/ErbB2(HER2) [19]. It is not yet clear whether different sets of substrates are phosphorylated by different PTKs and whether mitogenic signals converge at late common target proteins. The emerging selectivity of tyrphostins strongly suggests that at the early portion of the mitogenic signalling pathway different protein substrates are probably phosphorylated. However, the concept of "relaxed" specificity should be pursued. It is likely that tyrphostins can serve as tools to differentiate between these two possibilities and determine their relative importance.

Tyrphostins as molecular tools

Mitogenic as well as metabolic signals triggered by PTKs are the end result of the tyrosine kinase activity of these molecules. It is generally accepted that autophosphorylation of a growth factor receptor like the EGF receptor is the first event in a cascade leading to the mitogenic signal and the metabolic "awakening" of the cell. Likewise, the autophosphorylation of the insulin receptor is the first essential event in a cascade of events leading to a wide variety of metabolic signals. We have used tyrphostins to analyze signal transduction in the EGF receptor system and the insulin receptor system. In the EGF receptor system we have demonstrated that receptor autophosphorylation is inhibited only partially in intact cells, whereas the mitogenic signal and the phosphorylation of endogenous protein substrates are inhibited totally [12, 14, 17]. Under these conditions, the EGF receptor binds its growth factor and internalizes normally [17]. Thus, it seems that partially autophosphorylated receptor is recognized by the internalization apparatus.

When the effects of tyrphostins were examined in more detail, we found that they inhibit the EGF induced Ca^{2+} mobilization [21] concomitant to the inhibition of phosphatidyl inositol bisphosphate breakdown to inositol phosphates [20]. These findings support earlier findings which suggested that the EGF receptor activates phospholipase C, most probably by phosphorylating it on a tyrosine residue [21, 22]. Recently, using anti-PLC II antibodies, anti-EGF receptor antibodies, and anti-phosphotyrosine antibodies, we showed that the EGF receptor coimmunoprecipitates with PLC II and the latter becomes phosphorylated on tyrosine residues subsequent to activation by EGF of HER 14 cells, which harbour many EGF receptor molecules [21]. Tyrphostins, which were found to effectively inhibit EGF-receptor dependent phosphorylation of exogenous substrates as well as EGF receptor autophosphorylation, are also effective in inhibiting Ca^{2+} mobilization [21] and EGF-dependent inositol phosphate formation [20], and in blocking EGF-dependent phosphorylation of PLC II and its coimmunoprecipitation with the EGF receptor [21]. Interestingly, in PC12 cells, which possess much lower levels of EGF receptors, this "cross talk" with PLC is not found (Posner I and Levitzki A, unpublished observations). It is likely, but not proven, that the EGF receptor to PLC interaction occurs only in cells in which the receptor is overexpressed. Thus, the relative non-specificity of PTKs may be a function of their quantity within the cell, the potency of their kinase activity and their intracellular localization. In

Table 2. Differential inhibitory action of tyrphostins*

Compound	K_i (μM) EGFR	InSR	$p210^{bcr-abl}$
	10	1200	18
	2.2	ND	40
	1.0	ND	4.0
	2.0	410	ND
	0.85	640	9

* Data are from Refs. 12 and 14 and Anafi M, Gazit A, Gilon C, Ben-Neriah Y and Levitzki A, to be published. Abbreviations: EGFR, epidermal growth factor receptor; and InSR, insulin receptor.

malignant cells, therefore, in which PTK activity is frequently elevated, it may cause abnormal stimulation of biochemical pathways which are usually under the control of other signalling elements. In the particular case described (EGF receptor to PLC II cross talk), it is possible that such cross talk is only typical for cells with enhanced PTK activity. Thus, constant Ca^{2+} signalling induced by PTK growth factor receptors such as EGF [21] and PDGF [22] may be one of the many biochemical pathways which become aroused in the transformed cell. The demonstration that tyrphostins can block this cross talk may help identify the role of PTK to PLC interactions underlies their value as research tools. It will be of interest, for example, to examine whether the enhanced aerobic glycolysis typical of many transformed cells can also be inhibited by tyrphostins (see also below).

In another study [23], we demonstrated that a large group of PTK inhibitors which block the insulin-dependent kinase also inhibit insulin-dependent lipogenesis in the isolated rat adipocytes. Rather surprisingly these inhibitors were found not to inhibit the antilipolytic effect of the hormone. Strikingly, however, we found that one of the PTK inhibitors, t-Boc-tyrosine-amino malonic acid, inhibits both actions of insulin [23]. This inhibitor was also found to be the only one which effectively inhibits autophosphorylation of the insulin receptor. We therefore suggested that this finding supports the view that the antilipolytic effect of insulin and insulin-dependent lipogenesis are mediated by two separate signalling pathways. Lipogenesis seems to depend on the phosphorylation of intracellular target proteins, whereas the antilipolytic signal does not. Both pathways however, require as a first event, the autophosphorylation of the insulin receptor. The antilipolytic effect may be mediated by the interaction of the phosphorylated receptor with a biochemical effector system which can generate a soluble second messenger. It has been suggested time and again that some of the insulin effects are mediated by a soluble second messenger such as the inositol phosphoglycan produced by an insulin sensitive phospholipase C (for summary see Ref. 24). More recently we have shown (Zik Y and Levitzki A, unpublished experiments) that tyrphostins can effectively block the insulin-dependent activation of S6 kinases as well as the insulin-dependent tyrosine phosphorylation of p180, an intracellular substrate.

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