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Tyrosine kinases as targets for cancer therapy

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Abstract

Enhanced protein tyrosine kinase (PTK) activity correlates with the development of cancer and other proliferative diseases. The hypothesis that PTK inhibitors may be of value in the treatment of cancer led to the systematic synthesis of selective tyrosine phosphorylation inhibitors (tyrphostins) that show in vitro and in vivo anticancer activity. This review will provide an overview of research efforts in the development of tyrphostins such as AG 957, AG 1112, and AG 1318. Other tyrphostins discussed are AG 1478 and RG 13022, which are both epidermal growth factor receptor kinase inhibitors; AG 490, a Jak-2 kinase inhibitor; AG 1296, a PDGFR kinase inhibitor; and STI 571 (imatinib, Glivec®/Gleevec®; Novartis Pharma AG, Basel, Switzerland). STI 571 is now approved for the treatment of chronic myeloid leukemia and shows activity against gastrointestinal stromal tumors. The chemistry, kinetics, biological activity, and clinical potential of these compounds will be discussed.

Keywords: Tyrphostins; PTK inhibitors

1. History

In 1971, Unites States (US) President Richard Nixon signed The National Cancer Act to provide additional resources for a cure for cancer and alleviate bureaucratic entanglements that might slow research. This created a catalyst for the US and many other countries to make a massive investment into cancer research and, by 1980, major discoveries had been made. The most prominent was the dual realization that human cancer results from the activity of nonviral, endogenous oncogenes and that a major portion of these oncogenes code for protein tyrosine kinases (PTKs). PTKs are positioned in key crossroads within the cellular communication network and function as master switches. Their regulated signaling is pivotal to normal cell development and survival. Thus, it is no wonder that aberrant enhanced signaling emanating from PTKs converts these enzymes into dominant oncoproteins and results in the malfunction of cellular signaling networks. As a consequence of this malfunctioning, cancers and other proliferative diseases develop [1].

These insights led researchers to the hypothesis that signal transduction therapy may be a valid therapeutic modality for treating cancer and other diseases in which PTKs play a pivotal role [1–3]. Because PTKs represent a major portion of all oncoproteins, they take center stage as possible targets for cancer therapy. Therefore, researchers proposed designing tyrosine phosphorylation inhibitors (tyrphostins) of low molecular weight as prospective antiproliferative agents [2,4,5]. By the late 1980s, there was evidence that low-molecular-weight tyrphostins resembling tyrosine and erbstatin effectively inhibit the PTK epidermal growth factor receptor (EGFR) but are poor inhibitors of other PTKs like insulin, insulin receptor kinase, or protein kinase A [2]. This landmark finding was initially accepted with skepticism. According to the prevailing dogma at that time, the high degree of sequence homology between the active sites of protein kinases precluded the generation of selective small molecules to target specific protein kinases. However, we were encouraged by the early studies showing 1000-fold selectivity towards

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Table 1
Tyrphostins developed between 1988 and 2001

PTK	Disease(s) implicated	Trphostins generated	Year
EGFR	Cancers, psoriasis, papillomas	AG 213, RG 13022 [a], AG 1478 [b]	1988, 1991, 1994, 2001
Bcr-Abl	chronic myeloid leukemia (CML)	AG 957, AG 1112/AG 1318	1992, 1993-1994
Jak-2	Pre-B acute lymphoblastic leukemia (Pre B-ALL), multiple myeloma	AG 490, AGL 2355 [b]	1996, 2001
PDGFR	Glioblastomas, restenosis	AG 1295/6 [c], AGL 2043 [d]	1994, 1998
IGF-R	Cancers	AG 538, I-OMe-AG 538	2000

[a] First EGFR kinase inhibitor showing excellent efficacy in vivo [21].
[b] In combination with cisplatin [34,35].
[c] Compound showing excellent efficacy in inhibiting balloon-injury-induced stenosis in pigs and rats ([41,52] and Levitzki *et al.*, submitted).
[d] US Patent 6,358,954, March 19, 2002. Levitzki, A, Gazit A, Banai S, Golomb G, Gertz SD, Waltenberger J, Böhmer FD. PDGF receptor kinase inhibitory compounds. Their preparation, purification and pharmaceutical compositions including same.

EGFR vis-à-vis insulin receptor kinase with compounds of less than 300 Da [2].

In the early 1990s, we were also the first to show that tyrphostins that target the adenosine triphosphate (ATP)-binding domain can distinguish between EGFR/Her-1 and Her-2/neu, which are approximately 80% identical at the kinase domain [6]. By 1994, the feasibility of generating ATP mimics as selective PTK blockers was firmly established [7]. Mechanistic analysis of EGFR [7] and platelet-derived growth factor receptor (PDGFR) [8]. revealed that the binding of ATP and poly (Glu_6Ala_3Tyr) peptide (GAT) are independent events and are not sequential [9]. This mechanistic feature simplifies the kinetic analysis of the mode of action of novel inhibitors, which revealed that many tyrphostins compete with the substrate but are non-competitive inhibitors with respect to ATP. These agents were originally designed to be tyrosine mimics [2]. Interestingly, erbstatin was found to be a mixed-type competitive inhibitor, inhibiting the binding of both substrates (GAT and ATP) [7]. Subtle changes in the molecule produced a well-behaved structure-activity relationship (SAR) series where all the compounds were substrate competitive [2,4]. Within a short time, a number of reports appeared concerning selective PTK inhibitors that are ATP competitive [8,10–13]. These reports demonstrated that relatively small chemical changes alter the selectivity of molecules. For instance, quinazolines and quinoxalines, which have somewhat similar structures, inhibit different PTKs. Quinazolines are highly selective for EGFR [10,14]. whereas quinoxalines are highly selective for PDGFR [15]. Since then, it was only a matter of time before PTK inhibitors moved into the clinical arena. Table 1 highlights some of the most important tyrphostins developed since the mid-1980s.

2. Chemistry of PTK inhibitors

Tyrphostins, the first generation of PTK inhibitors, was found to cover a wide range of kinetic behavior. Some were substrate competitive, some ATP competitive, or bi-substrate competitive (competitive against the substrate as well as against ATP). Other compounds exhibited more complex kinetic behavior [7]. Most of the PTK inhibitors generated since have been ATP mimics. Interestingly, many tyrphostins that possess one aromatic ring and are substrate mimics[2,4,7,16]. are converted into ATP mimics once the nitrogen of the characteristic benzene malononitrile is incorporated into a second ring (Fig. 1) [7]. In our laboratory we moved from simple tyrosine mimics to two-ring systems as described in Fig. 1. Among the compounds developed over the years, few showed very good in vivo efficacy against various cancers and other diseases driven by excessive action of PTKs (Fig. 2 and Table 1).

Most of the compounds discovered by high-throughput screening efforts are ATP mimics that possess at least two aromatic rings. PTK inhibitors currently in development are ATP mimics (Fig. 3). This category includes STI 571 (imatinib, Glivec®/Gleevec®; Novartis Pharma AG, Basel, Switzerland) (Fig. 3), a drug recently approved for the treatment of chronic myeloid leukemia (CML) and currently undergoing the approval process for the treatment of inoperable gastrointestinal stromal tumors (GISTs) [17].

The feasibility of generating selective PTK inhibitors is due to the fact that, even in closely related PTKs, the minor differences in the kinase domains are sufficient to generate a difference of a few kilocalories in the binding energy of a small molecule to the site. Because 1.4 kcal/mol translates to a factor of 10 in binding affinity, it should not be a surprise that a change in a couple of hydrogen bonds and some hydrophobic interactions results in a difference of a few orders of magnitude in the affinity of an inhibitor to closely related kinases. This is illustrated by the comparison of the affinity of the Src family inhibitor PP1 to Hck as compared to its affinity to PKA. The IC 50 of PP1 to Src family kinases is 20–170 nM and above 50 μM to PKA. Thr 338 and Ala 403 within Hck ATP binding site are replaced by Met 120 and Thr 183 in PKA. These two residues are bulkier than Thr 338 and Ala 403 and “collide” with PP1. These small changes are sufficient to reduce markedly the affinity of PP1 to PKA [18].

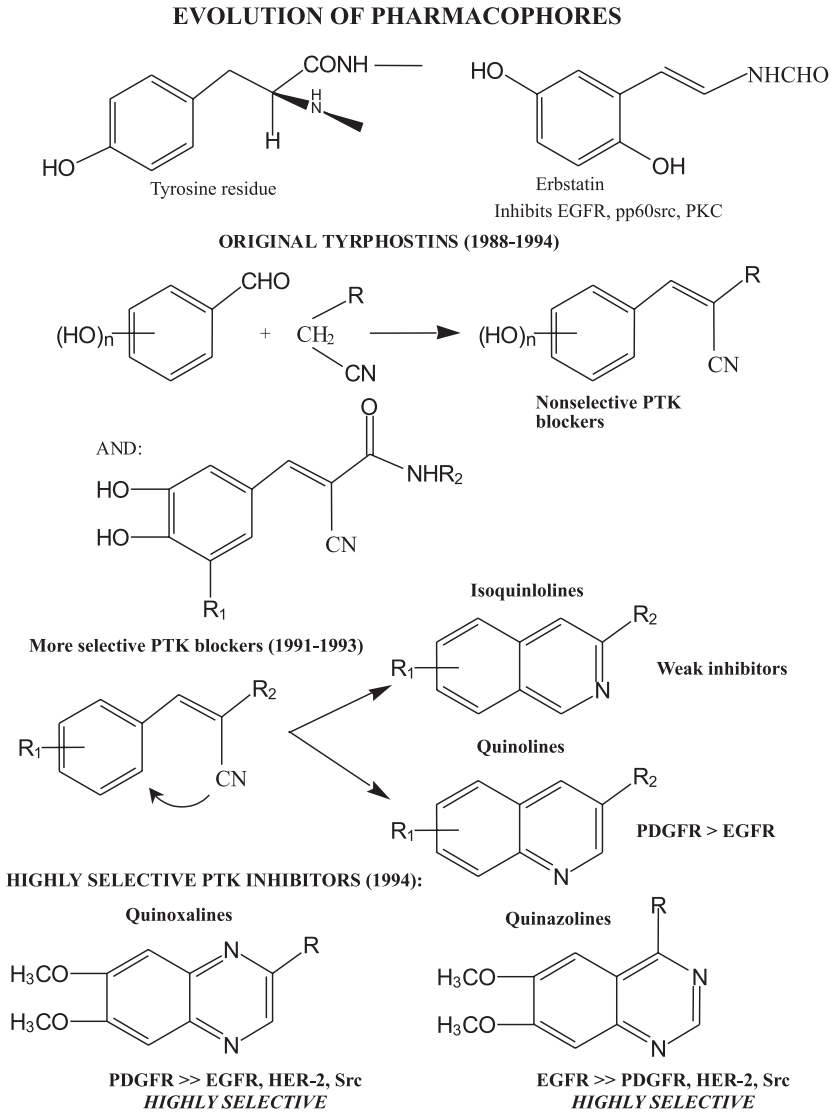

Fig. 1. The evolution of pharmacophores and their efficacy in vivo. Tyrphostins were designed on the basis of tyrosine and erbstatin and were all benzene malononitriles, many of which are substrate competitive. At a later stage, the N atom of the nitrile moiety was incorporated into a second ring, generating isoquinolines and quinolines. These compounds were found to be more selective but with rather weak affinities. The introduction of a second nitrogen, generating quinoxalines and quinozalines, improved affinity and selectivity tremendously.

3. The biological activity of tyrphostins

From the beginning of our research, it was quite clear that we needed to demonstrate the efficacy of tyrphostins in both cell cultures and in vivo. Early on, we were able to show that EGFR kinase inhibitors effectively block EGF-induced Ca^{2+}. release from intracellular stores without affecting bombesin- or bradykinin-induced Ca^{2+}. release [19]. The concentration of the tyrphostin AG 213 (RG 50864), at 100 μM, caused EGFR signaling to cease completely [19]. We were also able to utilize tyrphostins to study EGFR downstream signaling. We showed that AG 213 inhibits the phosphorylation and activation of PLC-γ (then known as PLC II) and phosphoinositide turnover [20]. These experiments established tyrphostins as useful reagents for the dissection of signal transduction pathways. The selectivity of tyrphostins regarding specific PTKs also established their non-toxicity. No adverse effects on the cells were noted, which suggested that this family of compounds might be non-toxic, even in high concentrations — as has been proven in later studies.

In 1991, it was demonstrated for the first time that the tyrphostin RG 13022, which inhibits EGFR, is able to significantly slow down the growth of an EGFR over-expressing tumor (squamous cell carcinoma in nude mice) and prolong the survival of nude mice that harbor the tumor [21]. Another important observation was that tyrphostins synergize with cytotoxic agents to inhibit the growth of Her-2 in over-expressing non-small-cell lung cancer (NSCLC) cells. The degree of synergism was found to be proportional to the level of Her-2/neu expression. It was therefore argued, for the first time, that the ef-

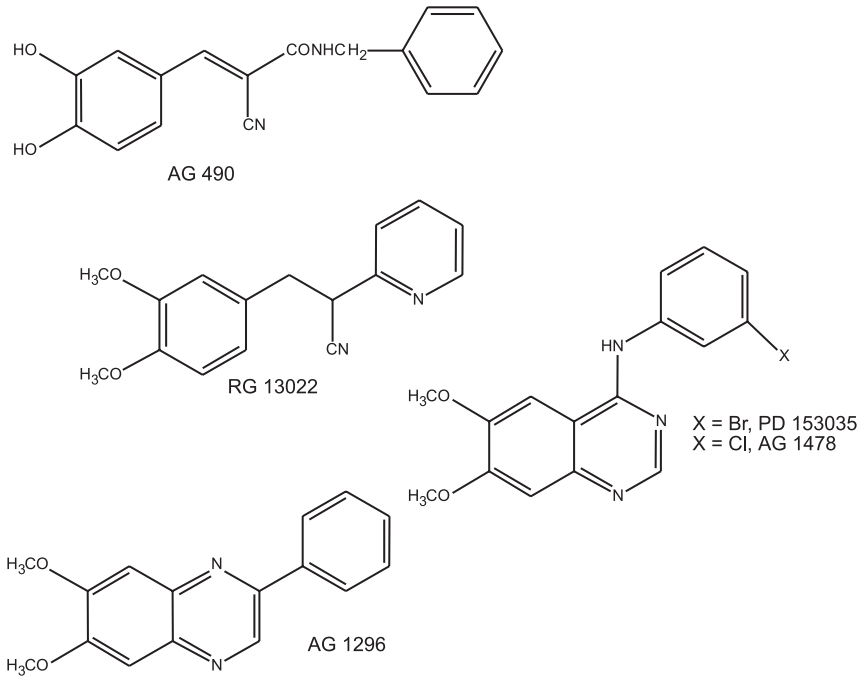

Fig. 2. Tyrphostins showing activity in vivo. RG 13022 was the first EGFR-directed tyrphostin possessing anti-tumor activity in vivo against human squamous cell carcinoma in nude mice [21]. The Jak-2 inhibitor eradicates human pre-B ALL from nude mice [32]. The quinazoline AG 1478 inhibits EGFR driven glioblastoma multiforme in nude mice [35]. The selective PDGF inhibitor AG 1296 inhibits balloon-induced restenosis in rats and pigs [52].

fect of tyrphostins could be optimized by combining them with cytotoxic drugs [22]. Today, it is understood that the main function of the Her-2/neu is to enhance anti-apoptotic signaling and thus protect cells that over-express Her-2 from stress or from pro-apoptotic signaling [23]. It seems that as the cancer progresses from low Her-2 expression of the oncoprotein to higher Her-2 expression, stress signaling is enhanced; but at the same time, the shield of anti-apoptotic signaling is also enhanced. Thus, when the anti-apoptotic shield is removed, the cells are more sensitive to genotoxic stress provided by the cytotoxic drugs cisplatin, etoposide, or doxorubicin [22]. Therefore, a combination of signaling inhibitors that block anti-apoptotic signaling and cytotoxic agents that trigger apoptosis can be an excellent therapeutic combination. As discussed below, such a combination is already being clinically tested for advanced brain glioblastoma multiforme.

4. Tyrphostins are moving to the clinic

Over the last 10 years it has been proposed that understanding signal transduction pathways and how they go awry would change how the medical community thinks about disease therapy in general and cancer therapy in particular [1,3,5,24]. Researchers suggested that signal transduction therapy would become an important therapeutic modality. Today, there are a number of new agents (signal transduction inhibitors) in clinical development. PTK inhibitors (tyrphostins) are the major portions of these agents (Table 1). This is not surprising because PTKs are the major portion of oncoproteins; therefore, from the beginning, they have been the primary targets for drug design. In our own laboratory we have developed a number of tyrphostins aimed at specific PTKs that have been identified as major players in a number of pathological situations (Table 1), with the aim of reaching the clinic. We developed AG 957, AG 1112, and AG 1318 as Bcr-Abl kinase blockers [25–27]. These compounds induce the death of K562 cells and purge Ph^+. cells from the blood of CML patients [28]. We did not further develop these compounds or attempt to optimize the compound due to lack of support. However, Novartis followed up on the subject and developed their own Bcr-Abl/PDGFR/c-Kit kinase inhibitor, STI 571 [29]. With vision and perseverance, they advanced STI 571 to successful treatment of early CML [30,31] and GIST [17], within 4 years of its discovery. CML is considered excellent for testing the hypothesis that PTK inhibitors are useful as anticancer agents for humans because, at the chronic phase, CML is almost solely driven by the oncoprotein Bcr-Abl. Thus, the likelihood that a potent Bcr-Abl kinase inhibitor would have a curative effect, even as a single agent, is a reasonable assumption. This assertion was bolstered by the finding that the Jak-2 kinase inhibitor tyrphostin AG 490 was found to eradicate Pre-B acute lymphoblastic leukemia (ALL) from severe combined immunodeficient (SCID) mice as a single agent in 1996 [32]. This finding was cited as a milestone in signal transduction therapy [33].

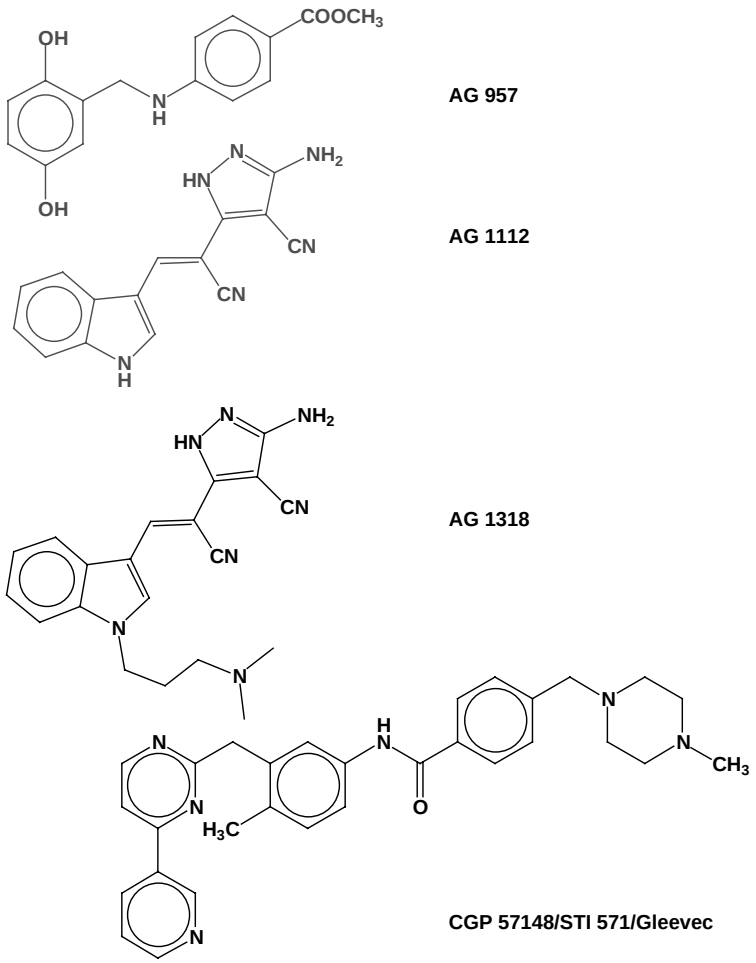

Fig. 3. Bcr-Abl kinase inhibitors. The potent and selective inhibitors of Bcr-Abl AG 957, AG 1112, and AG 1318 induce terminal differentiation and death of K562 cells. These compounds were not further developed for the clinic. CGP 53716B, known today as STI 571, is a potent and non-toxic selective PTK inhibitor, used for the successful treatment of patients with CML and GISTs.

Currently, our laboratory is involved in the clinical development of an EGFR kinase inhibitor, AG 1478 (Fig. 2), intended for the treatment of a highly malignant form of brain glioma. In this disease, EGFR is over-expressed and, at a late stage in the disease, the truncated ΔEGFR is also over-expressed. This truncated receptor confers high resistance to chemotherapy and radiotherapy. We have shown, in collaboration with Dr. Cavenee's laboratory at the Ludwig Institute for Cancer Research in La Jolla, California, that ΔEGFR induces the over-expression of the anti-apoptotic protein Bcl-X_L and the glioma cells that harbor this mutant receptor possess a very low apoptotic index and reduce the activity of caspase-3 [34]. These properties of the tumor cells are manifested both in tissue culture and in vivo (when the tumor cells are implanted in the mouse brain). When either the cells in tissue culture [34] or the tumor in vivo [35] are challenged with AG 1478, they become re-sensitized to cisplatin. Thus, AG 1478 synergizes to cause apoptosis in the glioma cells exposed to the combination of the two agents. This behavior is identical to the behavior of the NSCLC, which over-expresses Her-2/neu, as described earlier [22]. In vivo experiments, performed at the Ludwig Institute for Cancer Research in La Jolla and Melbourne, Australia in collaboration with us, demonstrate that AG 1478 synergizes with cisplatin and treatment of tumor-bearing mice with this combination prolongs their survival [35]. The AG 1478/cisplatin combination has recently been developed by the Ludwig Institute in La Jolla and Melbourne for clinical trials of brain glioblastoma multiforme, which were due to begin in January, 2002.

Another interesting molecule that has been developed is the Jak-2 inhibitor AG 490, which induces the death of Pre-B ALL cells. These cells express constitutively active Jak-2. As discussed earlier, AG 490 was found to eradicate the recurrent form of Pre-B ALL in SCID mice into which the disease had been engrafted [32]. This achievement was cited as a major advance in the proof of the efficacy of signal transduction therapy and has been considered the first successful test of its kind [33]. The compound is also highly effective against interleukin-6- (IL-6)/Jak-2-dependent multiple myeloma, both in cell culture [36] and

in vivo (data not shown). This study further demonstrated that AG 490 was highly active in combination with the Fas agonistic MAb CH-11 [36]. Interestingly, AG 490 also synergizes with IL-12, which significantly inhibits IL-6-driven multiple myeloma in nude mice [37]. More recently, AG 490 was also shown to effectively inhibit a subset of breast cancer cells derived from metastatic disease (Hua Yu, Moffitt Cancer Center, University of South Florida, Tampa, FL). Presumably, in metastatic disease, prolactin acts as an autocrine growth through Jak-2, contributing to the anti-apoptotic shield of these cells. Similar results are obtained for metastatic prostate cancer. The autocrine/paracrine IL-6 stimulation of Jak-2 is essential for the survival of metastatic non-androgen-dependent prostate cancer cells. Therefore AG 490 was found to induce apoptosis in these cells (unpublished experiments). Thus, it seems that Jak-2 emerges as a player in numerous cancers, although in the beginning this role was not appreciated. Without a doubt, Jak-2 inhibitors will eventually play an important role in cancer therapy as frequent components of anticancer drugs.

5. Drug design and the evolution of pharmacophores

Initially, it was thought that the best inhibitors for PTK would be tyrosine-mimics. Therefore, tyrphostins were designed [2] based on the model of tyrosine and the low-molecular-weight natural substance erbstatin, which blocks Src, EGFR, and PKC [38]. Within a short time, the classic Knoevenagel condensation yielded a large collection of benzene malononitrile tyrphostins [1,4,14,15,24,39,40]. Indeed, many of the compounds were found to be competitive with the substrate and non-competitive with ATP [7]. As the synthetic program progressed, it was discovered that a significant number of molecules are competitive with both the substrate and ATP [6,7] or exhibit more complex kinetics of inhibition, such as mixed-type competitive inhibition, etc. [8].

As the researchers moved to two-ring systems, such as the quinoxaline PDGFR kinase inhibitors (Fig. 1) [41], it was found that the pattern of inhibition is either ATP-competitive or mixed-type inhibition, depending on the state of the receptor. For the unphosphorylated, inactive form of PDGFR, AG 1296 is a pure ATP-competitive inhibitor. Once the PDGFR is fully activated and is auto-phosphorylated, AG 1296 becomes a mixed-competitive inhibitor [8]. Therefore, it seems that the kinase domain becomes altered once the receptor is auto-phosphorylated. Interestingly, a similar phenomenon in relation to Bcr-Abl: AG 957 exhibits a 13-fold higher affinity ($K_i = 0.75$ μM) to Bcr-Abl than to the proto-oncogenic form, c-Abl ($K_i = 10$ μM). It seems that once the oncoprotein has been activated, the kinase domain assumes a different conformation, which alters its interaction with the kinase inhibitor [25,42].

More recently, Kuriyan, in collaboration with our laboratory, elucidated the three-dimensional structure of inactive Hck in complex with the Src-family inhibitor PP1 [18]. In the structure, one clearly sees that PP1 occupies the ATP-binding site. Now we have found that PP1 drops out of competition with ATP when Src assumes a fully active state (data not shown). This finding hinders any efforts to improve on PP1 on the basis of the Hck-PP1 structure. All of the PTK inhibitors that have been reported on over the past 7 years are ATP competitive. Consequently, all the compounds currently in clinical and pre-clinical development are ATP-mimics. However, substrate mimics will become extremely useful in the future. They will most probably be more effective at lower doses because they would not have to compete against the high intracellular levels of ATP [43]. From the theoretical point of view, bi-substrate PTK inhibitors should work best [7]. With the current rapid advance in high-throughput screening and evaluation of new molecules, it will not be long before it is determined whether this assumption is correct.

6. PTK inhibitors — agents to prevent cancer?

Recent studies suggest that genistein, a natural PTK inhibitor present in soy beans, is responsible for a lower incidence of metastatic prostate cancer in countries where these beans are an important component of the diet [44]. A mechanistic study suggests that genistein induces the adhesion of prostate cancer cells, functionally antagonizing the first step in metastasis formation. It seems that the inhibition of focal adhesion kinase (FAK) activation and phosphorylation plays a role in the early prostate cell adhesion [45]. Phase I clinical trials for the prevention of metastatic cancer are underway. Another interesting example is the finding that EGFR kinase inhibitors induce growth arrest, apoptosis, and differentiation of HPV 16 immortalized keratinocytes. It seems that these inhibitors are effective because the over-expressed EGFR and its ligands in these cells drive their proliferation and survival. It has therefore been suggested that local application of EGFR kinase inhibitors to papilloma lesions may eradicate the virally transformed cells and thus block progression to cervical cancer [46,47]. These inhibitors also block the growth of psoriatic keratinocytes, which, like papilloma-infected cells, over-express EGFR and its ligands [48–51]. Future work will establish whether signal transduction inhibitors and PTK inhibitors (tyrphostins) will be utilized for cancer prevention.

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