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Protein kinase inhibitors — potential chemotherapeutic agents*

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Protein kinase inhibitors, widely exploited for elucidation of the biological functions of kinases, have more recently come under active consideration as potential chemotherapeutic agents for tumour and other diseases. A brief overview is presented of diverse approaches to the design and development of selective protein kinase inhibitors, and related problems such as donor and acceptor specificities, stereochemical aspects, emerging relationships between protein, sugar and nucleoside kinases. In particular, and contrary to popular belief that ATP-competitive inhibitors cannot be selective because of the close homology of the ATP catalytic sites, numerous examples are presented of such inhibitors which are both potent and selective for a given kinase or class of kinases. Some of these are undergoing preclinical trials. Attention is also directed to the role of cellular and viral protein kinases in the life cycle of viruses, and the potential of these enzymes, especially those encoded by, and essential for replication of, a given virus as targets for antiviral chemotherapy.

Protein phosphorylation, the most prevalent post-translational process in living cells, is catalyzed by protein kinases, comprising the largest known family of proteins. The number of such kinases in eukaryotic cells, identified by purification to homogeneity, by molecular cloning and, more recently, by PCR-based approaches, has increased from 75 in 1987 to more than 220 by April 1994, and this increase shows no signs of abating. It is estimated that vertebrate genomes may encode more than 2000 PK genes, i.e. as much as 3% of the complete genome [1]. More than 30% of intracellular proteins exist in a phosphorylated form.

The foregoing protein kinases play key roles in virtually every aspect of signal-transduction in eukaryotes, ranging from primary transmembrane signalling to control of transcription, translation and cellular metabolism. The search for, and use of, specific inhibitors of individual kinases was early recognized as an important element in attempts to define their biological functions. Bearing in mind the close links between constitutively activated signal transduction pathways and various pathological states, e.g. carcinogenesis [2], it is surprising that only in the past 4–5 years has attention

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Abbreviations: PK, protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PTK, protein-tyrosine kinase; CK-I and CK-II, protein kinases CK-I and CK-II (previously known as casein kinases 1 and 2); EGF-R, epidermal growth factor receptor; MLCK, myosin light chain kinase; cdk, cyclin-dependent kinase; NTP, nucleoside 5'-triphosphate.

been actively directed to the potential use of PK inhibitors as chemotherapeutic agents.

CLASSIFICATION OF PROTEIN KINASES

Protein kinases are phosphotransferases which transfer the γ-phosphate of an NTP donor (usually, but not always, ATP; see below) to an amino-acid side-chain. Although up to nine amino-acid residues have been shown to be acceptors, the majority of identified vertebrate kinases are embraced in two groups: (a) those which phosphorylate the target hydroxyl group on the β-carbon of serine and/or threonine, referred to as Ser/Thr kinases, and (b) those which phosphorylate the phenolic hydroxyl of tyrosine, referred to as protein-tyrosine kinases (PTK). It was subsequently found that some PKs are capable of phosphorylating all three amino acids, and these are referred to as "dual-specificity" protein kinases, recently reviewed by Lindberg *et al.* [3].

Some Ser/Thr kinases may be further classified by the nature of their activators, e.g. dependence on cAMP, Ca^{2+}/calmodulin, Ca^{2+}/ /phospholipid, etc. These possess a so-called regulatory domain containing a sequence motif denoted as the pseudo-substrate site which, in the resting state, interacts with, and suppresses the activity of, the catalytic domain. This "inhibition" is relieved when an activator or second messenger, e.g. cAMP, cGMP, Ca^{2+}, diacylglycerol, phorbol ester, binds to the regulatory domain.

Another type of dual specificity has been proposed for Ser/Thr kinases, based on the stereochemistry at the α-position of the residue undergoing phosphorylation. In a series of ongoing investigations by Lawrence and coworkers [4] on the active-site substrate specificity of such kinases towards synthetic peptide substrates with C-terminal alcohols, PKA and PKC both efficiently phosphorylated achiral residues. But, whereas PKA phosphorylated residues with the same stereochemistry as that in L-serine, PKC phosphorylated α-configurational isomers corresponding to both L- and D-stereoisomers. Extension of this approach to other members of the Ser/Thr kinase family, as well as to other kinases, should be of considerable interest, and probably of utility in the design of more specific inhibitors.

The foregoing does not take cognizance of the fact that the Ser/Thr kinases of group (a), above, phosphorylate a primary hydroxyl in Ser and/or a secondary hydroxyl in Thr. It is noteworthy, in this context, that nucleoside kinases, with possibly one reported exception, phosphorylate only the primary 5′-hydroxyl of nucleosides, but not the secondary 2′- and 3′-hydroxyls [5]. There is, actually, little quantitative information regarding the susceptibility to, and relative extent of, phosphorylation of Ser and Thr residues by a given Ser/Thr kinase. It would clearly be expedient to determine the effect of a Ser/Thr kinase inhibitor on the relative extent of inhibition of phosphorylation of the two residues, as well as of Tyr residues with a dual-specificity kinase.

One member of the Ser/Thr kinases, PKC, is involved in a particularly wide variety of diverse processes. It is not a single entity, and is comprised of at least 12 closely related polypeptides (isotypes). Such heterogeneity points to unique intracellular functions for the individual isotypes [6]. It is consequently of interest, as noted below, that some newly-developed PKC inhibitors are capable of discrimination between several of the isotypes.

Attention should also be directed, at this point, to the emergence of some relationship between protein kinases on the one hand, and sugar kinases and nucleoside kinases on the other. There are now several documented examples of sugar kinases with protein kinase activity, e.g. hexokinase 1 from rat brain undergoes autophosphorylation and also phosphorylates other proteins ([7] and references cited). Furthermore, the product of the *UL97* gene of human cytomegalovirus (HCMV), which is a protein kinase homologue, phosphorylates an unusual nucleoside analogue, *viz.* the antiviral acyclonucleoside Ganciclovir, apparently also stereospecifically ([8, 9] and references cited). This ability of some PKs to phosphorylate small monomeric non-peptide molecules is rather striking and invites further investigation.

TARGET SITES FOR INHIBITION OF PROTEIN KINASES

Screening procedures have been widely applied in the search for inhibitors, frequently, as in the case of antibiotics, in fermentation

broths, plant extracts, etc. Even when only partially succesful, they may still provide lead compounds for further development by synthetic procedures, e.g. staurosporine analogues from *Streptomyces* and other organisms. The synthetic procedures usually take into consideration the potential target sites in a kinase molecule, with a view to attainment of both potency and specificity, and include the following:

- (a) The binding sites for activator or second messenger-dependent Ser/Thr kinases.
- (b) The regulatory domain of Ser/Thr kinases. Synthetic analogues of these "pseudo-substrate" domains are capable of potent selective inhibition in *in vitro* systems. But their potential use *in vivo* is questionable because of their inability to traverse the cell membrane, as well as their susceptibility to proteases.
- (c) Structural analogues of the NTP phosphate donor, usually, but not always, ATP, and of the adenosine or even adenine moiety. Although NTP analogues do not readily traverse the cell membrane, they have proven useful in *in vitro* studies [8]. As shown below, structural analogues of the purine ring have been developed as effective and useful inhibitors.
- (d) Low-molecular mass analogues which mimic the target amino-acid acceptor, succesfully exploited in the design of PTK inhibitors [10].
- (e) Further development by synthetic methods of structural analogues initially discovered by virtue of their observed biological effects, e.g. purine analogue inhibitors of cdk kinases, halogenated benzimidazole inhibitors of CK-I and CK-II, structural analogues of the staurosporine aglycon as selective inhibitors of various protein kinases, discussed below.
- (f) Bisubstrate analogue inhibitors, which combine in a single molecule the structural elements of the NTP donor, with additional selectivity towards a given kinase provided by structural elements which mimic the amino-acid receptor moiety. The design of such inhibitors has been reviewed for various enzyme systems by Broom [11], and their applications to PTK inhibitors by Burke [10].
- (g) Computer 3D-database pharmacophore searches, combined with molecular modeling and chemical syntheses, an approach that culminated in the development of lead compounds which compete with phorbol esters that bind to and activate PKC [12].

Bearing in mind the large number of PKs with different specificities, establishment of selectivity for a given kinase is far from simple, e.g. Genistein, long known as an inhibitor of PTKs, was recently found to be a moderate inhibitor of protein-histidine kinase, a member of another class of PKs, the protein N-kinases, extensively reviewed by Matthews [13]. Furthermore, whereas Genistein is an ATP-competitive inhibitor of PTKs, its inhibition of protein histidine-kinase is non-competitive *vs* ATP [13], pointing to different mechanisms of inhibition in the two cases. Of additional interest, in this context, is a report that hydroxy-2-naphthalenylmethylphosphonate, an inhibitor of PTKs, but not of Ser/Thr kinases such as PKA and PKC, inhibits both Ser and Tyr phosphorylation by the dual-specificity human insulin receptor kinase [14].

HALOGENATED BENZIMIDAZOLE INHIBITORS OF CK-I AND CK-II

The ubiquitous protein kinases CK-I and CK-II were previously known as casein kinases I and II, because of their propensity to phosphorylate acidic proteins like casein. The term "casein kinase" is now considered inappropriate, inasmuch as casein is apparently not a physiological substrate.

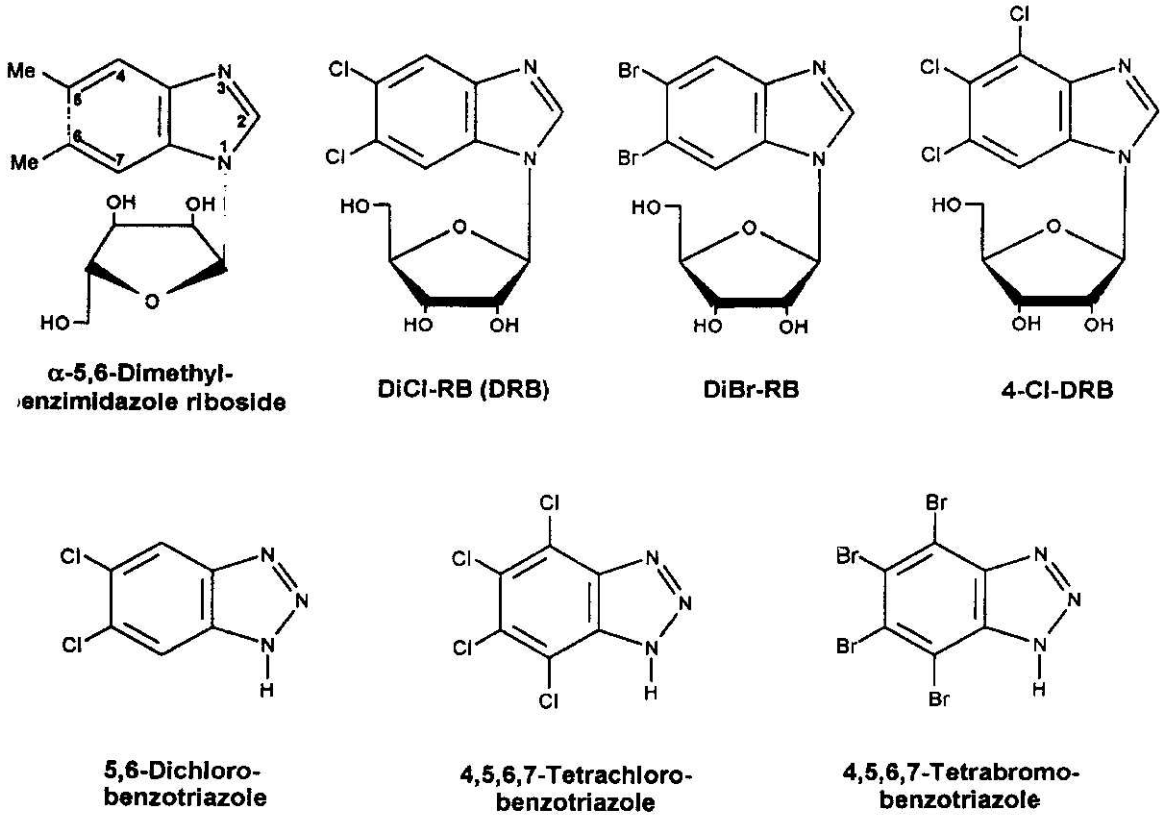
Notwithstanding the large number of known natural substrates of these enzymes, more than 100 for CK-II [15], many of them phosphorylated intracellularly by this enzyme, surprisingly little effort has been directed to development of specific inhibitors. Apart from several exceptions [8], a distinctive characteristic of CK-II, but not CK-I, is its known potent inhibition by polyanions, particularly heparin [16, 17]. But the high charge and molecular mass precludes the use of such molecules as intracellular inhibitors.

Halogenated benzimidazole inhibitors of CK-II were discovered in a roundabout manner through their biological effects. Following the demonstration in the 1950's that the α-anomer of 5,6-dimethylbenzimidazole riboside (see Scheme 1) is a key constituent of vitamin B_{12}, several structural analogues of this unusual

nucleoside were synthesized and their effects on cell proliferation examined. One of these, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB, see Scheme 1) was found to be an effective reversible inhibitor, initially shown to be due to inhibition of RNA synthesis, and subsequently to specific inhibition of mRNA, but not rRNA or tRNA, transcription.

The foregoing prompted investigations of the effects of various structural analogues of DRB (several of which are shown in Scheme 1) on mRNA transcription in an *in vitro* system, with the additional assumption that the ATP requirement for transcription indicated involvement of a protein kinase. Using a purified preparation of calf thymus CK-II, it was found that the inhibitory effects of various DRB analogues on transcription paralleled those on CK-II activity, e.g. the 5,6-dibromo analogue of DRB proved to be a 5-fold better inhibitor of both mRNA transcription and CK-II activity. Moreover, addition of CK-II partially reversed the inhibitory effects of these analogues on transcription. This was, in fact, the first demonstration, albeit indirect, of the role of a phosphorylation step in the transcription process [18]. The only partial reversal of the inhibitory effect of DRB, confirmed by Horikoshi *et al.* [19], is now interpretable by the fact that several protein kinases are involved in mRNA transcription.

The finding that DRB is an inhibitor of CK-II was, in turn, extended to an examination of the inhibitory properties of other halogenated benzimidazole glycosides *vs* rat liver CK-II [20]. It was then noted that these also inhibited CK-I, and that several weakly discriminated between the two enzymes. Further studies on the corresponding enzymes from plants (maize seedlings) [21] showed that a trichloro analogue of DRB (4-Cl-DRB, Scheme 1) was more potent than DRB, and that the 5,6-dichloro analogue of 2-azabenzimidazole (benzotriazole, Scheme 1), but not its riboside, exhibited a 10-fold discrimination between the two enzymes in favour of CK-II (see Table 1). This discrimination was further markedly enhanced with the tetra-



Scheme 1. α-5,6-Dimethylbenzimidazole riboside, a key constituent of vitamin B_{12}, and the lead compound which initiated development of halogeno benzimidazole glycosides, and halogeno 2-azabenzimidazoles (benzotriazoles) as inhibitors of protein kinases CK-I and/or CK-II.

Additional analogues may be found listed elsewhere [20–22]. DRB (DiCl-RB) = 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole; DiBr-RB = 5,6-dibromo analogue of DRB.

halogeno congeners of benzotriazole [22], as shown in Table 1.

All the foregoing inhibitors were competitive with respect to ATP, and GTP with CK-II, for which GTP can replace ATP as donor [16, 17]. And, despite the prevalent opinion that ATP-competitive inhibitors may lack specificity, several of the analogues were shown to be inactive or only feeble inhibitors of PKA, PKC and several protein tyrosine kinases [20, 22], one of which phosphorylates casein.

Table 1

Representative values of K_i for inhibition of protein kinases CK-I and CK-II from various sources by halogenated benzimidazole ribosides and halogenated 2-azabenzimidazoles (benzotriazoles).

Analogue[a]	K_i (μM)	
	CK-I	CK-II
5,6-DiCl-RB (DRB)	28	24
5,6-DiBr-RB	12	6
4,5,6-TriCl-RB (4-Cl-DRB)	8	4
5,6-DiCl-2-azaB	100	10
4,5,6,7-TetraCl-2-azaB	> 85	5
4,5,6,7-TetraBr-2-azaB	> 75	0.2–0.6

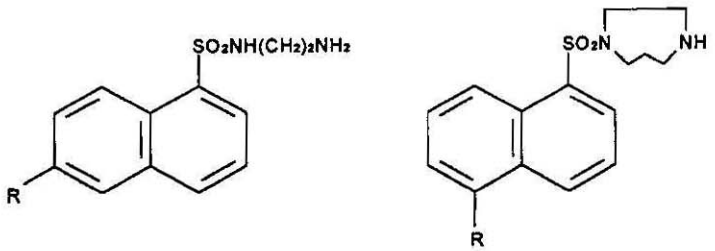
[a]See Scheme 1 for structures. RB = ribofuranosylbenzimidazole; 2-azaB = 2-azabenzimidazole.

It should be noted that, amongst the DRB analogues, fluoro-substituted compounds were only weakly inhibitory, replacement of a bromine by iodine enhanced inhibition [20], and an increase in the number of halogeno substituents was also effective [20–22]. This is reminiscent of the reports of Hidaka *et al.* [23] on monohalogeno naphthalene sulfonamide inhibitors of myosin light chain kinase (MLCK), where successive replacement of a hydrogen by Cl, Br, I led to progressively lower values of K_i (Scheme 2), ascribed to increasing hydrophobicity of the ring system, discussed elsewhere [21]. In retrospect, it appears likely that the effectiveness of these MLCK inhibitors could be further enhanced by introduction of additional halogeno substituents (see also article by Powis *et al.* in this number on halogeno naphthoquinone and quinoline quinone inhibitors of phosphatidylinositol-3-kinases, potential targets for anticancer drug development.)

The halogenated benzimidazole ribosides have been shown to inhibit mRNA transcription in the giant salivary gland cells of *Chironomus tentans* [24] and in HeLa cells [18]. We have recently found that the tetrahalogeno 2-azabenzimidazoles are effective inhibitors of phosphorylation of the low-molecular mass acidic surface proteins of the 60S ribosomes of yeast; and that this can be profited from to follow the role of CK-II in regulation of phosphorylation of these proteins in intact yeast cells (Szyszka, R., Grankowski, N. & Shugar, D., in preparation).

PURINE ANALOGUE INHIBITORS OF CYCLIN-DEPENDENT KINASES (cdk)

It should be noted that benzimidazole and 2-azabenzimidazole are analogues of the purine ring. It had elsewhere been reported that 6-dimethylaminopurine is an inhibitor of cell division in embryos, leading to identification of N^6-(Δ^2-isopentenyl)adenine as an inhibitor of



R	K_i (μM)
H	48
Cl	13
Br	7
I	3

R	K_i (μM)
Cl	3.8
Br	1.7
I	0.3

Scheme 2. Two series of halogeno naphthalene sulfonamide inhibitors of myosin light chain kinase (MLCK), showing the progressive decrease in inhibition constants (K_i) on replacement of H by Cl, Br, I. Data from ref. [23].

p34/cyclin B kinase [25]. Bearing in mind the essential role of cyclin-dependent kinases (cdk) in regulation of the cell division cycle, and the reported implication of natural cdk inhibitors in causes of cancer, Veselý *et al.* [25] conducted a structural-activity relationship study on more than 80 structural analogues of purine, all of which were tested against 35 widely different highly purified PKs. This culminated in the finding that 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (code-named Olomoucine, see Scheme 3) is a relatively specific inhibitor of several cdk kinases, with IC_{50} values in the low micromolar range. By contrast, N^6-(Δ^2-isopentenyl)adenine is a more general inhibitor with IC_{50} values in the range 50–100 μM.

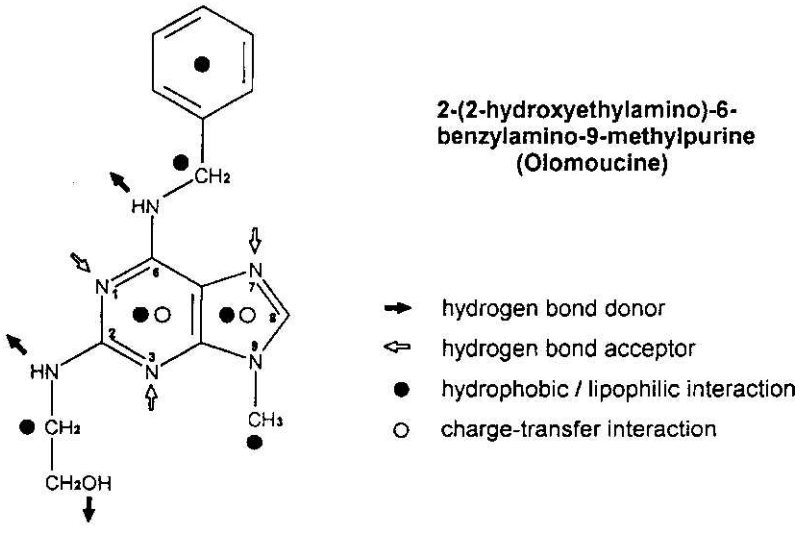
Amongst the 35 kinases tested, only 5, proline-directed kinases of the cdk family, were significantly inhibited by micromolar concentrations of Olomoucine, thus underlining its specificity, and notwithstanding that it was, like the other compounds, competitive with respect to ATP. It was inactive *versus* other key cell cycle enzymes such as nucleoside diphosphate kinase, DNA topoisomerases I and II, DNA polymerases α and δ, and protein-tyrosine phosphatases cdc25A and pyp3.

Further structure-activity relationship studies, with Olomoucine as lead compound, may well result in more potent inhibitors with K_i values in the nanomolar range. Preliminary analyses by X-ray diffraction of cdk2/Olomoucine crystals demonstrated location of the purine ring moiety in the hydrophobic pocket which binds ATP in the crystal structure of cdk2, consistent with Olomoucine being competitive *vs* ATP. Furthermore, it was found that the purine ring N(7) must be free, presumably to act as a hydrogen bond acceptor; and that an alkyl, but not a sugar, substituent at N(9) is essential (see Scheme 3).

In the foregoing, Olomoucine was found to inhibit *in vitro* the M-phase-promoting factor activity and DNA synthesis in metaphase-arrested *Xenopus* egg extracts, as well as the essential replication factor (licensing factor) ensuring DNA replication only once in each cell cycle. *In vivo* the compound inhibited the G2/M transition in the starfish oocyte. Also cited are results of preliminary tests of inhibition of certain steps in the cell cycle which show promise for development of Olomoucine as an antitumour agent and, possibly, in Altzheimer's disease.

Further structure-activity relationship studies might well consider replacement of the purine ring of Olomoucine by such ring systems as benzimidazole, 2-azabenzimidazole and pyrazolopyrimidine rings. And, bearing in mind the significant role of halogenation on the inhibitory properties of the benzimidazole and benzotriazole inhibitors of CK-I and CK-II, and of the naphthalene sulfonamide inhibitors of MLCK (described above), halogenation of the benzene ring of Olomoucine might well prove to be a promising new lead for more potent inhibitors.

The foregoing stands in contrast to another, somewhat more sophisticated, approach to the design of orally therapeutically active non-peptidal low-molecular mass ligands which interact with macromolecules such as the somatostatin receptor, the angiotensin receptors, etc.,



Scheme 3. Structure of Olomoucine, a potent and selective inhibitor of cyclin-dependent protein kinases. The arrows and symbols indicate various sites which may interact with the enzyme molecule, but without consideration of conformational features due to rotation of the substituents about single bonds. See text for further details. Adapted from ref. [25].

and are built up on a central core (such as the adenine ring in Olomoucine, above) denoted as the scaffold. To the latter are then attached substituents which mimic peptide side-chains and their locations derived from X-ray diffraction structures of the macromolecule. For example, β-D-glucose was selected as the scaffold for design of a mimic of a peptide β-turn, leading to development of blocking agents of the somatostatin, β-adrenergic and other receptors of therapeutic interest, reviewed recently by Hirschmann *et al.* [26].

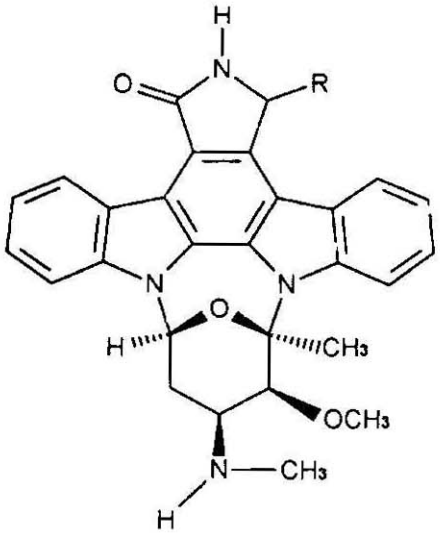
STAUROSPORINE OFFSPRING INHIBITORS

Staurosporine is a microbial alkaloid initially isolated as a *Streptomyces* fermentation product, and then shown to be a very potent inhibitor of PKC, with an IC_{50} of 3 nM. The same strain also produced the equally effective inhibitor UCN-01. An additional member of this family, all with an indolocarbazole ring system (Scheme 4), designated as K252a, is a poor inhibitor of PKC, but quite effective against phosphorylase kinase. However, despite their potency, all three are relatively non-selective [27].

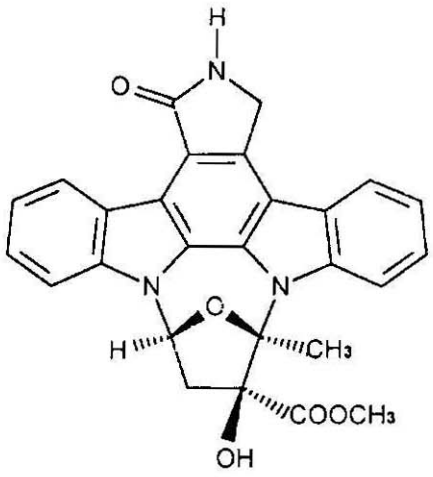
Some attempts have been made to modify or/and enhance the selectivity of the foregoing compounds by chemical modification, e.g. Nakanishi *et al.* [28] prepared KT5926, the C(14)-n-propoxy congener of K252a. This inhibited MLCK competitively with respect to ATP, with K_i approx. 18 nM, hence similar to the K_i for K252a. But, unlike the latter, which inhibits other kinases to a similar extent, the K_i values of KT5926 were much higher with PKA (1200 nM), PKG (160 nM) and PKC (725 nM), so that it is markedly more selective for MLCK, reflected also by results of biological studies with the use of both inhibitors.

Staurosporine is a good inhibitor of the proteolytically generated catalytic fragment of PKC, with no effect on binding of phorbol esters to the regulatory domain, in line with the finding that K252a competes with ATP, but not with Ca^{2+} or phospholipid. It follows that these inhibitors interact with the ATP binding site on the catalytic domain common to protein kinases.

The most comprehensive analysis of the differing susceptibilities of PKs to staurosporine is that of Meggio *et al.* [29], who conducted a systematic study on 20 kinases, most with IC_{50} values in the low nanomolar range. For all the enzymes, staurosporine was an ATP-competitive inhibitor. By contrast, inhibition with respect to the phosphate acceptor was uncompetitive with Ser/Thr kinases, and non-competitive with PTKs, pointing to different mechanisms of catalysis by the two classes of enzymes. Computer modeling, based on the crystal structure of PKA, in conjunction with sequence analysis, indicated that the low sensitivity to staurosporine of one of the enzymes, CK-II, is due to the bulkiness of three residues, Val^{166}, Phe^{113}, Ile^{174}, which are homologous to Ala^{70},



R = H Staurosporine
R = OH UCN-01



K-252a

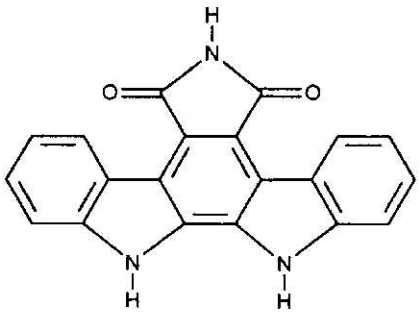
Scheme 4. Structures of staurosporine and related microbial fermentation products, all three of which are potent, but reltively non-specific, inhibitors of protein kinases.

Met^{120}, Thr^{183} in PKA, a kinase sensitive to staurosporine. The latter three residues are either conserved, or replaced by less bulky ones, in kinases sensitive to staurosporine. A chemically modified analogue of staurosporine, less effective *versus* staurosporine-sensitive kinases, proved more effective *versus* CK-II, but less so *versus* a CK-II mutant in which His^{160} was replaced by aspartic acid. It was inferred from the foregoing that the catalytic sites of PKs are sufficiently divergent so that an ATP-competitive inhibitor can exhibit selectivity, in accord with experimental results presented throughout the present text.

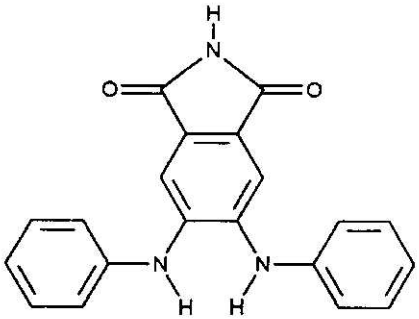
A turning point in the now widespread use of staurosporine as a lead compound for development of selective kinase inhibitors was the observation that, on removal of the sugar moiety of K252a, the resulting indolocarbazole aglycon (Scheme 5) still retained moderate inhibitory activity, suggesting that this rigid aromatic ring system interacts with kinases, probably in the hydrophobic pocket where the adenine moiety of ATP is bound ([27] and references cited). This finding has since been, and continues to be, exploited to develop structural analogues of the indolocarbazole ring system (Scheme 5) with remarkable selectivity and potency *versus* widely different PKs, despite the fact that they are ATP-competitive, and illustrated by the following examples.

Bisindolylmaleimide inhibitors of PKC

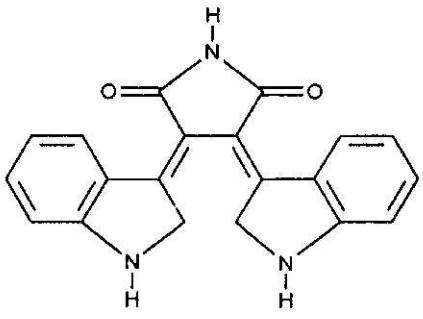
The potential of PKC as a target for anticancer treatment has been recently reviewed [30]. In concerted, and ongoing, efforts to develop highly specific inhibitors of PKC for therapeutic purposes, including treatment of autoimmune diseases such as rheumatoid arthritis, Davis *et al.* ([31] and references cited) selected as the lead compound a non-planar analogue of the staurosporine aglycon. The latter, which is planar, was considered a potential intercalator of DNA, hence with possible attendant cytotoxicity. The lead compound decided upon was *bis*indolylmaleimide (Scheme 5), for which molecular mechanics calculations pointed to a significant barrier to coplanarity, confirmed by X-ray diffraction. Subsequent chemical modifications led to three compounds with IC_{50} values in the nanomolar range, and with a several-hundred-fold selectivity relative to PKA and phosphorylase kinase (Scheme 6). The compounds were also shown to inhibit PKC within intact platelets and T-cells, and to be good *in vitro* inhibitors of antigen-driven T-cell



Staurosporine aglycon



***Bis*anilinophtalimide**



***Bis*indolylmaleimide**

Scheme 5. Structures of the aglycon of staurosporine (see Scheme 4), a planar molecule, and of the structurally related, but non-planar, bisanilinophthalimide and bisindolylmaleimide, both of which have been employed for development of more selective inhibitors of protein kinases. See text for further details.

proliferation, with IC_{50} values comparable to those for inhibition of intracellular PKC.

All inhibitors were ATP-competitive, again confirming ability to attain selectivity with such inhibitors. The claim for high selectivity towards PKC would, however, have been more convincing if they had been tested against a wider variety of enzymes (see next section).

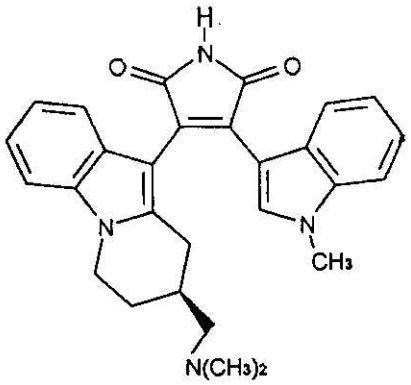
Noteworthy is that two of the inhibitors exhibited some discrimination between five PKC isotypes. These did not include PKC δ, which would have been extremely interesting, inasmuch as this isotype has since been reported to accept GTP as the phosphate donor six-fold more effectively than ATP for autophosphorylation, with concomitant incorporation of a larger amount of phosphate. Moreover, phosphopeptide mapping demonstrated that different sites were phosphorylated with ATP and GTP [32].

*Bis*anilinophthalimide inhibitors of EGF-receptor protein-tyrosine kinase

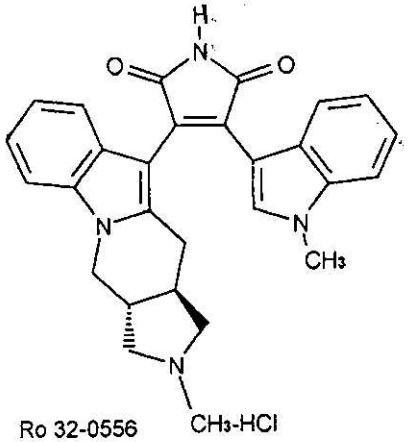
In a manner analogous to the approach of Davis *et al.* [31], Trinks *et al.* [33] and Buchdunger *et al.* [34] selected as lead compound the staurosporine aglycon structural analogue *bis*anilinophthalimide (Scheme 5) which, surprisingly, led to a series of potent and selective inhibitors of the epidermal growth factor receptor (EGF-R) protein-tyrosine kinase (PTK), and correspondingly poor inhibitors of PKC. It should, however, be noted that the IC_{50} values for these were in the micromolar range, as contrasted with those for the *bis*indolylmaleimide inhibitors, above, which were in the nanomolar range.

In contrast to the staurosporine aglycon, which is planar, and the *bis*indolylmaleimides, which are in a bowl-shaped conformation, the *bis*anilinophthalimides were found to exhibit an asymmetrical propellor-shape conformation. Since all three classes are ATP-competitive inhibitors, the conformational differences between them probably contribute to their different selectivity profiles. Hence, notwithstanding the pronounced sequence homology of the ATP-binding regions of protein kinases, ATP-competitive inhibitors are once again shown to be capable of selectivity. This may be further rationalized by the fact that ATP is usually weakly bound (K_i = 2–20 μM), and that even minor amino-acid substitutions in the vicinity of the ATP binding pocket may modify the enzyme surface, and hence affinity for an inhibitor, as also underlined elsewhere [8, 29].

The most active and selective amongst the above inhibitors, designated as DAPH-1, had an IC_{50} below 1 μM for inhibition of the EGF-R holoenzyme from A431 cells and the purified recombinant enzyme; whereas for a wide variety of other kinases the IC_{50} ranged from 16 to



Ro 32-0432



Ro 32-0556
Ro 32-0557

	IC_{50} (nM)		
	PKC	PKA	Phosphorylase kinase
Ro 32-0432	17	22400	15600
Ro 32-0556	8	5500	3800
Ro 32-0557	5	2800	1700

Scheme 6. Three structurally related analogues of the bisindolylmaleimide shown in Scheme 5, and their IC_{50} values for inhibition of protein kinases PKC, PKA and phosphorylase kinase.
Note the high selectivity for PKC. Data from ref. [31] and references cited.

> 500 μM, with somewhat lower values for two PKC isotypes, PKC α (6 μM) and PKC β_2 (5 μM). DAPH-1 concentrations for inhibition of EGF-R PTK activity *in vitro* and in intact cells were comparable, pointing to efficient transport across the cell membrane. The compound was also shown to exhibit antitumour activity *in vivo* at well-tolerated doses.

Additional structural analogues of the staurosporine aglycon

Interest in modified staurosporine aglycon analogues as PK inhibitors continues to hold the stage, largely directed to more potent and selective inhibitors of PKC. Hendricks *et al.* [35] have described a series of aryl-indolylmaleimides, one of the most active of which, with an IC_{50} = 3 nM as compared to 500 nM for PKA and totally inactive *vs* $p60^{src}$ PTK, exhibited antitumour activity in several *in vitro* and *in vivo* models.

Kleinschroth *et al.* [36] prepared a number of lactam and imide indolocarbazoles which led to inhibitors of PKC in both classes of compounds, with fairly good discrimination *versus* other PKs, e.g. one code-named Go-7852, with IC_{50} = 0.03 μM (PKC), 24 μM (PKA), 9.4 μM (PKG), 5.5 μM (MLCK), 100 μM (PTK). This compound possessed the additional advantage in that it contains an aminoalkyl function permitting preparation of a water-soluble hydrochloride salt. No biological data were given, but another compound in this series, Go-6976, was earlier reported to inhibit human immunodeficiency virus type 1 (HIV-1) induction from latent U1 cells at non-toxic concentrations [37].

ANILINOQUINAZOLINE INHIBITORS OF EGF-R PTK

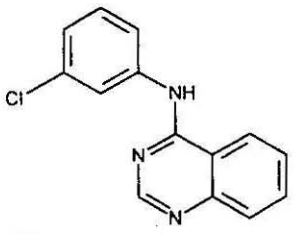
Two such series of inhibitors have been developed, simultaneously and apparently independently, in both instances by pharmaceutical research groups as potential antitumour agents.

One approach [38] was based initially on an investigation, by kinetic studies, of the catalytic mechanism of the enzyme. The resulting information, implying that the enzyme forms a ternary complex with ATP and the peptide substrate, was followed by a study of the pH- and temperature-dependence of the reaction, pointing to requirement of an ionized carboxylate (pK = 5.3) and the protonated form of another group with pK = 9. The mechanism derived suggested that the carboxylate of Asp^{813} facilitates deprotonation of the tyrosine phenolic hydroxyl of the peptide substrate. This leads to nucleophilic attack of the γ-phosphate of ATP which, in turn, interacts with a protonated side chain of the enzyme, presumed to be the guanidium group of Arg^{817}. The resulting postulated 3D-structure of these interacting species was used to formulate a query in a search for inhibitors in a 3D-database of predicted structures, *viz.* compounds that mimic the ATP γ-phosphate and the tyrosyl aromatic ring and its phenolic hydroxyl, all of which react with the enzyme during catalysis. This led to identification of inhibitors which served as lead structures for a 2D-search of a larger database, culminating in identification of 4-(3-chloroanilino)quinazoline (CAQ, see Scheme 7) with a K_i approx. 16 nM, and competitive *versus* ATP. However, the only criterion for its selectivity was the observation that, at 100 μM, it inhibited PKC by a mere 6%.

Assuming an intracellular ATP concentration of 2 mM (probably an underestimate, 5 mM would be more realistic), the measured K_i approx. 16 nM permitted prediction of an IC_{50} approx. 3 μM, compared to an observed value of 1 μM, for inhibition of EGF-stimulated growth of two cell lines, KB and normal rat kidney. Similar CAQ concentrations inhibited EGF-dependent phosphorylation of proteins in KB cells, consistent with a link between inhibition of the kinase and EGF-stimulated growth.

Independently of the foregoing, a compound code-named PD 150035, also an anilinoquinazoline derivative (Scheme 7) was reported as a member of a series of PTK inhibitors, but without details [39]. It was claimed to inhibit the EGF-R PTK with the remarkably low IC_{50} approx. 30 pM, hence several orders of magnitude more potent than CAQ (16 nM, see above) and such widely used PTK inhibitors as Erbstatin, Genistein and sulfonylbenzoyl nitrostyrene. An IC_{50} approx. 30 pM is comparable to the concentration of the enzyme used for its measurement, so that steady-state Michaelis-Menten kinetics cannot be applied to measurement of K_i. Use of equations for tight-binding inhibitors gave a K_i approx. 5 pM. Less potent analogues of the series, not specified, were

PD 153035



CAQ

Scheme 7. Structures of anilinoquinazoline inhibitors of the EGF-R protein-tyrosine kinase. See text for details.

ATP-competitive, suggesting that PD 150035 is also competitive with respect to ATP.

When tested against six different recombinant PTKs, the compound had little effect on five of them at a concentration of 50 μM, and an IC_{50} about 2 μM against the sixth, testifying to its selectivity. It was not, however, tested against Ser/Thr kinases. It was shown to be a highly specific inhibitor of EGF-dependent cellular processes, e.g. the IC_{50} for inhibition of EGF-dependent mitogenesis was 0.08 μM.

Presumably further information will shortly be forthcoming. Meanwhile, a comparison between PD 150035 (K_i approx. 5 pM) and CAQ (K_i approx. 16 nM) is quite striking, inasmuch as the former differs from the latter only by replacement of a Cl by a Br, and the two methoxy substituents on the quinazoline benzene ring. Recalling the enhanced inhibitory properties conferred on halogeno benzimidazole inhibitors of CK-I and CK-II, and on halogeno naphthalene sulfonamide inhibitors of MLCK, on replacement of H by Cl, Br, I (see above), it may well turn out that the Br substituent on PD 150035, in place of the Cl substituent on CAQ, may contribute significantly to the higher potency of the former. It would probably be useful to synthesize additional halogeno congeners of both these compounds.

PROTEIN KINASES AS TARGETS FOR ANTIVIRAL AGENTS

The role of protein phosphorylation in the life cycle of viruses has been a topic of widespread interest since the disclosure that the product of the transforming gene of Rous sarcoma virus exhibits protein kinase activity. Less attention has been directed to these enzymes, both of cellular and viral origin, as potential targets for antiviral chemotherapy. The first comprehensive review on viral protein kinases (and phosphatases), only two years ago [4] considered the subject of viral PKs "still in its infancy". It is presently, however, a rapidly burgeoning field with promising prospects for pinpointing new targets for antiviral agents [9, 40], depending on the extent to which a viral kinase differs in specificity from its cellular counterpart.

Role of cellular kinases

Replication of many viruses is frequently dependent on the intervention of cellular PKs. Perhaps the best-known example is that of nonsegmented negative-strand viruses, exemplified by vesicular stomatitis virus (VSV), the genome of which encodes five proteins. The VSV nucleocapsid consists of three separable components: the N-RNA template, comprising the N-protein wound around the RNA genome; the L protein, which is the viral RNA polymerase; and a protein P which, following intracellular phosphorylation, combines with the L protein to confer transcriptional activity on the latter. Full transcriptional activity can be reconstituted by recombining the isolated purified components, i.e. N-RNA, phosphorylated P and the L protein. The key step is the phosphorylation of the P protein by cellular CK-II [41, 42]. Albeit some controversy exists as to whether transcriptional activation is a single or two-step process, i.e. initial phosphorylation of P by cellular CK-II, followed by additional phosphorylation by L-associated kinase activity, there is general agreement that VSV replication is absolutely dependent on cellular CK-II [41, 42].

It is, however an open question as to whether a CK-II inhibitor (e.g. those listed in Table 1, above) would be effective as an inhibitor of VSV replication in infected cells, bearing in mind the ubiquity of CK-II, and its involvement in many metabolic processes in the host cells [16]. A more appropriate target might well be the presumed L-associated kinase activity.

Several PK inhibitors have, indeed, been shown to modulate viral replication. One example, already referred to above, is the inhibition of HIV-1 induction from latent U1 cells

by a staurosporine analogue-derived inhibitor of PKC [37]. Several other PKC inhibitors have been reported to inhibit Epstein-Barr virus (EBV) replication, as well as viral DNA synthesis, in P3HR1 cells [43], but the most effective, staurosporine, is far from being a specific inhibitor of PKC (see above). Genistein, a PTK inhibitor, was claimed to effectively repress replication of herpes simplex virus type 1 (HSV-1) in Vero cells, accompanied by the expected marked reduction in phosphorylation of tyrosine residues of specific viral polypeptides [44], but the authors themselves recognised that Genistein is also an inhibitor of DNA topoisomerases I and II, which are involved in the regulation of DNA synthesis (see article by Kaufmann, S.H. & Hancock, R., in this number).

Numerous reports also describe modulation of viral replication by cellular levels of cAMP and cGMP, and by inhibitors of cyclic nucleotide phosphodiesterases [5]. These effects are, as expected, host cell-dependent. No cAMP- or cGMP-dependent protein kinase of viral origin has hitherto been identified. However, the cyclic phosphate of the acyclonucleoside Ganciclovir (GCV), a potent broad-spectrum agent against DNA viruses, the mechanism of action of which is as yet not clarified, has been postulated to act as a structural analogue of the second messenger cGMP [9, 45].

Role of viral kinases

Clearly the most appropriate targets would be viral-encoded protein kinases, with special emphasis on those essential for viability, e.g. the two identified Ser/Thr protein kinases of vaccinia virus, coded for by its B1R and P10L genes [46]. It is to be anticipated that such kinases will differ in specificity from their cellular Ser/Thr kinase counterparts, since they have evolved differently, and their genes are quite distinct [40], and hence would be expected to behave differently towards inhibitors. In this context it is worth nothing that viral-encoded nucleoside kinases (so-called viral thymidine kinases) display a much broader specificity than the corresponding cellular enzymes, permitting the design of highly specific inhibitors virtually inactive against cellular thymidine kinases ([5] and references cited).

Relevant also to the foregoing is the finding that Ganciclovir (GCV), referred to above, and which is an active agent against human cytomegalovirus (HCMV), but only after its intracellular phosphorylation, is phosphorylated in infected cells, not by a viral thymidine kinase (the HCMV genome does not contain an open reading frame for a thymidine kinase), but by a protein kinase homologue encoded by the HCMV *UL97* gene [47, 48], and that mutations in this gene, with resultant impaired phosphorylation of GCV, confer resistance to the latter [49]. Furthermore, GCV contains two prochiral hydroxyls and, apparently, is phosphorylated stereospecifically at the pro-(S) hydroxyl. Moreover, a synthetic peptide substrate of cellular PKA with a terminal serinol containing prochiral hydroxyls is also phosphorylated stereospecifically, like GCV, at the pro-(S) hydroxyl ([8, 9] and references cited). No protein substrate has as yet been reported for the *UL97* gene product.

The ability of the viral protein kinase homologue to phosphorylate a low-molecular mass (and even non-peptide) substrate, already referred to above, recalls the observation of Foulkes *et al.* [50] that the product of v-*abl*, which encodes the PTK activity of Abelson murine leukemia virus, is capable of phosphorylating peptides consisting of as few as three (Lys-Tyr-Lys) or even two (Arg-Tyr) residues at levels not far inferior to that of an octapeptide substrate, Angiotensin II.

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