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## PROTEIN KINASES AS DRUG TARGETS

In the present issue of *Dosis*, the reader will be introduced to protein kinases (PKs), particularly those being studied in the Pro-Kinase Research Consortium. During the Helsinki Drug Research 2006 congress, a broader view of PKs and, especially, PKs as drug targets, will be given by leading scientists in the field.

### **Protein kinases; localization and function in the cell**

Protein kinases form the largest single family of enzymes, numbering over 500 and accounting for almost 2% of the proteins encoded by the human genome (Cohen, 2002a). PKs catalyse protein phosphorylation, an important mean by which cell functions are regulated (in concert with protein dephosphorylation catalysed by protein phosphatases). Phosphoproteins are the main effectors in intracellular signal transduction of chemical or hormonal stimuli leading, in most cases, to changes in gene-expression and sustained cellular responses (Cohen, 2002b).

The amino acid residues in the target (substrate) proteins that are phosphorylated by the PKs are serine, threonine and tyrosine. Therefore, PKs are classified as either serine/threonine (Ser/Thr) or tyrosine (TK) kinases, respectively. Protein kinases are expressed in various cellular compartments, i.e. on the cell surface (or the plasma membrane), the cytoplasm, as well as within intracellular organelles, including the nucleus.

Receptor tyrosine kinases (RTKs) are plasma membrane PKs that function as receptors for various extracellular mediators (hormones or other transmitters). This can lead to activation of an intracellular kinase-cascade causing activation of several other kinases in a concerted action and, finally, induce changes in cellular functions. An example of RTK is the insulin receptor, the activation of which causes the glucose transporter to be translocated from intracellular sites to the cell membrane, a prerequisite for glucose intake (Watson & Pessin, 2001).

Cytoplasmic PKs can be activated by intracellular second messengers which, in turn, are mostly generated by activation of G-protein coupled receptors (GPCRs) in the plasma membrane by extra-cellular mediators. Well-known examples for such processes are the activation of protein kinase A (PKA) by cyclic AMP (cAMP), and the activation of protein kinase C (PKC) by diacylglycerol (DAG). PKA can also be negatively regulated by a cell surface receptor via an inhibitory G-protein. In this case, the activity of PKA is decreased as a consequence of receptor activation. In addition to activation through GPCRs, the intracellular kinases can be activated by upstream PKs as well.

An interesting family of PKs is the cyclin-dependent protein kinases (CDKs) that include both protein activators and protein inhibitors. The CDKs is an important class of kinases because their physiological peptide modulators can serve as templates for development of peptidomimetic chemical drugs. The CDKs are of key importance in the control of cell cycle and also in the function of the nervous system (Knockaert et al., 2002).

Thus, PKs can be activated by extracellular and intracellular messengers, and they can be activated by other kinases. Their activation leads to modification of cellular responses and regulation of huge variety of physiological events such as function of ion channels, regulation cell cycle, regulation of nerve cell plasticity, to name a few.

### **Protein kinases as drug targets**

Aberrant protein phosphorylation is involved in the mechanism of many disease processes, e.g. the development of cancer. PKs are also major players in the signalling of cytokines and other mediators of inflammation. Interestingly, in non-mammalian cells, such as in certain parasites (e.g. *Leishmania*), PKs may differ in structure from those of mammalian cells (Doerig et al. 2002), and are therefore most interesting targets for PK

inhibitors (for a comprehensive review see Pechan and Jaffe, in this issue). Thus, drugs affecting protein kinases (or more widely protein phosphorylation) may be of great importance in future treatment strategies of a large variety of diseases.

The cell surface kinases are attractive drug targets. The receptor tyrosine kinases have an extracellular ligand binding domain (regulatory domain) and intracellular protein kinase domain (catalytic domain) that are linked via the cell membrane perturbing domain. The intracellular protein kinase activity is increased when a specific ligand binds to the extra-cellular domain. In principle, it is possible to develop either small molecule drugs or biological drugs which would behave as agonists or antagonists in the ligand binding domain of such PKs (Bennasroune et al., 2004).

RET tyrosine kinase is a transmembrane TK activated by the endogenous activator Glial cell-line Derived Neurotrophic Factor (GDNF) (Airaksinen & Saarna, 2002). More precisely, GDNF binds to the co-receptor GFRalpha1 and the GDNF-GFRalpha complex activates the RET-TK. In the brain, GDNF can restore dopaminergic function of the brain nigro-striatal system that is degenerated in Parkinson's disease. Indeed, GDNF has shown promising effects in experimental models of Parkinson's disease (Eslamboli, 2005), but clinical trials have been controversial (Sherer et al., 2006). It is obvious that chemical agonists of this receptor would be of great interest, at least for experimental medicine. Another interesting example is Multiple endocrine neoplasia (MEN), a disease where RET TK is constitutively active due to a genetic abnormality. As a result of this unregulated RET-TK activity, MEN patients have multiple endocrine tumours. Hence, RET-TK is an obvious drug target that is also studied within the Pro-Kinase Research Consortium.

A major breakthrough in protein kinase clinical pharmacology was the discovery of imatinib (Glivec® trade name by Novartis), an inhibitor of the tyrosine kinases BCR-ABL, c-KIT, and the platelet-derived growth factor receptor (PDGF) TK. Imatinib exhibits high efficacy in the clinic in the treatment of tumours that possess constitutively active forms of c-ABL, c-KIT, or PDGF and, importantly, it turned out to be superior

to the other available drug therapies. Among the tumours that can be treated successfully by imatinib are Philadelphia-chromosome-positive (Ph-positive) chronic myeloid leukemias (Abl-Bcl), gastrointestinal stromal tumours (c-KIT), and PDGF-positive leukaemias. Imatinib is well tolerated, but some tumour cells develop resistance to it (Klein et al., 2005). Imatinib also highlighted the importance of discriminating between the active and inactive conformations of tyrosine kinases when designing inhibitors. This drug binds to the ATP-binding site of the tyrosine kinase and thus its site of action is intracellular. Recently, allosteric inhibitors of the Abl-Bcl kinase have been discovered (Adrian et al., 2006). Many monoclonal antibodies directed to the extracellular ligand-binding domain of receptor tyrosine kinases have been raised for therapeutic use (Bennasroune et al., 2004). An example of these is trastuzumab (Herceptin®, Genentech). Carlo Gambacorti-Passerini and Leonardo Scapozza will summarize tyrosine kinases as drug target in more detail in this Dosis.

The cytoplasmic PKs are obvious drug targets as well. The CDKs control cell division by controlling cell cycle in its various phases. The regulation is complex and involves both activation and inhibition of CDKs. The CDK5 isozyme is an important regulator of nerve cell function. Several compounds affecting CDKs are in clinical trials for cancer and other diseases (Knockaert et al., 2002).

One of the classical PKs is the family of protein kinase C (PKC) isozymes. These kinases are cytoplasmic when inactive, but upon activation by DAG (with or without calcium), they are translocated to cell membranes (Parker & Murray-Rust, 2004). These PKs are essential players in signal transduction and may have significance in diseases such as cancer and diabetic vascular complications. It is possible to develop activators and inhibitors targeting the regulatory domain of PKCs (see Yli-Kauhaluoma et al. in this issue). Even so, the most promising compounds that have been developed so far target the ATP binding site of these enzymes (Goekjian & Jirousek, 2001). Also, the anchoring of the PKCε to RACK2 is a possible target for drug intervention (Hofmann, 2004). For more details, see Garczarczyk et al. in this issue. PKC isozymes as drug targets have been in the focus of research

for a quarter of century. A compound inhibiting the PKC $\beta$  isozyme had promising activity against diabetic complications. Several lines of evidence suggest a role for PKC in modulating agents for the treatment of leukaemia (see Wang et al. in this Dosis).

### Drug binding sites on protein kinases

Regulatory domain of PKC isoenzymes. PKC-regulatory domain is unique to PKC and is not found in other PKs. The C1-domain of the regulatory domain of PKC is the binding site of DAG, the physiological activator of the enzyme. The C1-domain of various PKC-isoenzymes is an attractive drug-target, since the drugs would probably have specificity for PKCs over other kinases, and it would also be possible to discover PKC-isozyme-selective inhibitors. For details, see Yli-Kauhaluoma et al. in this issue.

Catalytic domain of various PKs. The catalytic domain of various PKs is highly conserved, especially the ATP binding site. On the other hand, the region surrounding the *sensu stricto* ATP binding site (the so-called "pharmacophore") is quite variable from one kinase to another, and can be exploited for the specific binding of ligands that will act as selective inhibitors competing with ATP (Noble et al., 2004). Indeed, most of the currently available PK-inhibitors, such as imatinib (Glivec<sup>®</sup>) and LY333531, a PKC $\beta$ -selective inhibitor, are targeted to the ATP binding site of the catalytic domain (see Goekjian in this issue)

Anchoring proteins. The receptors for activated C-kinase, RACK1 and RACK2, bind to PKC $\epsilon$  and translocate it to the site of catalytic activity (Mackay & Mochly-Rosen, 2001). Several peptides inhibit the binding of PKC $\epsilon$  to RACK1 or RACK2 (Hool, 2005). The PKC $\epsilon$ -derived octapeptide HDAPIGYD has been shown to be a PKC $\epsilon$  agonist and prevents the heart from ischemic damage (Schechtman et al., 2004).

### Pro-Kinase Research Consortium

In the Pro-Kinase Research Consortium, much emphasis has been paid to the characterization of the role of selected PKs in signal transduction pathways and in various disease models, such as proliferation of tumour cells, functions of immune cells and vascular cells, the parasitic

infection leishmaniasis, and also in the physiology of the central nervous system.

The Consortium is organized as five sub-projects, Work Packages (WP1-WP5). In WP1, several hundred extracts have been prepared from cyanobacterial and fungal strains, as well as from plants. Pure compounds from the extracts have been isolated and preliminary activity screening has been done. The WP2 is concentrating on virtual screening of known chemicals libraries and on developing new computer programs for virtual screening. Several hundred compounds targeted to various PKs have been synthesised in WP 3, in groups oriented to synthetic organic chemistry. They use modern methods of organic synthesis to prepare compounds that are based on natural product screening (WP1) or virtual screening of known chemicals (WP2). These synthesis products can be tested in various protein kinase binding and activity assays to find hit molecules. This provides essential feed-back information for the molecular modellers and synthetic chemists to refine the chemical structure of the found hits within the iterative process of drug discovery and development.

A number of the novel compounds that show biological activity in various assay systems utilising purified kinase enzyme preparations have been found. Several recombinant protein kinase domains have been expressed and used in the screening for ligand binding. Indeed, there are more than 150 different kinase activity assays available within the Consortium, thus the binding affinity and biological activity of potential protein kinase inhibitors/activators can be screened with the help of extensive collaboration. Rational drug design relies on three dimensional models of proteins and more specifically of the active site – the ligand binding site. In order to determine the crystal structure of the critical binding sites of different kinases, large quantities of various protein kinases have been produced in cell cultures. Structures of different kinase/inhibitor complexes and a novel protein kinase have been solved. (WP4)

In WP5, various model systems for a range of diseases have been utilised to test therapeutic potential of novel compounds of both natural and synthetic origin. The diseases include cancer (particularly leukaemia and also breast, glioma and

gastric tumours), epilepsy, heart disease and post-stroke neurodegeneration, leishmaniasis and inflammatory disease. The protein kinases that have been the focus of the project include tyrosine kinases (receptor and cytoplasmic), Protein kinase C, MAP kinases and Cyclin Dependent Kinases.

A recent article by Vieth et al. (2005) summarised the status of protein kinase targeted drug development in the USA. At that time (June 2005), there were 76 different compounds that were active in clinical trials. Most of them were for oncological diseases. It is obvious that we need small molecule chemical substances that are rather selective to one or several kinases.

Opposite to a major pharmacological principle, protein kinase inhibitors may not necessarily need to be specific to a single PK but, rather, could inhibit several "correct" kinases simultaneously. This is of course a major challenge to rational drug discovery and, indeed, there is an increasing need for better *in vitro* and *in vivo* models of diseases to test such compounds.

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## YHTEENVETO

## PROTEIINIKINAASIT LÄÄKEVAIKUTUKSEN KOHDEMOLEKYYLEINÄ

Proteiinikinaasit (PK) ovat suurin yksittäinen entsyymiperhe, jossa on yli 500 proteiinia. Se kattaa lähes 2 % ihmisen genomien koodaamista proteiineista. PK:t katalysoivat proteiinien fosforylaatiota, joka on tärkeä vaihe kemiallisessa tai hormonaalisessa solunsisäisessä signaalinkujetuksessa johtaan muutoksiin geenien ilmentymisessä ja pitkäkestoissa soluvasteissa. PK:t fosforyloivat kohdeproteiinin seriini-, treoniini- tai tyrosiini-aminohappotähteitä. Tämän vuoksi PK:t luokitellaan joko seriini-/treoniinikinaaseiksi tai tyrosiinikinaaseiksi. Proteiinikinaaseja esiintyy joko solukalvoilla, sytoplasmassa tai solunsisäisissä organelleissa, esim. tumassa.

PK:t ja proteiinifosforylaatio ovat tärkeässä roolissa monissa sairauksissa, esim. syövän kehittämisessä tai tulehdusreaktioissa. On havaittu myös, että parasiittien (esim. Leishmania) solujen PK:t poikkeavat rakenteeltaan nisäkässolujen vastaavista, ja ovat näin hyvin kiinnostava lääkevaikutuksen kohde. Proteiinikinaasien rakenteissa on useita potentiaalisia yhdisteiden sitoutumiskohtia, kuten PKC isoentsyymien säätelyosa ja katalyyttisen osan ATP:n sitoutumiskohta, joita tutkitaan lääkevaikutuksen kohteina. Myös ns. proteiinikinaaseihin ankkuroituvat proteiinit ovat kiivaan tutkimuksen kohteina.

EU:n rahoittama Pro-Kinase Research Consortium, jonka koordinaattorina Helsingin yliopiston farmasian tiedekunta (Prof. Raimo K. Tuominen) toimii, karakterisoi tiettyjen PK:ien roolia signaalien kuljetusreiteissä ja erilaisissa sairausmalleissa, kuten kasvainsolujen jakaantumisen, immuuni- ja verisuonten solujen toiminnassa, leishmaniaasissa ja myös keskushermoston toiminnassa. Konsortion partnerit eristävät luonnonaineista ja syntetisoivat yhdisteitä, jotka voisivat toimia joko PK-inhibiittoreina tai aktivaattoreina. Pienimolekyylisten yhdisteiden, jotka olisivat selektiivisiä joko yhdelle tai useammalle proteiinikinaasille, kehittäminen on erittäin tärkeää. Lisäksi on olemassa yhä suurempi tarve paremmille *in vitro* ja *in vivo* -sairausmalleille, joissa voitaisiin näitä yhdisteitä testata. Tämä on suuri haaste lääkekehitykselle. Pro-Kinase Konsortio pyrkii ratkaisemaan näitä kysymyksiä. Hanke on viisivuotinen ja EU rahoittaa sitä 15 miljoonalla eurolla. Tämän käsilläolevan DOSIS-teemanumeron kirjoittajat ovat Pro-Kinase Konsortiumin partnereita.

► **Prof. Raimo K. Tuominen**

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# PROTEIN KINASE C ISOZYMES

## - TARGETS FOR POTENTIAL THERAPIES

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### INTRODUCTION

Protein kinase C (PKC) is a family of serine/threonine-specific protein kinases. The PKC isoenzymes can be classified into three groups: i) the conventional (cPKCs)  $\alpha$ ,  $\beta$ ,  $\beta$ II, and  $\gamma$  (require negatively charged phospholipids, diacylglycerol or phorbol ester, and calcium for optimal activation), ii) the novel (nPKCs)  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\eta$ /L (mouse/human) and  $\mu$  (require negatively charged phospholipids, diacylglycerol or phorbol ester, but no calcium), and iii) the atypical (aPKCs)  $\lambda$ / $\iota$  (mouse/human) and  $\zeta$  (do not require calcium,

diacylglycerol or phorbol ester, but only negatively charged phospholipids for optimal activity) (Nishizuka, 1995; Newton and Johnson, 1998). The PKC isoenzymes (**Figure 1**) are characterized by four conserved (C1 – C4) and five variable (V1 – V5) domains (Stabel and Parker, 1991; Azzi et al., 1992; Hug and Sarre, 1993; Stabel, 1994). The regulatory domain consists of the C1 and the C2 region. C1 contains the pseudo-substrate region that can inhibit the enzyme by binding to the catalytic site (C4). C1 also contains

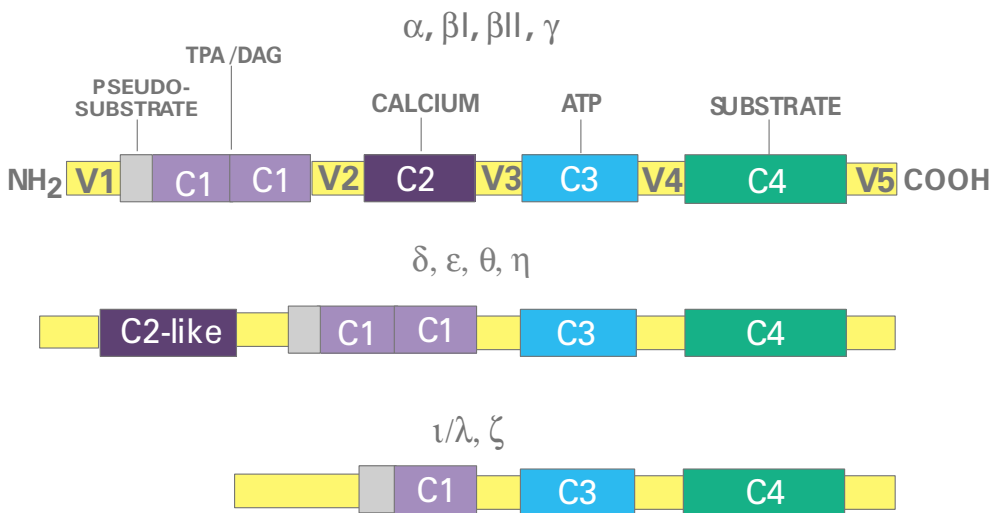


Figure 1. Structures of the PKC isoenzymes and their domains

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tandemly repeated cysteine-rich regions to which diacylglycerol (DAG), phorbol esters and bryostatins can bind. cPKCs and nPKCs contain two zinc fingers in the phorbol ester binding site, aPKCs are characterized by a single zinc finger. C2 contains the calcium binding region present only in cPKCs but not in nPKCs and aPKCs. Between the C2 and the C3 region the so called hinge region is situated, which serves as cleavage site for calpain and trypsin during degradation. The C3 region is believed to be the ATP binding site and the C4 region the catalytic site.

Investigations into the expression of distinct PKC isoenzymes in various tissues revealed a highly variable tissue distribution. PKC $\alpha$  and PKC $\zeta$  are ubiquitously expressed. Brain contains all isoenzymes, whereas others such as skin and skeletal muscle contain only a few (Blobe et al., 1994). Such a different pattern of expression suggests that the PKC isozymes play different roles in the tissue of expression.

PKC isoenzymes seem to play important roles in activation of signal transduction pathways leading to synaptic transmissions, the activation of ion fluxes, secretion, proliferation, cell cycle control, differentiation and tumor proliferation. PKC has become of major interest as target for therapeutic intervention in a range of different diseases (Nixon, 1997; Goekjian and Jirousek, 1999). PKC is the intracellular receptor for tumor promoting phorbol esters (Castagna et al., 1982; Niedel et al., 1983; Leach et al., 1983). Short term exposure of intact cells with phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activates PKCs (by binding to the diacylglycerol-binding site), long term exposure down-regulates PKC activity. Phorbol esters are able to promote tumor formation. Therefore, it was presumed that activation of PKC by TPA induces tumors and inhibition may reduce carcinogenesis or inhibit tumor growth. However, investigations revealed that the situation is more complicated: For example, bryostatin, a PKC modulator with properties similar to those of TPA (Blumberg, 1991; Kennedy et al., 1992; Szallasi et al., 1994) does not induce tumor formation. PKC modulators such as miltefosine (hexadecylphosphocholine), bryostatin, safingol, midostaurin (PKC412, CGP41251) and UCN-01 (7-hydroxystaurosporine) are used in the clinic or are in clinical evaluation as antitumor agents.

However, PKC is not the only target of these compounds. They also interfere with other targets (Hofmann, 2004).

The different PKC isozymes complicate the situation. Their exact functions are not known at present. In this review potential roles of the different PKC isozymes and possible effects of their modulation are discussed.

### **PKC $\alpha$**

PKC $\alpha$  has been implicated in a variety of cellular functions including proliferation, apoptosis, differentiation, motility, and inflammation. However, the responses induced by activation or overexpression of PKC $\alpha$  vary depending on the types, and sometimes conditions, of cells. For example, in some types of cells, PKC $\alpha$  is implicated in cell growth. In contrast, it may play a role in cell cycle arrest and differentiation in other types of cells. PKC $\alpha$  exhibits proapoptotic or antiapoptotic functions in different cell types (Nakashima, 2002).

Hearts of PKC $\alpha$ -deficient mice are hypercontractile, whereas those of transgenic mice overexpressing PKC $\alpha$  are hypocontractile. Adenoviral gene transfer of dominant-negative or wild-type PKC $\alpha$  into cardiac myocytes enhances or reduces contractility, respectively (Braz et al., 2004). PKC $\alpha$  transgenic mice exhibit severe intraepidermal neutrophilic inflammation, disruption of the epidermis and upper hair follicles when treated topically with TPA, conditions that characterize several human cutaneous diseases such as pustular psoriasis and acute generalized exanthematous pustulosis (Cattaison et al., 2003).

PKC $\alpha$  was found to be activated *in situ* in a significant number of human breast tumors (Ng et al., 1999). In contrast, down-regulation of PKC $\alpha$  in breast carcinomas was found by Kerfoot et al. (2004). PKC $\alpha$  overexpressing MCF-7 cells exhibited a significant reduction in estrogen receptor expression (Ways et al., 1995; Manni et al., 1996) and PKC $\alpha$  levels may especially be increased in breast cancer patients with low or negative estrogen receptor levels (Lahn et al., 2004). PKC $\alpha$  has been shown to activate telomerase in human breast cancer cells, which may represent an essential step in the maintenance of proliferation in human cancers (Li et al., 1998). On the other hand, increased

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expression of PKC $\alpha$  led to cessation of growth, induction of differentiation in B16 melanoma cells, and to inhibition of proliferation in K562 cells (Gruber et al., 1992).

Cell lines derived from high-grade gliomas expressed higher levels of PKC $\alpha$  than did cell lines derived from low-grade gliomas. In glioblastoma-derived cell lines PKC $\alpha$  was mainly expressed in the cytosolic fraction, indicating an inactive state of the enzyme. The PKC modulator bryostatin 1 specifically down-regulated PKC $\alpha$  in glioblastoma-derived cell lines. However, this was not associated with significant growth inhibition illustrating that PKC $\alpha$  seems not to be essential for proliferation (Zellner et al., 1998). In models of melanoma, PKC $\alpha$  activation is typically associated with increased tumor cell proliferation, invasiveness and decreased differentiation (Lahn and Sundell, 2004).

Decreased PKC $\alpha$  seems to be of major importance for the development of colorectal cancers (Suga et al., 1998). Transfection of CaCo-2 cells with PKC $\alpha$  in the antisense orientation resulted in enhanced proliferation and decreased differentiation (Scaglione-Sewell et al., 1998). Human HT-29M6 colon cancer cells transfected with an activated form of PKC $\alpha$  showed decreased proliferation and increased invasion due to alterations in cell adhesion. When these cells were xenografted into athymic mice, higher expression of activated PKC $\alpha$  led to a reduction in tumor size (Batlle et al., 1998). PKC $\alpha$  was decreased in clear cell renal carcinoma versus normal tissue (Brenner et al., 2003).

Elevated levels of PKC were found in thyroid cancers if compared to normal thyroid tissue (Hagiwara et al., 1990; Hatada et al., 1992). In human thyroid cancers (Prevostel et al., 1995; Prevostel et al., 1997) and in pituitary cancers (Alvaro et al., 1993; Alvaro et al., 1997) point mutations in PKC $\alpha$  were detected. However, 11 human pituitary tumors showed a normal wild-type sequence of PKC $\alpha$  DNA (Schiemann et al., 1997). A single point mutation in D294 was found in a subpopulation of thyroid and pituitary tumors with more invasive phenotype, suggesting a role in tumor progression (Alvaro et al., 1993). A PKC $\alpha$ -D294G mutant was found to be unable to bind to cellular membranes tightly despite the fact that it translocates to the membrane as efficiently as the wild-type PKC $\alpha$  upon treatment of phorbol ester.

The impaired membrane binding is associated with this mutant's inability to transduce several antitumorigenic signals. Thus, the PKC $\alpha$ -D294G seems to be a loss-of-function mutation. Therefore, developing selective activators instead of inhibitors of PKC $\alpha$  was suggested for the treatment of certain endocrine tumors (Zhu et al., 2005).

Treatment of mice bearing U-87 (Yazaki et al., 1996), T-24 bladder carcinoma, A549 lung carcinoma or Colo 205 colon carcinoma xenografts with antisense phosphorothioate oligonucleotides directed against PKC $\alpha$  (ISIS 3521, LY900003, aprinocarsen) resulted in suppression of tumor growth (Dean et al., 1996). Recent phase I and phase II trials in patients with colorectal adenocarcinomas (Cripps et al., 2002), breast and ovarian cancers (Lahn et al., 2004), NSCLC (Vansteenkiste et al., 2005), high grade lymphomas (Advani et al., 2005) and hematologic malignancies (Lahn et al., 2006) did not show promising results.

## **PKC $\beta$**

PKC $\beta$ I and PKC $\beta$ II are generated by alternative splicing from a single gene (Kawakami et al., 2002). Elevated expression of PKC $\beta$ II seems to be an important early event in colon cancer development (Gokmen-Polar 2001). PKC $\beta$  knockout mice showed that this gene is important for B cell development and activation (Leitges et al., 1996). A dramatic increase in the angiogenic response to oxygen-induced retinal ischemia in transgenic mice overexpressing PKC $\beta$ II and a significant decrease in retinal neovascularization in PKC $\beta$  null mice was observed (Suzuma et al., 2002). Hyperglycemia leads to PKC $\beta$ I and II activation, which experimentally has been shown to contribute to the development and progression of diabetic microvascular complications (retinopathy, nephropathy and neuropathy). Animal and human studies using ruboxistaurin mesylate (LY333531), a novel, selective inhibitor of PKC $\beta$ , have shown a delay in the progression and, in some cases, reversal of diabetic retinopathy, nephropathy and neuropathy (Joy et al., 2005). The compound enhanced the antitumor activity of fractionated radiation, carboplatin, gemcitabine, paclitaxel, BCNU and 5-FU. The ruboxistaurin analogue, LY379196, suppressed

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the growth of neuroblastoma cells and enhanced the antiproliferative activity of taxol and vincristine *in vitro*. The analogue LY317615 enhanced the antitumor activity of 5-FU and BCNU in tumor bearing mice (Teicher et al., 1999).

### **PKC $\gamma$**

PKC $\gamma$  is restricted to the brain and spinal cord (Hug and Sarre and 1993). In normal animals, peripheral nerve injury produces a persistent, neuropathic pain state. Mice that lack PKC $\gamma$  displayed normal responses to acute pain stimuli, but they almost completely failed to develop a neuropathic pain syndrome after partial sciatic nerve section, and the neurochemical changes that occurred in the spinal cord after nerve injury were blunted (MalMBERG et al., 1997). In mice lacking PKC $\gamma$ , impaired motor coordination, but intact eyeblink conditioning, a form of component movement learning (Chen et al., 1995), spatial learning deficits, impaired hippocampal long-term potential (Abeliovich et al., 1993a, 1993b; Ramakers et al., 1999), and increased ethanol consumption and behavioural impulsivity (Bowers and Wehner, 2001), were observed. An analysis of PKC $\gamma$  knockout mice supports the notion that the loss of the PKC $\gamma$  gene may protect the functional  $\mu$ -opioid receptors from degradation in the spinal cord and produce antinociception (Narita et al., 2001). Thus, PKC $\gamma$  might be a potential target for prevention and therapy of persistent pain or alcohol problems. PKC $\gamma$ -activating or inhibiting compounds are not available at present.

### **PKC $\delta$**

PKC $\delta$  is expressed ubiquitously among tissues. It is activated, in addition to by phospholipids, by caspase 3-cleavage (Emoto et al., 1995; Ghayur et al., 1996) or tyrosine-phosphorylation (Kikkawa et al., 2002). In contrast to other PKC isozymes, PKC $\delta$  is involved in tumor suppression and induction of apoptosis (Perletti et al., 1999; Gschwendt, 1999; Kikkawa et al., 2002). Transgenic mice overexpressing PKC $\delta$  in the epidermis were found to be resistant to skin tumor promotion by TPA (Reddig et al., 1999). PKC $\delta$  knock-out mice, due to reduced apoptosis, exhibited an increased smooth muscle cell

accumulation (Leitges et al., 2001) and increased proliferation of B cells leading to autoimmune disease (Mathis and King, 2002; Miyamoto et al., 2002; Mecklenbrauker et al., 2004).

Rottlerin was reported to be an inhibitor of PKC $\delta$  (Gschwendt et al., 1994). However, the compound also inhibits other kinases. It seems to be a mitochondrial uncoupler and may inhibit PKC $\delta$  indirectly (Kikkawa et al., 2002). Bistratene A, an activator of PKC $\delta$ , induced translocation of PKC $\delta$  to the nucleus and induced growth arrest in G2/M in HL-60 cells (Griffiths et al., 1996). Therefore, activation of this isozyme seems to increase antitumor activity and activation of apoptosis. An activation of PKC $\delta$  seems to be responsible for the antitumor activity of the adriamycin-derivative *N*-benzyladriamycin-14-valerate (AD 198) (Lothstein et al., 2001). The anthracyclins adriamycin and daunorubicin are essential components of first-line chemotherapy in the treatment of solid and hematopoietic tumors. The compounds intercalate into DNA and inhibit topoisomerase II religation. Their efficacies are impeded by serious side effects (cardiotoxicity). To obtain derivatives lacking these disadvantages, compounds with alkylesters at C-14 have been synthesized, among them AD 198 (*N*-benzyladriamycin-14-valerate) and valrubicin (*N*-trifluoroacetyl adriamycin-14-valerate). These alterations resulted in an increase in lipophilicity and rapid cellular penetration. Both compounds are localized in the perinuclear region and showed poor DNA-intercalation and interference with topoisomerase II. AD 198 shows a spatial homology with TPA and DAG and binds to the C1 region of PKC. In intact cells, AD 198 induced the translocation of PKC $\alpha$  and PKC $\delta$  in murine NIH3T3 cells (Roaten et al., 2001). The compound seems to induce apoptosis by activation and cleavage of PKC $\delta$ , but may also interact with other C1-containing proteins such as chimaerins, Ras-GRP or PKD.

### **PKC $\epsilon$**

PKC $\epsilon$  is expressed in many tissues but abundantly in neuronal, hormonal and immune cells. In neuronal cells, PKC $\epsilon$  seems to be important for proliferation and differentiation (Hundle et al., 1995; Fagerstrom et al., 1996), alcohol sensitivity (Choi et al., 2002), nociceptor

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function (Cesare et al., 1999; Dina et al., 2000) and cell death and survival (Jung et al., 2004). Overexpression of PKC $\epsilon$  in NIH3T3 cells and Rat 6 embryo fibroblasts caused increased growth rates, growth to higher cell densities in monolayer, growth in soft agar and tumor formation in nude mice (Mischak et al., 1993; Cacace et al., 1993), indicating that it is an oncogene (Perletti et al., 1996).

Mice overexpressing PKC $\epsilon$  in the skin exhibited reduced papillomas but enhanced carcinomas (Jansen et al., 2001; Li et al., 2005). A modest increase in overexpression of PKC $\epsilon$  in transgenic mice exhibited protection against myocardial ischemia (Ping et al., 2002), whereas a marked increase exhibited cardiac hypertrophy and failure (Takeishi et al., 2000). Targeted disruption of the PKC $\epsilon$  gene blocked cardioprotection caused by ischemic preconditioning (Gray et al., 2004) and led to decreased hypoxic pulmonary vasoconstriction (Littler et al., 2003). Compared to wild-type mice, PKC $\epsilon$  knock-out mice also exhibited reduced macrophage activation (Castrillo et al., 2001; Aksoy et al 2004), reduced stress hormones, decreased anxiety-like behaviour (Hodge et al., 2002), decreased hyperalgesia (Khasar et al., 1999), less alcohol consumption, and greater acute sensitivity to alcohol (Choi et al., 2002, Newton and Messing, 2006).

PKC $\epsilon$  inhibitors might be potential drugs against certain tumors, anxiety, alcoholism, cardiac hypertrophy or pain. PKC $\epsilon$  activators might be beneficial for prevention of myocardial ischemia or activation of macrophages.

Receptors for activated C kinases (RACKs) are proteins interacting with PKCs. RACK2 interacts with activated PKC $\epsilon$  (Csukai et al, 1997). A short peptide (EAVSLKPT), corresponding to the amino acids 14 – 21 of the V1 region of PKC $\epsilon$ , interferes with the PKC $\epsilon$ /RACK2 interaction and selectively inhibits the translocation of PKC $\epsilon$  by binding to RACK2 (Johnson et al., 1996). It has been shown that this peptide inhibits protection from hypoxia-induced cell death of cardiac myocytes (Gray et al., 1997).

The peptide HDAPIGYD corresponding to a part of the RACK sequence that binds to PKC $\epsilon$ , selectively translocates PKC $\epsilon$  from cytosolic to particulate subcellular fractions, a hallmark of PKC activation (Dorn et al., 1999; Mackay and Mochly-

Rosen, 2001). Activation of endogenous PKC $\epsilon$  in hearts of diabetic mice promoted the survival phenotype and inhibited the negative inotropic properties of chronic hyperglycemia (Malhotra et al., 2005). Combined treatment of the PKC $\epsilon$  activator peptide and a PKC $\delta$  inhibitor reduced ischemia-reperfusion injury and decreased the resulting graft coronary artery disease induced by prolonged ischemia (Tanaka et al., 2005).

Recently the group of O'Brian described that the active-site cavity of human PKC $\epsilon$  harbors a redox-sensitive cysteine residue, (Cys452), which inactivates the kinase when modified by thiol-disulfide exchange with small disulfides, such as cystamine, cystine and disulfiram or *N*-ethylmaleimide (O'Brian et al., 2006). Cys452 is only conserved in 11 out of the approximately 500 human protein kinases.

### **PKC $\eta$**

PKC $\eta$  is strongly expressed in skin and lung but only slightly in spleen and brain. It is highly expressed in epithelial tissues, especially in squamous epithelia. PKC $\eta$  is unique in that it is specifically activated by cholesterol sulfate and sulfatide, sulfated metabolites of cholesterol and cerebroside, respectively. PKC $\eta$  overexpression induces G1 arrest and differentiation in keratinocytes. PKC $\eta$ -induced differentiation is accompanied by the transcriptional activation of transglutaminase I, a key enzyme in squamous differentiation, and involucrin, a precursor of cornified envelopes. In keratinocytes, PKC $\eta$  associates with the cyclin E/cdk2/p21 complex and inhibits the cdk2-kinase activity, leading to G1 arrest. Cholesterol sulfate inhibits the promotional phase of skin carcinogenesis. Moreover, PKC $\eta$ -knock-out mice showed a much higher sensitivity to carcinogenesis, suggesting that PKC $\eta$  is negatively involved in tumor promotion through stimulation of keratinocyte differentiation. In addition to epithelial cells, recent studies revealed that PKC $\eta$  acts as a key regulator in early B-cell development. Although the functions of PKC $\eta$  in other cell types are not yet fully elucidated, available evidence indicates that this particular isoform plays a crucial role in the signaling of cell differentiation in a cell-type-specific manner (Kashiwagi et al., 2002).

In PKC $\eta$ -deficient mice wound healing on the

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dorsal skin, particularly reepithelialization, was significantly delayed. These mice exhibited increased susceptibility to tumor formation in two-stage skin carcinogenesis by single application of 7,12-dimethylbenz(a)anthracene for tumor initiation and repeated applications of TPA for tumor promotion (Chida et al., 2003). A clear correlation of PKC expression with tumor progression in clear cell renal cell carcinoma was observed (Brenner et al., 2003). Compounds selectively modulating PKC $\eta$  are not known at present.

### **PKC $\theta$**

PKC $\theta$  demonstrated a relatively restricted expression pattern, i.e. in skeletal muscle, lymphoid organs and hematopoietic cell lines, in particular T cells (Baier, 2003). T cell receptor-induced activation is blocked in T cells from two independent PKC $\theta$  knockout mouse lines. PKC $\theta$  is essential for the activation of mature T cells (Sun et al., 2000). Therefore, PKC $\theta$  appears to be a prime target for novel anti-inflammatory or immunosuppressive therapies.

PKC $\theta$  is necessary for induction of the interleukin-2 gene because the transcription factors AP-1 and NF-kappaB, which are essential for interleukin-2 gene promoter activation, are main targets of PKC $\theta$ . Therefore, given the essential role of PKC $\theta$  in interleukin-2 production, interleukin-2 gene regulation by PKC $\theta$  could also be of therapeutic interest. Recent studies revealed that PKC $\theta$  provides an important survival signal that protects leukemic T cells from Fas- or UV-induced apoptosis. These findings and the constitutive localization of PKC $\theta$  in the membrane of some leukemic T cells suggests that it plays a role in leukemic T cell survival and/or proliferation, and that selective PKC $\theta$ -inhibitory strategies may facilitate elimination of malignant T cells (Villalba and Altman, 2002). Duensing et al. (2004) showed that PKC $\theta$  is a diagnostic marker for gastrointestinal stromal tumors.

### **PKC $\iota$**

The human PKC $\iota$  and its mouse ortholog PKC $\lambda$  exhibit an amino acid identity of 98%. This isozyme is involved in cell polarization (Suzuki et al., 2001), fundamental for development and

tissue functions. PKC $\lambda$  knock-out mice die at an early embryonic stage (Suzuki et al., 2003). PKC $\iota$  was found to be overexpressed in the vast majority of primary human non-small cell lung cancer (NSCLC) cells and in primary NSCLC tumors. Elevated PKC $\iota$  expression levels in tumors predict poor survival. Therefore, PKC $\iota$  expression profiling might be useful in identifying early-stage NSCLC patients at elevated risk of relapse. Disruption of PKC $\iota$  signaling with a dominant negative PKC $\iota$  blocks the transformed growth of human NSCLC cells harboring PKC $\iota$  gene amplification. Thus, PKC $\iota$  seems to be an attractive target for development of novel, mechanism-based therapeutics to treat NSCLC (Regala et al., 2005). The PKC $\iota$ -RAC1-PAK-MEK-ERK pathway seems to be responsible for cell transformation. In this signal transduction, a complex of PKC $\iota$ -RAC1-PAR6 is involved. Aurothioglucose, a gold compound used clinically to treat rheumatoid arthritis, and the related compound aurothiomalate, were identified as inhibitors of PKC $\iota$ -PAR6 interactions. Aurothioglucose inhibited the tumor growth of NSCLC A549 cells in nude mice (Stallings-Mann et al., 2006).

### **PKC $\zeta$**

PKC $\zeta$  is activated by phosphatidylinositol-3,4,5-trisphosphate (PIP $_3$ ). This PKC isozyme associates with other signaling and scaffold proteins and is involved in the mitogen-activated protein kinase, ribosomal S6 protein kinase and NFkB signaling (Hirai and Chida, 2003). The prostate androgen response-4 (Par-4) protein has been shown to inhibit and the lambda-interacting protein (LIP) to activate PKC $\zeta$ . The level of Par-4 correlated with growth inhibition and apoptosis (Moscat and Diaz-Meco, 2000). In PKC $\zeta$  knock-out mice the activation and survival of isolated purified cultures B cells are severely impaired (Martin et al., 2002).

## **PERSPECTIVES**

The PKC $\alpha$ -specific antisense oligonucleotide aprinocarsen (ISIS 3521) did not show promising results as an antitumor agent. The PKC $\beta$ -specific inhibitor ruboxistaurin mesylate (LY333531) is in

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phase III clinical trials. It has shown a delay in the progression of diabetic retinopathy, nephropathy and neuropathy. The experimental antitumor drug AD 198 is believed to exert its antitumor activity by activation of PKC $\delta$ . For the other PKC isozymes, no isozyme-specific small-molecule inhibitors or activators are available so far. Such modulators would be important for the elucidation of the exact functions of PKC isozymes. With such compounds also investigations into their possible therapeutic potential could be performed.

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# ONCOGENIC TYROSINE KINASES: THE BCR-ABL MODEL

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## INTRODUCTION

Tyrosine kinases (TK) and related molecules comprise over 100 different genes. Approximately two thirds represent receptor TK, the other third being non receptor TK. TK regulate important cellular signalling pathway, from the transduction of extracellular signals, to the regulation of key biological processes such as cell proliferation and apoptosis (Manning et al., 2002; Noble et al., 2004).

TK can turn themselves into the engine of malignant cell transformation. Their relationship to cancer varies in the different diseases. They range from cases with a known, clear and causal relationship, to examples in which such a relationship is more circumstantial. A recent review describing seven different oncogenic TK is available (Gambacorti-Passerini, 2004).

In this review, the model TK Bcr-Abl will be presented as an example of an oncogenic TK, in which the delineation of the molecular lesion (Ph-chromosome) and the assessment of the causal association between the TK and the disease phenotype (principally Chronic Myeloid Leukemia) led to the discovery and clinical development of a highly successful treatment. The clinical and the molecular aspects of Bcr/Abl inhibition will be highlighted.

## ONCOGENIC TYROSINE KINASES: THE BCR-ABL MODEL

Chronic Myeloid Leukemia (CML) is a myeloproliferative disease, initially characterized by an abnormal expansion of clonal hematopoiesis still capable of achieving terminal differentiation (Goldman & Melo, 2003). CML exhibits a characteristic biphasic clinical course. The initial chronic phase, which lasts on average five years, originates as an indolent disease. It is invariably followed by the development of an acute leukemia, termed blast crisis that is marked by the emergence within the clonal hematopoiesis of fully transformed cell clones arrested at an early stage of differentiation, either myeloid or lymphoid.

For many years, the presence of a Ph-chromosome in the cells of the CML patients has been the only cytogenetic abnormality known to be associated with a specific malignant disease in humans (Nowell & Hungerford, 1960). Later it was recognized that the Ph-chromosome is the result of a reciprocal translocation between the long arms of chromosome 9 and 22, t(9;22)(q34;q11) (Rowley, 1973). Finally, in the eighties, the molecular defect associated with this cytogenetic abnormality was identified, and it was established that the Ph-chromosome results in

the juxtaposition of parts of the *BCR* and *ABL* genes, which are normally located respectively on chromosome 22 and chromosome 9, to form a new hybrid *BCR-ABL* gene (Groffen et al., 1984; Heisterkamp et al., 1985). The corresponding chimeric protein (P210; Ben-Neriah et al., 1986) has a causative role in neoplastic transformation of pluripotent stem cells, giving rise to the expansion of the myeloid compartment which characterizes the chronic phase of the disease. However, in spite of the fact that the structural organization and the molecular biology of the *BCR-ABL* gene as well as that of the normal *ABL* and *BCR* genes have been subjects of intensive investigation in the last thirty years, many questions concerning the mechanisms by which the hybrid gene is formed and transforms the hemopoietic stem cells still remain unanswered. Moreover, a further degree of complexity is represented by the fact that the Ph-chromosome may also be found in leukemias other than CML and associated with a wide spectrum of hematological phenotypes, ranging from that of apparently "de novo" acute lymphoblastic leukemia to that, on the opposite side, of indolent chronic myeloproliferative disorders (Melo, 1996; Saglio et al., 1997).

In the late 1980s, the data accumulated on the role of *BCR-ABL* in onset and progression of CML indicated *BCR-ABL* as the most attractive target for molecularly targeted therapy approaches. Therefore, attempts to decrease the amount of the *BCR-ABL* transcripts and/or to inhibit the TK activity of the oncoprotein were initiated and this process finally ended with the discovery and the development of imatinib mesylate (Buchdunger et al., 1996). This small chemical compound inhibits the kinase activity of Bcr-Abl at nanomolar concentrations and was shown to stop cellular growth and to induce apoptosis of leukemic cells both *in vitro* and *in vivo* (Druker et al., 1996; Gambacorti-Passerini et al., 1997; Deininger et al., 1997; le Coutre et al., 1999). The importance of imatinib goes beyond the exceptional therapeutic results obtained with its use in CML and in other Ph-positive leukemias, and should represent the real starting point of the so called "molecularly targeted therapy" (Figure 1).

The X-ray structure of Abl in complex with imatinib revealed its molecular mechanism of action (Nagar et al., 2002). Imatinib, (4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamid (IUPAC)), that has an IC50 value of 38 nM, captures

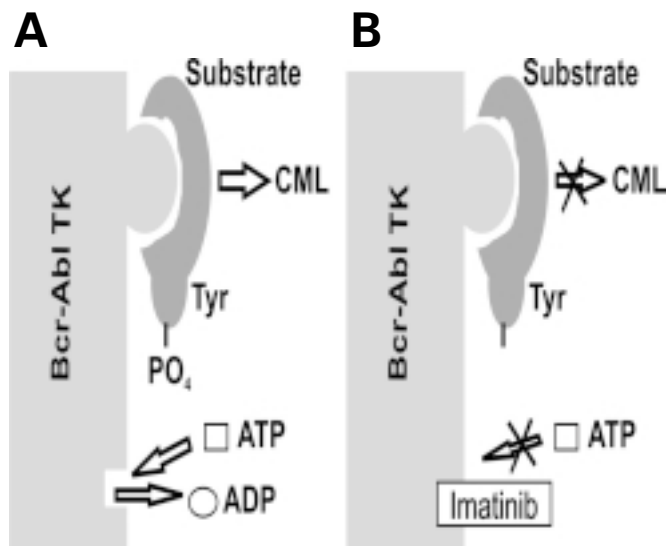
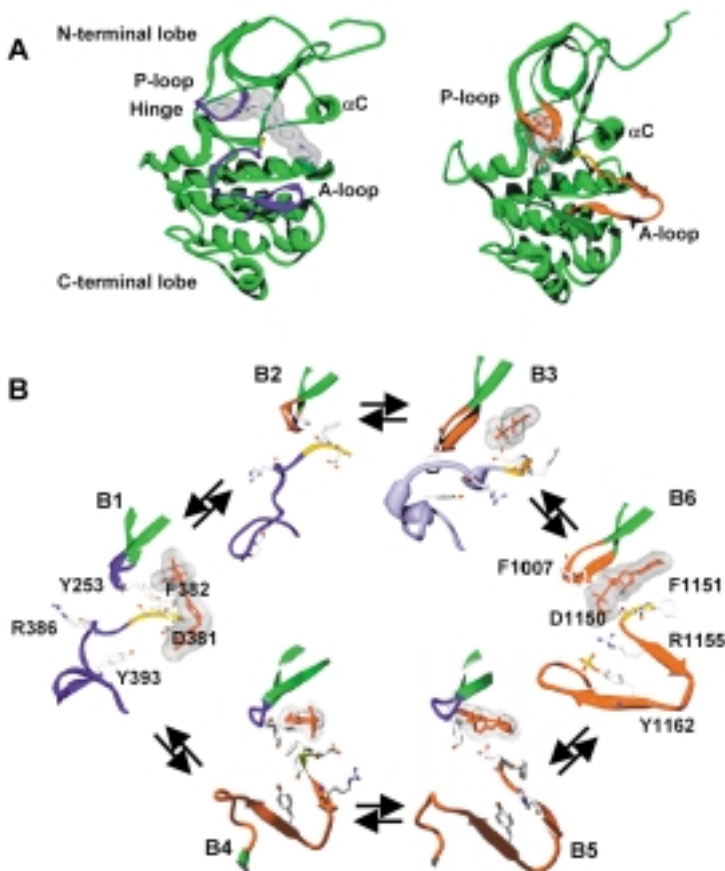


Figure 1. Principle of the Bcr-Abl based molecularly targeted therapy. A) The constitutively active Bcr-Abl tyrosine kinase transfer phosphate from ATP to tyrosine residues of various substrates inducing excess proliferation and inhibition of apoptosis of myeloid cells characteristic of CML. B) Imatinib inhibits the kinase activity by blocking the binding of ATP to the protein, thus preventing CML.



**Figure 2. Conformational plasticity: a multiple pathway from "Off-state" to "On-state" and vice versa.**

**A)** On the left, the X-ray structure of Bcr-Abl tyrosine kinase domain in complex with imatinib (1IEP; Nagar et al., 2002) represents the inactive "Off-state" conformation of tyrosine kinase with the nucleotide binding loop (P-loop) in a peculiar conformation and the activation loop (A-loop) inserted into the substrate binding pocket that prevent binding of ATP and the substrate. On the right, the X-ray structure of insulin receptor tyrosine kinase domain (1IR3; Hubbard, 1997) in complex with an ATP analog represents the active "On-state" conformation with an elongated P-loop and an open A-loop that allow ATP and substrate binding. The  $\alpha$ -helix C ( $\alpha$ C) that experiences significant movement along the transition from "Off-state" to "On-state" is labelled. The hinge region (Hinge) connecting the N-terminal lobe with the C-terminal lobe is labelled. All ligands, ATP and inhibitors binding to the ATP pocket of the kinase domain form hydrogen bonds with the hinge region (Hinge) (Moretti et al., 2006). The position of the conserved and catalytically important DFG motif lying at the beginning of the A-loop is shown in yellow. Imatinib and the ATP analog are depicted by capped sticks and their molecular surface is shown. The P-loops and A-loops having a conformation closer to the "Off-state" one are coloured in blue, while those presenting a more "On-state" conformation are shown in red. Cyan is used in cases where the loops assume a conformation in-between "Off-state" and "On-state".

**B)** Close up on the P-loop, A-loop and DFG motif of different X-ray structures showing the existing variability in conformation going from the "Off-state" (B1) and the "On-state" (B6). The tyrosine on the A-loop in the "On-state" is phosphorylated. In the "Off-state" conformation, the Phe of the DFG points towards the ATP binding site, while in the "On-state" it is tilted 180°. The colour code is the same as for panel A. The DFG as well as important aligned amino acids of the P-loop and A-loop are shown as colour coded sticks and labelled for (B1) and (B6). Amino acid numbering refers to Abl (B1) and IR (B6). The X-ray structures shown here are: B1: Abl in complex with imatinib (1IEP; Nagar et al., 2002), B2: Insulin receptor kinase apo (1IRK; Hubbard et al., 1994), B3: HCK ligated to PP1 (1QCF; Schindler et al., 1999), B4: Abl complexed with PD173955 (1M52; Nagar et al., 2002), B5: Abl in complex with VX-680 (2F4J; Young et al., 2006), B6: Insulin receptor kinase ligated with an ATP analog (1IR3; Hubbard, 1997). Because of the orientation of the DFG, conformation of B2 and B4 are considered more close to B1, while B3 and B5 are more similar to B6.

a specific inactive conformation of Abl in which the activation loop (A-loop) mimics the substrate and the Phe of the DFG motif points towards the ATP binding site (**Figure 2**). Imatinib forms 6 hydrogen bonds with the protein and several hydrophobic contacts with amino acids forming an extended selectivity pocket favoured by the closed conformation of the A-loop. The nitrogen of the pyridine moiety forms a hydrogen bond with the hinge region that is characteristic for tyrosine kinase inhibitors (Moretti et al., 2006). In addition, the amino group of the 2-phenylaminopyrimidine moiety builds a hydrogen bond with the gatekeeper amino acid Thr315 (Nagar et al., 2002).

The scenario of treatment options for CML and other types of Philadelphia chromosome positive (Ph+) leukemias is entering a new phase, thanks to the development of Imatinib (Goldman & Druker, 2001). For the first time, a specific and relatively non-toxic drug is able to induce durable remissions in most patients with Ph+ leukemias, and cytogenetic responses in the majority of CML patients in chronic phase. Imatinib has been under clinical investigation for almost 8 years. During this time, over 50.000 patients, mostly affected by CML or Ph+ALL, have received imatinib.

Patients with acute leukemia (defined as the presence of > 30% blasts in either the bone marrow or the peripheral blood) showed a high (> 50%) rate of initial haematological responses: 52% of the patients with "myeloid" BC-CML and 60% of the patients with Ph+ ALL or "lymphoid" BC-CML showed reduction in blast values corresponding to a haematological response (Ottmann et al., 2002; Sawyers et al., 2002). Patients included in this category present either

"de novo" acute leukemias (Ph+ ALL or AML) or blast crisis CML. The therapeutic effects obtained in such patients are, however, short-lived (3–6 months) and quickly followed by relapse and resistance to imatinib in almost all patients. Although patients affected by the myeloid blast crisis CML seem to fare slightly better than the patients with ALL or lymphoid blast crisis CML, the present overall evaluation of imatinib in this category of patients can be considered as a "qualified failure".

In patients with accelerated phase leukemia ((AP), defined as blasts between 15 and 30%, basophiles > 20%, or platelet counts < 100 k/mm<sup>3</sup>), durable major cytogenetic responses can be obtained, but only in approximately 25% of patients, although the treatment is probably able to prolong survival even in the absence of a cytogenetic response (Talpa et al., 2002).

Patients affected by CML and treated with imatinib in late CP (defined as < 15% blasts, < 20% basophiles and with platelet count > 100 k/mm<sup>3</sup>), who are intolerant, resistant or refractory to interferon treatment, respond better than the patients in more advanced phases. Major cytogenetic responses (MCyR) were initially obtained in 64% of 450 patients who started imatinib in late CP, at a median time of 34 months after the diagnosis of CML (Kantarjian et al., 2002). This trial now reached the 5 year of median follow up, and the results were recently presented (Gambacorti et al., 2005a). This population of late CP-CML patients is the first one, in which the treatment with imatinib has proved to be able to change the biological history of the disease. At 60 months, 81% of patients who reached a MCyR

Table 1: Time to AP/BC, yearly event rate (from Gambacorti et al., 2005a)

Year since start of treatment	All (n=454)	MCyR at any time (n=304)	No MCyR at any time (n=150)	MCyR at 3 months (n=148)	No MCyR at 3 months (n=295)
1 <sup>st</sup>	7.8%	2.6%	18.6%	2.7%	8.9%
2 <sup>nd</sup>	6.0%	0.3%	21.1%	0.7%	8.9%
3 <sup>rd</sup>	7.2%	3.6%	20.6%	2.3%	10.2%
4 <sup>th</sup>	7.3%	4.0%	24.7%	2.5%	10.6%
5 <sup>th</sup>	7.3%	4.4%	28.1%	1.8%	11.6%

are still in cytogenetic remission, and 92% are alive (**Figure 3** and **Table 1**). The early reach of cytogenetic remission is evidenced in **Figure 3** as an important prognostic sign, and the relapse rate (a bona fide indicator of the development of resistance to imatinib) among patients in cytogenetic response continues to range between 1 and 2% (compared with a historical value of 20-25%). These data indicate that, given the average age at which CML is diagnosed (45-50 years) and the average life expectation, and considering the present yearly risk of relapse, the majority of CML patients will die of other causes, and not of CML.

These early results were later confirmed in a subsequent trial, in which imatinib demonstrated a clear superiority as a first line treatment of CML when compared with the combination of Interferon beta and Ara-C (O'Brien et al., 2003).

In this protocol, monitoring patients by quantitative PCR for the presence of the Bcr/Abl transcript allowed to obtain further information on how imatinib affected CML biology. In fact, if patients reach at least 3 log unit reduction of Bcr-Abl transcript present, their yearly risk of relapse is further reduced to less than 0.5%. However, the available data allow also to state that the vast majority of treated patients (80-90%) remain positive for the presence of Bcr-Abl transcript for at least the first 2 years of treatment (Hughes et al., 2003). When the level of Bcr-Abl positive cells falls below 1/10,000-1/100,000, even PCR fails to detect the transcription. Until recently, the discontinuation of imatinib was regularly followed by relapse of CML, even in patients who tested negative at the time of drug suspension (Cortes et al., 2004), indicating that PCR negativity only meant that the number of residual leukemic cells

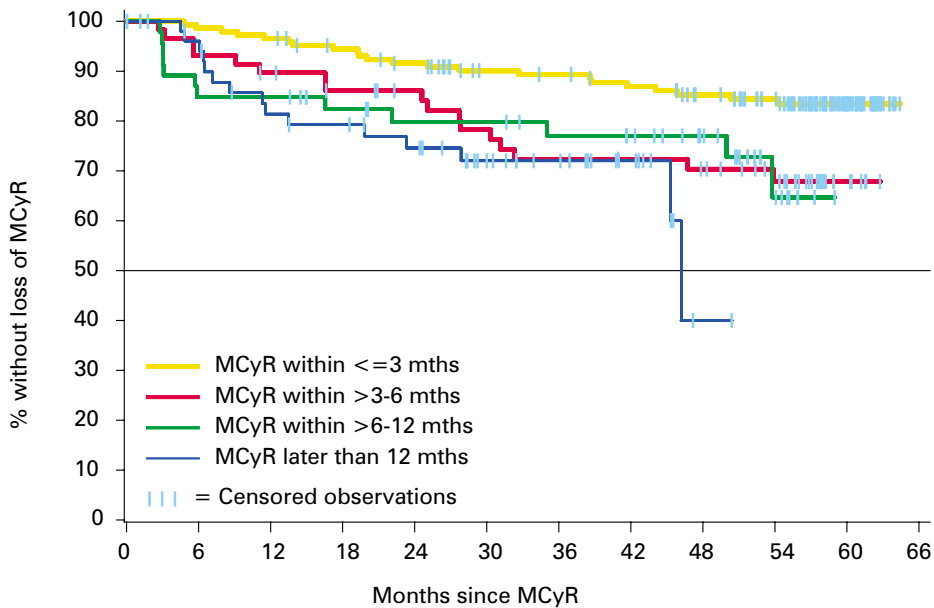


Figure 3. Duration of major cytogenetic responses (MCyR) after reaching MCyR (from Gambacorti et al., 2005)

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was below the PCR detection limit.

Very recent data, however, have changed this view, at least in part. Two recent reports indicate that the discontinuation of imatinib in patients, who achieved a sustained PCR negativity, was not followed by disease relapse in approximately 50% of cases (Merante et al., 2005; Rousselot et al., 2005). Moreover, a recent presentation on the molecular monitoring of patients enrolled in the IRIS trial (Goldman et al., 2005), indicated that the amount of transcript decreases gradually, up to 4 years after imatinib initiation. In this report, the number of patients who achieved >4 log unit reduction of Bcr-Abl transcript (i.e. PCR negative or almost negative) after 4 years of treatment reached the value of 41%. Therefore, an important issue connected to the long term follow up of CML patients on imatinib treatment is whether the treatment could be interrupted at a certain point. This issue remains unresolved at present.

In addition to Ph+ leukemias, important clinical responses were also obtained in patients with malignancies caused by structural alterations of the PDGFR beta gene (Magnusson et al., 2002; Apperley et al., 2002). Imatinib is also active against gastrointestinal stromal tumours (GIST), a rare form of non-epithelial gastrointestinal cancer that frequently presents activating mutations of the c-KIT gene, another target of imatinib. In this group of patients, objective response rates of 53% and 54% were obtained (Apperley et al., 2002; van Oosterom et al., 2001), with therapeutic effects often observed within a few days of treatment (Joensuu et al., 2001). These responses appear to be durable and linked to the presence of c-Kit mutations in the tumour: risk of progression after imatinib treatment was 8 times higher in patients lacking c-Kit mutations than in patients carrying mutated c-Kit (44% vs 5.8%; Heinrich et al., 2002).

During the successful clinical trials, resistance to imatinib emerged particularly in patients with acute leukemias, but it is a potential issue also in patients in chronic phase. The molecular mechanism of resistance has been identified in Bcr-Abl gene amplification and mutations in the catalytic kinase domain of the gene (for review; Gambacorti-Passerini et al., 2003). The mutation of the gatekeeper amino acid threonine into a isoleucine (T315I) has been depicted as the

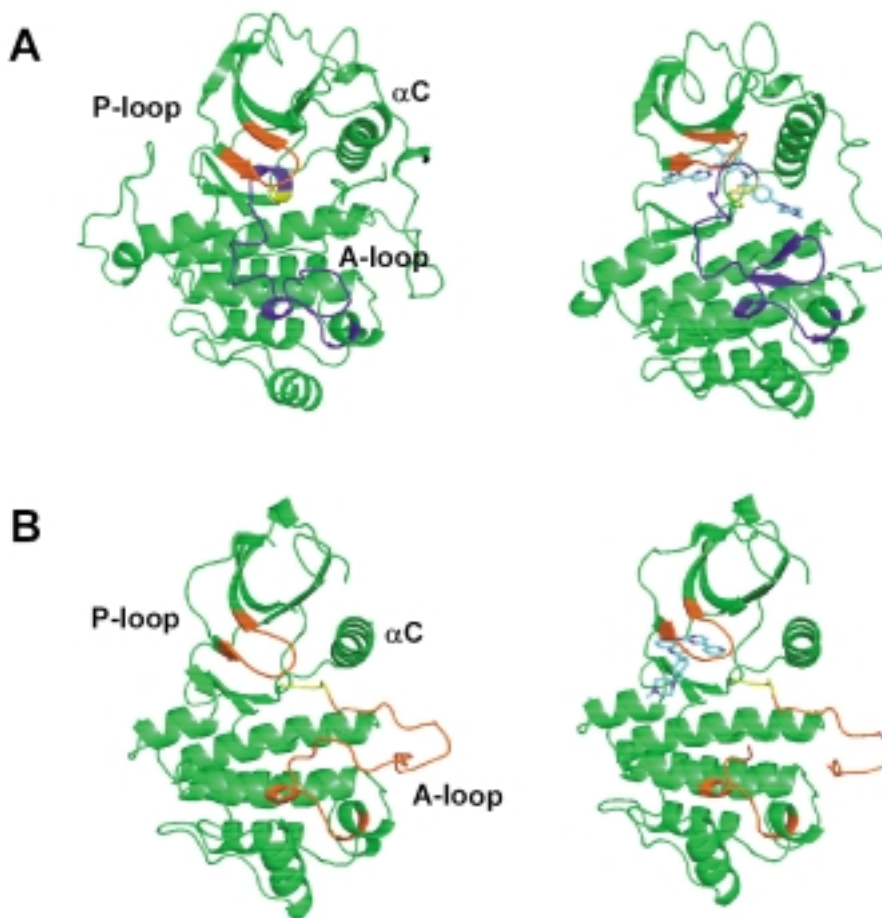
predominant one in patients (Gambacorti-Passerini et al., 2003). This has prompted intense research to find new compounds able to overcome the resistance problem, such as BMS-354825 (Shah et al., 2004), SKI-606 (Golas et al., 2003) and AMN107 (Weisberg et al., 2005). BMS-354825 and SKI-606 are dual Src and Abl inhibitors, and are currently undergoing clinical evaluation. Both inhibit *in vitro* and *in vivo* several imatinib-resistant Bcr-Abl mutants, but not the T315I. Their activity profiles suggest that both compounds have less stringent conformation requirements for binding than imatinib, and they are thought to share the binding mode and binding conformation of PD173955 (**Figure 2B**) (Gambacorti-Passerini et al., 2005b; Thaimattam et al., 2005).

Very recently, an ATP competitive inhibitor, VX-680, ligating the "On-state" conformation of Abl (**Figure 2B**) and moderately inhibiting the T315I variant of Abl, has been reported (Young et al., 2006). A recent example of a potent ( $IC_{50}$  in the low nanomolar range) and specific small molecule inhibitor targeting the peptide substrate pocket of Abl is ON012380 (Gumireddy et al., 2005). ON012380 inhibits the T315I mutation, because it targets a different site of the protein compared to the inhibitors competing with ATP (Gumireddy et al., 2005). The first allosteric inhibitor of Bcr-Abl (GNF-2), a 4,6-disubstituted pyrimidine derivative, has been shown to inhibit Bcr-Abl-dependent cell proliferation with submicromolar  $IC_{50}$  without inhibition of the Abl kinase activity *in vitro* (Adrian et al., 2006).

The development of imatinib, the search of more potent Abl inhibitors, the knowledge acquired at the molecular, structural and cellular level represent not only a new phase for the treatment of CML, but also a bench example for other oncogenic tyrosine kinases (Blume-Jensen & Hunter, 2001). The conformational plasticity of tyrosine kinases (Huse & Kuriyan, 2002) represents a great opportunity for therapeutic intervention, because it offers the possibility of exploiting more putative binding sites that are present or formed along the trajectory going from the "Off-state" to the "On-state" (**Figure 2**), as shown by the Bcr-Abl model case. Crystallographic studies of apo and imatinib complexed structures of c-Kit and Spleen kinase SyK (**Figure 4**) clearly show that the inhibitor binds

following the conformational selection model (Bosshard, 2001). According to this model, a certain part of the protein is distorted in the sense that a number of alternative conformations are available. Selective binding of the ligand to a protein molecule in one of these conformational states causes a perturbation of the equilibrium

between these states. In the case of tyrosine kinases, the two extreme states are the "Off-state" and "On-state" (**Figure 2**). Thus, we face a situation of having one protein with a series of targetable sites. Nevertheless, the number of targetable sites available within the protein and their stability in time remain to be discovered.



*Figure 4. The conformation targeted by the inhibitor exists already in its apo form. A) On the left, the crystal structures of c-Kit tyrosine kinase in its apo form showing a clear "Off-state" inactive conformation stabilized by the juxtamembrane domain (1T45; Mol et al., 2004). On the right, c-Kit in complex with imatinib (1T46; Mol et al., 2004) exhibits the same "Off-state" conformation like its apo structure. Imatinib assumes the same binding mode as in Abl TK. B) The structure on the left shows the apo form of the spleen tyrosine kinase (SyK) (1XBA; Atwell et al., 2004) that is characterized by the "On-state" conformation. The same conformation is also assumed once ligated with imatinib (right, 1XBB; Atwell et al., 2004). In this case, the binding mode of imatinib differs from the one observed within Abl. For the protein, the same colour code as in Figure 2 has been used. Imatinib is depicted in cyan stick. The same view angle was used for the pictures.*

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## CONCLUSIONS

In conclusion, the story of the CML-Bcr/Abl-*imatinib* "connection" points out to the need of clearly identified molecular targets, causally related to transformation, if effective anti-neoplastic treatments are to be developed. It also points out the need for increased knowledge on stability of single conformations (targetability of the conformation) as well as new chemical scaffolds for exploiting the targetable sites offered by each tyrosine kinase. Therefore, Bcr-Abl stands as a model oncogenic TK for the development of targeted treatments in other diseases. It will remain to be seen, if such a model will be followed by the major drug developers.

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# PROTEIN KINASES AS THERAPEUTIC TARGETS IN LEUKAEMIA

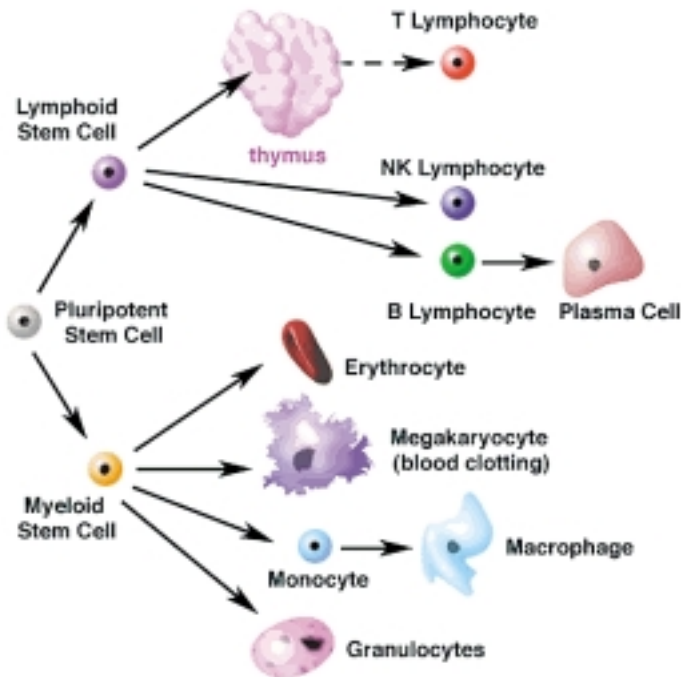
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*Figure 1. Haemopoiesis*

*Blood cells are derived in the bone marrow from a common precursor stem cell. Progenitor cells commit early on to either the myeloid or lymphoid lineages. Lymphoid progenitors differentiate to produce T and B-lymphocytes. Myeloid progenitors differentiate to produce leukocytes, erythrocytes and megakaryocytes. (Figure kindly provided by Dr C M Bunce).*

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## WHAT IS LEUKAEMIA?

All blood cells develop in the bone marrow from a common precursor, the pluripotent haemopoietic stem cell (HSC), via various progenitor cell stages (**Figure 1**). As the HSC differentiates towards a mature blood cell, proliferative capacity is lost and the phenotype of the mature cell is acquired. This process of haemopoiesis is tightly regulated by soluble and stromal cell associated factors, e.g. SCF, GM-CSF, IL3, which control the proliferation, differentiation and survival of the progenitor cells and mature blood cells (Dexter & Spooncer, 1987). Haemopoiesis is also highly flexible and can respond to changing needs very rapidly. For example at times of microbial infection, the output of neutrophils can increase three fold under the influence of factors such as GM-CSF, and under conditions of low oxygen tension the release of erythropoietin stimulates increased production of erythrocytes. Homeostasis is eventually restored, once the need for extra blood cells has passed, by the removal of excess cells by apoptosis.

Leukaemia is derived from the Greek word meaning literally "white blood" and refers to cancers of the haemopoietic system. In leukaemia the normal balance between proliferation, differentiation and apoptosis of blood cells and their progenitors in the blood and bone marrow is distorted, leading to an abnormal accumulation of immature precursors. In the lymphoid lineage cells can undergo a further round of expansion and differentiation in the secondary lymphoid

organs in response to antigen challenge, and this can give rise to a distinct class of diseases called the lymphomas, which for the sake of brevity will not be considered here. The presence of large numbers of abnormal progenitor cells in the bone marrow causes a suppression of the growth and development of normal progenitor cells and, as a consequence, symptoms of leukaemia include neutropenia, thrombocytopenia and anaemia. The leukaemias are classified according to the lineage affected and the rate of progression of the diseases. The four main types are thus: Acute Lymphoblastic Leukaemia (ALL), Acute Myeloid Leukaemia (AML), Chronic Lymphocytic Leukaemia (CLL) and Chronic Myeloid Leukaemia (CML). In general, the disease course is quite indolent for the chronic leukaemias and patients survive for many years with the disease, whilst in the acute leukaemias disease onset and progression is rapid. The leukaemias can be subdivided further (for example there are 8 AML sub-types), dependent upon the lineage involved and/or the stage of differentiation in which the cells are arrested, but a more detailed description of the leukaemias and their classification is beyond the scope of this article.

There are approximately 6,000 new cases of leukaemia per year in the UK, of which almost half are CLL and one third are AML (**Table 1**). In general, leukaemia is a disease of the elderly, though ALL occurs predominantly in children. Whilst ALL is almost entirely curable (80–85% remission rate in children), the prognosis for adult leukaemia is poorer, due in part to the need for

*Table 1. Annual incidence of new cases of leukaemia and lymphoma in the UK.*

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Acute lymphoblastic leukaemia	650
Acute myeloid leukaemia	2,000
Chronic lymphocytic leukaemia	2,750
Chronic myeloid leukaemia	750
Hodgkin's lymphoma	1,400
Non-Hodgkin's lymphoma	8,450
Myeloma	3,300
Myelodysplastic syndromes	3,250
Myeloproliferative disorders	1,900
Aplastic anaemia	130
<b>Total (rounded)</b>	<b>24,500</b>

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aggressive chemotherapy regimes which are not well tolerated in the elderly (Lowenberg et al., 1999; Schoch et al., 2004). There is thus a pressing need for new therapies for leukaemia, especially as combination therapies that might synergise with existing chemotherapies to enhance efficacy in the older patients. The predominant therapies for the treatment of leukaemia aim to either overcome the differentiation block or to induce growth arrest and apoptosis in the leukaemic cells. The most successful differentiation therapy is the treatment of one type of AML (M3 sub-type, Acute Promyeloid Leukaemia) with all trans retinoic acid (ATRA). AML-M3 is associated with a chromosomal translocation (t15:17) resulting in the expression of the fusion protein PML-RAR $\alpha$ , which is more resistant to the differentiating effects of retinoic acid than the normal retinoic acid receptor RAR $\alpha$ . The insensitivity of the leukaemic cells to endogenous retinoic acid can be overcome by treatment of the patients with ATRA. For induction of growth arrest and apoptosis, a variety of chemotherapeutic agents are used. Classically this involves giving cytarabine (a deoxycytidine analog) by continuous infusion for 7 days and an anthracycline antibiotic such as daunorubicin or idarubicin, i.v. for 3 days. Postremission therapy is usually then in the form of high-dose chemotherapy, or chemo-radiotherapy with either allogeneic or autologous hematopoietic stem cell transplantation (Yates et al., 1982; Dillman et al., 1991).

However, most of these chemotherapeutic agents are non-specific and damage normal healthy cells as well as malignant cells and, thus, more targeted "smart" therapies are now the aim for the pharmaceutical industry. In this respect the success of Imatinib, a small molecule kinase inhibitor, which targets a fusion protein BCR-ABL present in CML, has provided proof that understanding the biology of leukaemia can lead to the development of targeted and effective drugs. In this respect, genetic alterations to haemopoietic transcription factors may be key factors in leukaemogenesis (Look, 1997), but it is the aberrant activity of signalling pathways regulating cell proliferation and apoptosis that may be most amenable to therapeutic modulation (Martinelli et al., 2005; Lydon & Druker, 2004).

Thus, in addition to the production of overactive tyrosine kinases by chromosomal mutations, exemplified by BCR-ABL, it has also become clear that targeting serine/threonine protein kinases with a known role in proliferation and/or apoptosis is also a rational strategy. Both of these options will be discussed here.

## **TYROSINE PROTEIN KINASES AND LEUKAEMIA**

Tyrosine kinases play an essential role in the signalling pathways involved in the control of cellular proliferation. The number of small molecule protein kinase inhibitors entering clinical development is increasing following the success of imatinib (9,10) and the majority of these are targeted at tyrosine kinases that are overactive in different leukaemias and lymphomas. In many cases the aberrant activity has arisen as the result of a chromosomal translocation that has resulted in the expression of a fusion protein with enhanced tyrosine kinase activity. Examples of this genetic alteration are BCR-ABL present in CML (11) and the NPM-ALK protein found in Anaplastic Large Cell Lymphoma (12). Small molecule inhibitors of NPM-ALK are also now in pre-clinical development and are discussed elsewhere in this issue.

BCR-ABL and CML: The Philadelphia chromosome occurs in 95% of CML patients and arises from a reciprocal translocation of chromosomes 9 and 22. This results in the fusion oncogene *BCR-ABL* whose protein product shows enhanced tyrosine kinase activity (Ben Neria et al., 1986). Whilst most CML patients express a 210 kDa splice variant of BCR-ABL, a 190 kDa BCR-ABL protein is also expressed in ALL and is associated with a much more aggressive disease (Ren, 2005; Faderl et al., 2002). The fact that BCR-ABL translocation occurs in 95% of patients, and is sufficient to cause the initial chronic stage of the disease, made it an attractive target for drug design. STI-571 (Glivec, Gleevec, Imatinib) - a dual inhibitor of v-Abl and PDGF-R kinases, is a 2-phenylaminopyrimidine identified by high throughput screening of compound libraries and optimisation of active compounds based on the inhibition of protein kinases *in vitro*. Subsequent preclinical tests showed that STI-571 could suppress the proliferation of BCR-ABL

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expressing cells both *in vitro* and *in vivo* (Druker et al., 1996). In colony-forming assays of peripheral blood or bone marrow from patients with CML, there was a 92–98% decrease in the number of bcr-abl colonies formed, but no inhibition of normal colony formation. The drug has consequently undergone clinical trials in CML and has been highly effective, with a majority of patients achieving a sustainable haematological remission (Druker et al., 2001; Peng et al., 2004). Unfortunately, Imatinib has proved less useful in patients who have progressed to the terminal blast phase of CML. This is frequently due to the emergence and expansion of clones of cells that express mutated forms of BCR-ABL which show decreased sensitivity to imatinib (Cowan-Jacob et al., 2004), and this has led to attempts to design second generation compounds targeting the BCR-ABL mutants. Much effort has focussed upon compounds able to bind to the active conformation of the ABL kinase domain. The rationale here is that as imatinib binds the inactive conformation then resistance BCR-ABL mutants are less likely to arise with a drug targeting the active kinase, since any mutations would have to be able to reduce binding of the inhibitor to the active site without affecting basic kinase activity, i.e. ATP-binding or phosphate transfer. However, while compounds able to target the active conformation of ABL have been developed, such as the pyridopyrimidines (Lombardo et al., 2004), these lack the degree of selectivity to be achieved with compounds that target the inactive conformation. Second generation compounds targeting BCR-ABL are therefore still predominantly those that bind the inactive conformation. AMN107 is in clinical development and is 20–30 times more potent than imatinib. Despite being of the same class of compounds as imatinib, it has been shown to be effective against the most prevalent BCR-ABL mutants, with the exception of the T3151 mutation (reviewed in Manley et al., 2005).

A second approach is to develop dual specificity agents that target BCR-ABL and other tyrosine kinases that contribute to the transformed phenotype of the imatinib resistant cells. Src family kinases include nine structurally-related tyrosine kinases, many of which are expressed in haemopoietic cells and regulate multiple cellular functions including cell

migration, proliferation, differentiation and survival. They appear to contribute to Bcr-Abl mediated leukemogenesis in that they lie downstream in BCR-ABL signalling cascade (Dai et al., 2004; Donato et al., 2003) and inhibition of their activity may thus provide synergy with BCR-ABL inhibition to overcome imatinib resistance. Several Src and ABL dual inhibitors have been developed and are in preclinical or early clinical studies, the most advanced compounds being BMS-354825, AZD05340 and SKI-606 (Martinelli et al., 2005). BMS-354825 is in phase II clinical trial in imatinib resistant CML patients.

Flt3 and AML: In addition to reciprocal chromosomal translocations, other mutations have also been described that have resulted in over active tyrosine kinases. Probably the best studied with respect to leukaemia are the mutations to the FMS like tyrosine kinase 3, Flt3, gene. The Flt-3 gene encodes a receptor tyrosine kinase containing two intracellular tyrosine kinase domains which plays important role in proliferation, differentiation and survival of normal haemopoietic cells (Gilliland & Griffin, 2002). Flt-3 is expressed by early myeloid and lymphoid progenitors as well as on malignant haemopoietic cells. Human Flt-3 ligand occurs mainly as a type 1 transmembrane protein that can be cleaved proteolytically to release soluble Flt3L. It is expressed by most tissues, including haemopoietic tissues and bone marrow fibroblasts, as well as on many transformed hematopoietic cell lines (Rosnet et al., 1996; Lisovsky et al., 1996). In contrast, Flt3 is found mainly in early haemopoietic progenitor cells and activation of Flt-3 by its ligand promotes the growth of early progenitor cells *in vitro* and *in vivo* via activation of the PI3 kinase and RAS signaling pathways (reviewed in Stirewalt & Radich, 2003). Flt-3 mutations occur in approximately 35% of AML patients and are associated with a poor prognosis. Mutations are either internal tandem duplications or mis-sense point mutations. Internal tandem duplications were first reported in the juxtamembrane domain of Flt-3 in AML by Nakao and colleagues in 1996, who suggested that these mutations may play an important role in the pathogenesis of AML (Nakao et al., 1996). They analysed the flt3 gene in 30 patients with acute myeloid leukemia (AML) and 50 with acute lymphoblastic leukemia (ALL) and

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found five patients with extended flt3 transcripts, which essentially included a duplication of the juxtamembrane tyrosine kinase domain. Two different mis-sense point mutations occur in the tyrosine kinase domain of Flt-3, resulting in the constitutive activation of the receptor and downstream signalling pathways such as RAS (Stirewalt & Radich, 2003). Given the high frequency of activating *Flt-3* mutations in patients with AML, Flt-3 represents an attractive target for selective therapeutic inhibition.

Four Flt-3 inhibitors are currently in clinical trials, namely SU11248, PKC-412, MLN518 and CEP-701 (Stirewalt & Radich, 2003). Results to date have been modest, but have shown promise with transient reduction in peripheral blood leukaemic blasts, but with only occasional reduction in bone marrow blasts and a very low incidence of haematological responses (Stirewalt & Radich, 2003; Stone et al., 2002). However, this may reflect the fact that AML is an aggressive and heterogenous disease and unlike BCR-ABL, Flt-3 mutations alone are not enough to cause AML. It is therefore more likely that drugs targeting Flt-3 will show better efficacy in combinatorial therapy.

### ***Jak kinases and leukaemia***

Jaks are a family of four tyrosine kinases (Jak1, Jak2, Jak3, Tyk2). Jak2 has been associated with leukaemia, since a chromosomal translocation forming the TEL-JAK2 fusion protein that results in constitutive kinase activity was detected in B- and T-lineage ALL (Lacronique et al., 1997; Peeters et al., 1997). An activating Jak2 mutation (JAK2V617F) has also been closely linked to BCR/ABL negative myeloproliferative disorders (Tefferi & Gilliland, 2005). More recently, a BCR-Jak2 fusion gene was observed in an imatinib resistant CML -like patient (Faderl et al., 2005). These findings indicate that Jak tyrosine kinases could be promising targets for leukaemic therapies. In this context, WP-1034, a novel inhibitor of Jak signaling, is a potent inhibitor of AML cell proliferation (Meydan et al., 1996) and systemic administration of AG-490, another selective Jak kinase inhibitor, in a xenograft animal model of AML lead to complete tumor regression (Steelman et al., 2004).

## **SERINE/THREONINE PROTEIN KINASES AND LEUKAEMIA**

The pivotal role of serine/threonine protein kinases in the regulation of cell proliferation and apoptosis would suggest that they had considerable appeal as pharmacological targets across a broad spectrum of pathologies including cancer. However there has been long-standing skepticism with regard to the targeting the serine/threonine protein kinases. This relates primarily to concerns over the possibility of developing compounds with sufficient specificity among this class kinases and also the wisdom of targeting a single kinase to treat a disease in which several signaling pathways and cellular processes are altered. Despite these concerns and following the success of imatinib, several agents targeting serine threonine kinases have reached clinical phase testing. The current status of attempts to target serine/threonine kinases, is reviewed briefly here.

### ***Protein Kinase C and leukaemia***

Protein Kinase C (PKC) is a family of at least 12 isoenzymes and there is reasonable evidence that these play differential roles within the cell, including regulation of proliferation, differentiation and apoptosis (Lord & Pongracz, 1995). They therefore represent logical therapeutic targets and indeed PKC has received the most attention to date with regard to therapeutic modulation, not only for cancers but also for diabetes. Although there are currently few modulators of PKC that show isoenzyme selectivity, the gradual publication of the crystal structures of the regulatory domains of key PKC isoenzymes, should lead rapidly to the synthesis of such compounds. With respect to anti-cancer therapies, it is PKC- $\alpha$  and PKC- $\delta$  which appear to represent logical targets, as both have been implicated in the regulation of apoptosis, and there are now several reports of increased or decreased activity of these kinases in association with haematological malignancies. In CLL, although cells do not have increased PKC activity, inhibition of PKC induces CLL cell apoptosis (Barragan et al., 2002; Kitada et al., 2000). More recently, it has been shown that expression of

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dominant-negative PKC- $\alpha$  in normal haemopoietic progenitor cells resulted in cells that phenotypically resembled human CLL cells (Nakagawa et al., 2006), suggesting a role for this isoenzyme in tumour suppression. Our own recent studies have shown that primary CLL cells express PKC- $\alpha$  and that compounds which selectively activate this isoenzyme can induce apoptosis in these cells (K. Wang, unpublished observations). Possible downstream targets of PKC- $\alpha$  include bcl-2, with some authors reporting that phosphorylation of bcl-2 leads to reduced functioning of this key anti-apoptotic protein (Yamamoto et al., 1999). However, caution is required as other groups have suggested that PKC- $\alpha$  mediated bcl-2 phosphorylation leads to enhanced anti-apoptotic activity. Work from Deng et al. (2001) showed that Bcl2 phosphorylation by ERK 1/2 and PKC  $\alpha$  kinases, either at Ser70 residue or at multiple Thr69, Ser70, and Ser89 sites, positively regulated Bcl2 antiapoptotic function. However, other investigators have shown that c-Jun NH2-terminal kinase-mediated multiple-site Bcl2 phosphorylation hinders Bcl2 survival function in paclitaxel-induced apoptosis (Yamamoto et al., 1999).

PKC- $\delta$  has also been proposed as an anti-cancer target. The majority of studies have shown that this kinase has significant pro-apoptotic functions (Lord & Pongracz, 1995; Emoto et al., 1996). Our own studies have shown that a plant derived broad range PKC activator, 3 ingenyl angelate (PEP005), is a potent anti-leukaemic agent and that its effects are mediated via PKC- $\delta$  (Hampson et al., 2005). PEP005 induced apoptosis in human primary AML cells in the low nanomolar range, with no effects on normal myeloblasts up to two log concentrations higher. PEP005 is already in phase II clinical trial for non-melanoma skin cancer and is due to enter phase I clinical trials for AML late in 2006 (Hampson et al., 2006). Interestingly, PKC- $\delta$  has also been implicated in the development of CLL, but in this case PKC- $\delta$  was constitutively activated downstream of PI-3-kinase (Ringshausen et al., 2002). Small molecule inhibitors of either PI-3-kinase (Ly294002) or PKC- $\delta$  (Rottlerin) were potent inducers of apoptosis in CLL cells in vitro (Ringshausen et al., 2002; Ringshausen et al., 2006). These data emphasise the need to define the biology of the individual leukaemias and that a protein kinase modulator effective in one leukaemia will not necessarily be effective in all leukaemias.

In addition to PEP005, several broad range PKC activators and inhibitors have reached clinical trial stage. 12-O-Tetradecanoylphorbol-13-acetate (TPA) entered phase I clinical trial for AML in the US (Strair et al., 2002) and Bryostatatin 1 (NSC 339555), a macrocyclic lactone PKC activator has been tested in phase I clinical trials for refractory acute leukaemia (Cragg et al., 2002). PKC inhibitors UCN-01 and PKC412 have also been tested in phase I and II clinical trials for B-CLL (Byrd et al., 2001; Virchis et al., 2002).

### ***The Ras-Raf-Mek-Erk pathway and leukaemia***

The Mitogen Activated Protein Kinases (MAPKs) are a family of evolutionarily conserved, ubiquitously expressed serine/threonine kinases that play an important role in the signal transduction involved in a number of cellular processes including proliferation, differentiation and apoptosis. Mammalian cells express at least three distinctly regulated groups of MAPKs. These are, the extracellular signal-related kinases (ERK), consisting of ERK1 and ERK2 (p42, p44), the Jun amino-terminal kinases (JNK), consisting of JNK1/2/3, and the p38 proteins consisting of p38 $\alpha/\beta/\delta$ . MAPKs are activated in a complex MAPK signalling cascade, the core of which is an evolutionarily conserved unit of three protein kinases activated in sequence (reviewed in Chang & Karin, 2001). The activation of the ERK pathway is regulated via a signalling cascade initiated at the level of the small GTP-binding protein Ras. This cascade involves the sequential activation of Raf, Mek1/2 and Erk1/2. The fact that the Ras/Raf/MEK/ERK pathway promotes proliferation and is anti-apoptotic, makes it another potential therapeutic target. Importantly, it has been shown that a conditionally active form of MEK1 can cause the autocrine transformation of haemopoietic cells, leading to cytokine independence (Blalock et al., 2000), suggests that this pathway may be involved in leukemogenesis. Moreover, several studies have shown that Mek1/2 and Erk1/2 are constitutively activated in primary human acute myeloid leukaemia cells (Kim et al., 1999; Milella et al., 2001). In addition, mutations in Ras leading to its constitutive activation have been detected in around 20–30% of human ALL and AML (Blalock et al., 2000; Farr et al., 1988).

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A variety of approaches have been used to target this pathway therapeutically. For example, farnesylation of Ras is necessary for its membrane localisation and cell transforming activity. Consequently farnesyltransferase inhibitors (FTIs), such as R115777 have been developed to inhibit Ras activity. R115777 has shown promise when administered as a single agent in patients with haematological malignancies such as MDS, CML and AML (Kurzrock et al., 2004; Cortes et al., 2003; Karp et al., 2001). 3 out of 28 MDS patients treated with R115777 responded (2 with complete remission and 1 partial remission). 7 out of 22 patients with CML (6 in chronic phase, 1 in advanced phase) achieved complete or partial hematologic response with R115777 and four of these had a minor cytogenetic response. However, responses were transient, with a median duration of 9 weeks (range, 3–23 weeks). In a phase 1 trial of the same agent for AML, clinical responses occurred in 10 (29%) of the 34 evaluable patients, including 2 complete remissions. Although mutations of Raf have been found in a number of human cancers – mainly melanomas (Davies et al., 2002), we are not aware of such mutations in leukaemias. However, a small molecule inhibitor of Raf, BAY 43-9006, has shown anti-leukemic effects against cell lines in vitro associated with MCL-1 downregulation (Rahmani et al., 2005). MEK mutations are also uncommon, although as stated above, several studies have shown that Mek1/2 and Erk1/2 are constitutively activated in primary human acute myeloid leukaemia cells. Treatment of leukaemia blasts with the MEK inhibitors PD98059 or PD184352 caused growth inhibition and induced apoptosis (Milella et al., 2001). Treatment of blasts with MEK inhibitors can also sensitise them to drug induced cytotoxicity from a variety of established chemotherapeutic agents including Ara-c (Milella et al., 2001), UCN-01 (Dai et al., 2001), ST1571 (Yu et al., 2002) and lovastatin (Wu et al., 2004).

### **PI-3 kinase/Akt signaling pathway and leukaemia**

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays an important role in the proliferation, differentiation and survival of hematopoietic cells (Steelman et al., 2004). This pathway is negatively regulated by the

phosphatases PTEN, SHIP-1 and -2. Mutations and hemizygous deletions of PTEN have been detected in some primary acute leukemias and non-hodgkin's lymphoma, and many hematopoietic cell lines lack or have low PTEN protein expression (Dahia et al., 1999). Recently, it was found that Akt was phosphorylated and activated in the majority of primary AML cells, and that constitutive activation of the PI3K/Akt pathway is necessary for the survival and proliferation of AML cells and proliferation (Min et al., 2003; Kubota et al., 2004). In addition, constitutive phosphorylation of Akt is associated with poor prognosis in AML (Min et al., 2003). PI3 kinase/Akt have also been shown to be involved in arsenic trioxide, imatinib and Ara-C resistance in leukaemia patients (Tabellini et al., 2005; Burchert et al., 2005; Grandage et al., 2005). These findings may provide new therapeutic strategies for leukaemia, although drugs targeting these pathways are still in preclinical development.

### **SUMMARY**

Both tyrosine and serine/threonine protein kinases represent significant and rational targets for anti-leukaemic therapeutics. Significant progress has been made with tyrosine kinase inhibitors, exemplified by the BCR-ABL inhibitor imatinib and its success in the treatment of CML. The current literature suggests that exploitation of novel and more selective agents that activate or inhibit serine threonine kinases may form the next wave of novel anti-leukaemics to progress to clinical usage.

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# STAUROSPORINE AS AN EARLY LEAD: AN OVERVIEW OF KINASE INHIBITORS INSPIRED BY THE INDOLOCARBAZOLE ALKALOIDS

► **Peter G. Goekjian**

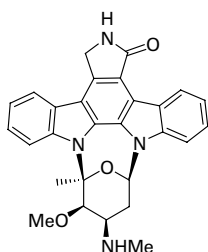
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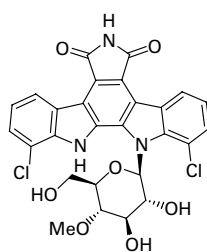
## ABSTRACT

As one of the very early "hits" in the now vast domain of protein kinase inhibitors targeting the ATP-binding site, staurosporine has been the inspiration for an impressive array of selective kinase, topoisomerase, and p-glycoprotein inhibitors. The current review will seek to present an overview of the different classes of inhibitors that have evolved from the original structure. These include staurosporine derivatives, indolocarbazole monoglycosides, substituted indolocarbazoles, bisindolylmaleimides, macro-

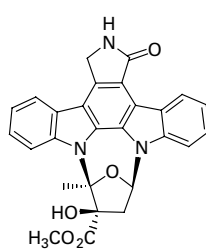
cyclic bisindolylmaleimides, dianilinophthalimides, 4-arylcarbazoles, heteroarylo[a]carbazoles and aryl[o]carbazoles, and azaindolocarbazoles. Potent and selective inhibitors have been discovered against PKC, GSK-3 $\beta$ , myosin light chain kinase, checkpoint kinases, cyclin dependent kinases, flt-3, DAPK, EGFR, VEGFR, PDGFR, and PIM1, as well as non-kinase targets such as topoisomerase I, PARP-1, and p-glycoprotein based on the original indolocarbazole lead structure.



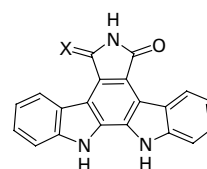
staurosporine



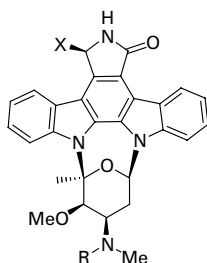
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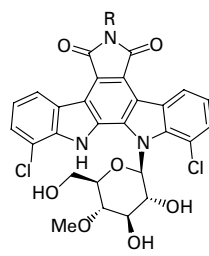
K252a



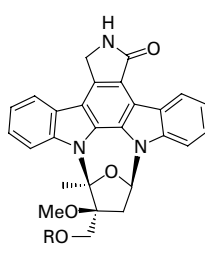
K252c: X = H,H  
Arcyriaflavin A: X = O



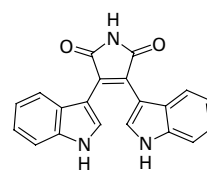
midostauroin: X = H, R = PhCO-  
UCN-01: X = OH, R = H



BMY-27557-14  
R = (CH<sub>2</sub>)<sub>2</sub>NEt<sub>2</sub>



CEP 751: R = H  
CEP 2563: R = CO(CH<sub>2</sub>)<sub>2</sub>NHLys



Arcyriarubin A

## INTRODUCTION

### Indolocarbazole alkaloids

The indolocarbazole alkaloids are a family of glycosylated natural products bearing a [2,3-a]indolo[3,4-c]pyrrolo[1,2-b]carbazole chromophore. The indolocarbazole aglycon is linked by either one or two *N*-glycoside bonds to a pyranose or furanose sugar. Staurosporine (STP) and UCN-01 are bis-*N*-glycosides of D-daunosamine, while rebeccamycin is a mono-*N*-glycoside of 4-*O*-methyl- $\beta$ -D-glucopyranoside. K252a is the bis-*N*-glycoside of a furanose sugar related to parasaccharinic acid, bearing a carboxymethyl group at the 3'-position. The aglycons are also natural products, the lactam K252c and the imide arcyriaflavin A, as well as the bisindolylmaleimide arcyriarubin A.

The indolocarbazole lactam or imide moiety turns out to be a surprisingly effective mimetic of adenine. As a consequence, the indolocarbazole alkaloids show a wide range of biological activities, including against many protein kinases, topoisomerase 1, and the P-glycoprotein (PGP) efflux pump. Several close derivatives of the indolocarbazole alkaloids have made it into clinical trials, in particular UCN-01, midostaurin, CEP751/CEP2563 prodrug, and BMY-27557-14 (Prudhomme, 2003; Goekjian & Jirousek, 2001).

### Staurosporine

Staurosporine was first isolated by Omura et al. from the bacterium *Streptomyces staurosporeus* in 1977 (Omura et al., 1977), although the absolute stereochemistry was revised in 1982 (Furusaki et al., 1982). The unusual bis-*N*-glycoside structure has attracted the interest of synthetic chemists, and several total syntheses have been reported (Link et al., 1996; Wood et al., 1997). Related structures have been isolated from natural sources with some regularity (**Figure 1**). Various analogs of STP bearing a hydroxyl group on the indole rings have been identified, in particular 11-hydroxy, 3-hydroxy, and 3,11-dihydroxystaurosporines (Kinnel & Scheuer, 1992; Cantrell et al., 1999; Schupp et al., 1999). The 10-methoxy analog, TAN 999, has been isolated from *Nocardiodopsis dassonvillei* C-71425 (Tanida et al., 1989). Oxidized forms of the imide ring, UCN-01, UCN-02, and 7-oxostaurosporine, have also been isolated from *Streptomyces* (Takahashi et al., 1987; Takahashi et al., 1989a; Takahashi et al., 1989b). Finally, a number of variations of the sugar ring have been observed, in particular methylated or demethylated forms (Schupp et al., 1999; Schupp et al., 2002; Cai et al., 1996), analogs based on 2-deoxy hexose and hexose sugars (Osada et al., 1990; Hernandez et al., 2000), and 4'-nitro, 4'-oxime, and 4'-

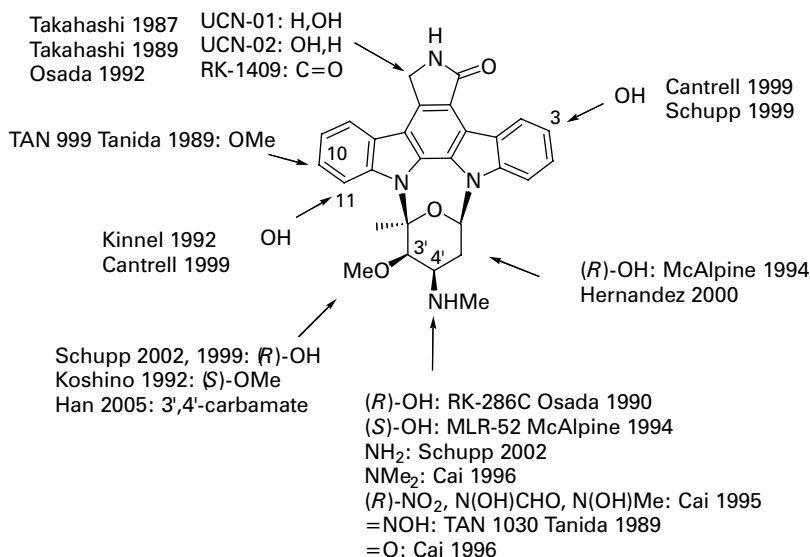


Figure 1. Natural products related to staurosporine

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hydroxylamino analogs of staurosporine (Tanida et al., 1989; Cai et al., 1995).

### Biological activity

Staurosporine was originally identified as an antitumor antibiotic with hypertensive activity in rats. It was later identified as a potent protein kinase C (PKC) inhibitor in 1986 (Tamaoki et al., 1986). In fact, STP turned out to be a remarkably promiscuous inhibitor: in a recent screen, STP was shown to inhibit 110 out of 119 protein kinases (Fabian et al., 2005). It also inhibits P-glycoprotein (PGP; Wang et al., 2003), but not topoisomerase I (Prudhomme, 2004).

### Crystal structures of STP complexed to protein kinases

A number of crystal structures of STP complexed to protein kinases have been published (Vulpetti et al., 2005; Jacobs et al., 2005; Atwell et al., 2004; Xu et al., 2004; Jin et al., 2004; Brown et al., 2004; Yamakawa et al., 2004; Zhang et al., 2004; Holton et al., 2003; Bertrand et al., 2003; Underwood et al., 2003; Zhao et al., 2002; Johnson et al., 2002; Zhu et al., 1999; Lamers et al., 1999; Prade et al., 1997; Toledo et al., 1997; Lawrie et al., 1997). Staurosporine acts as a true ATP analog, in which the lactam provides the key hydrogen bond donor-hydrogen bond acceptor motif of the adenosine ring to the hinge backbone amides. The hydrogen bond acceptor, and to a lesser extent the hydrogen bond donor to the backbone, have been found to be a universally conserved feature in ATP-competitive kinase inhibitors. The central aromatic ring of the indolocarbazole overlaps with the purine ring, while the two indole moieties fill hydrophobic pockets within the ATP-binding site. The pyranose bis N-glycoside oxygen matches the ribose furanose ring oxygen of ATP (Prade et al., 1997; Lawrie et al., 1997).

A notable feature of the crystal structures is that STP binds to the catalytically active conformation of the kinase, in which the activation loop is in the open conformation, and the catalytic DFG motif maintains the aspartic acid oriented towards the ATP-binding site (Prade et al., 1997). By comparison, Glivec has been shown to bind to a conformation with a closed activation loop,

and the DFG motif having the aspartate oriented away from the ATP-binding site (Atwell et al., 2004; Gambacorti-Passerini et al., 2005). The binding site being more open and more conserved in the catalytically active form might help to explain the lack of selectivity of this compound.

### **Staurosporine as a lead compound**

The lack of selectivity of STP leads predictably to high toxicity, and STP itself clearly has no potential as a therapeutic agent. Nonetheless, as the original "hit" in the now vast domain of protein kinase inhibitors targeting the ATP-binding site, it has been the inspiration for an impressive array of selective kinase, topoisomerase, and PGP inhibitors. The current review will seek to present some of the compounds that have evolved from the original STP structure (Omura et al., 1995; Prudhomme, 1997; Gescher, 1998; Gescher, 2000; Prudhomme, 2005).

### "PKC inhibitor": true target or historical accident?

Although STP has long been referred to as a PKC inhibitor, this should be seen more as a historical accident than a biological result. Indeed, the discovery of protein kinase C as the cellular targets of tumor promoters such as phorbol esters by Kikkawa et al. (1983) launched an intensive search for potent inhibitors (Tamaoki et al., 1986; Solomon et al., 1985; Sano et al., 1985; O'Brian et al., 1985; Shoji et al., 1985; Inagaki et al., 1985; Besterman et al., 1985; Kawamoto et al., 1984; Hidaka et al., 1984; Sanchez et al., 1983; Sahyoun et al., 1983; Taffet et al., 1983). It is now clear that STP is a very general kinase inhibitor, and can be considered a lead compound for most kinases. Selective inhibitors of many kinases, including PKC, cdk's, trkA, checkpoint kinases, flt-3, VEGFRs, EGFR, and PDGFR, as well as non-kinase targets PGP, PARP-1, and topoisomerase I, have been developed based on the indolocarbazole alkaloids (*vide infra*). For this reason, the current review will not focus particularly on PKC inhibitors, but on inhibitors structurally related to staurosporine. Of course, such a definition is *per se* subjective, and the limits of the current discussion are thus somewhat arbitrary. Structural modifications of the indolocarbazole system (**Figure 2**) have led to

the bisindolylmaleimide, dianilinophthalimide, 4-arylcarbazole, aryl[o]carbazole, and azaindolocarbazole classes of inhibitors.

The current review will not focus strongly on the biological activity of the inhibitors. The issue of potency and selectivity, while of course central to the discussion of ATP-competitive kinase inhibitors, is better addressed elsewhere in the current issue. Furthermore, inhibition constants measured in different assays are not comparable, and reliable conclusions can therefore only be made based on results obtained with the same assay. The focus of this article will therefore be more on the various structural modifications of the STP system than on the biological results. Finally, somewhat arbitrarily, analogs of K252a will not be discussed here, despite the obvious importance of these compounds. Indeed, the K252a story is a fairly coherent one, and the

reader is referred to recent publications on this topic (Prudhomme, 2005; Mucke, 2003; Saporito et al., 2002; Moffat et al., 2005; Gingrich et al., 2005; Schneider et al., 2005).

## STAUROSPORINE ANALOGS

### *N*4'-substituted derivatives

A large series of *N*-alkylated and *N*-acylated analogs have been prepared, which will not be listed here in detail (Brazzell et al., 2000; Griffin et al., 2003; Nomura et al., 1996; Lewis et al., 1996; Regenass et al., 1995; Murakata et al., 1994; Wacker, 1994; Miyamoto et al., 1993). 4'-*N*-benzoyl staurosporine, midostaurin (CGP41251), is an inhibitor of PKC, Flt-3, TGFR, VEGFR-1 and P-glycoprotein, and has made it to phase II clinical trials for chronic lymphocytic leukaemia and non-

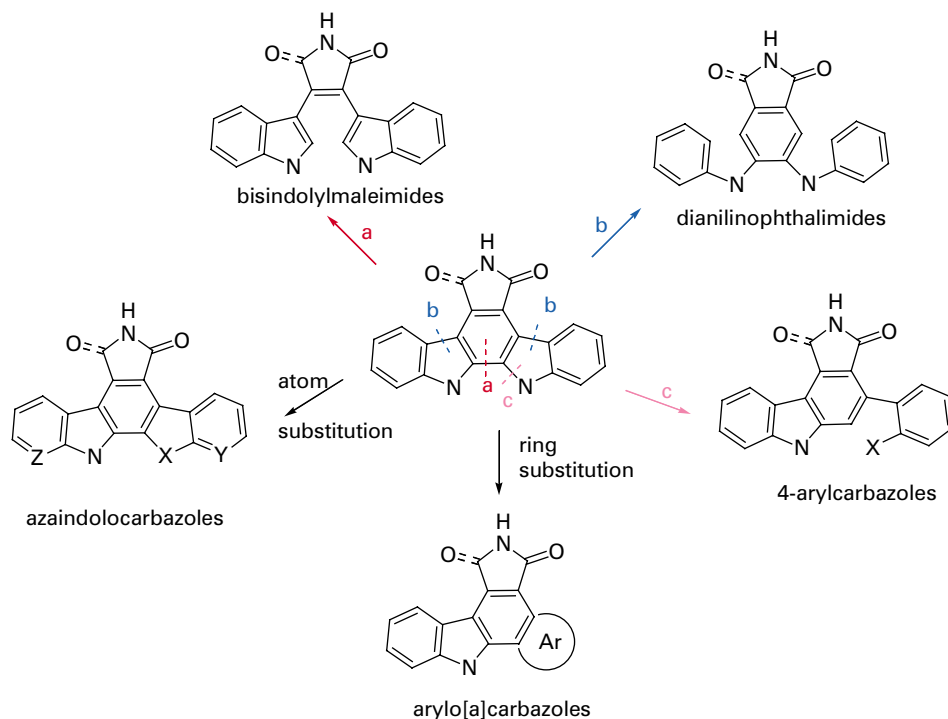


Figure 2. Inhibitor design based on the indolocarbazole structure

Hodgkin's lymphoma (Goekjian & Jirousek, 2001). A Phase I clinical trial in combination with 5-fluorouracil has recently been reported. Recruiting is currently underway for Phase I clinical trials in combination with daunorubicin and cytarabine, as well as Phase II trials in aggressive systemic mastocytosis and mast cell leukemia (<http://clinicaltrials.gov>).

A study of the biological activity of a series of STP derivatives substituted at *N4'*, *N6*, and *C7*, was reported by Caravatti et al. (1994) *N4'*-acylation reduces potency, but improves selectivity, while substitution of the imide abolishes all kinase activity. 7-oxostaurosporine shows a similar activity profile to staurosporine. The 7(*R*)-hydroxy analog, UCN-01, maintains potency against PKC, yet improves selectivity. However, the 7(*S*)-hydroxy epimer, UCN-02, is considerably less potent. The 7-oxo-5-hydroxy analog is somewhat less potent, but shows a similar stereochemical discrimination between hydroxy epimers.

### Substitution of the indole rings

The most extensive studies have involved the substitution of the indole benzenoid rings, and an important collection of substituted analogs have been prepared. The most common substitution has been at the 3 and 9 positions (pyrroloindolocarbazole numbering,

corresponding to a 5-substituted indole), including fluoro, bromo, iodo, hydroxy, alkoxy, nitro, amino, acylamino, alkyl, acyl, carboxy, vinyl, and acetylene (Murakata et al., 2002; Saulnier et al., 2002; Kanai et al., 2001; Yamada et al., 1996; Tamaoki et al., 1994). The tetrafluoro analogs have been prepared by Saulnier et al. (1998), while tetrahydrostaurosporine analogs have been investigated by Zimmermann (1999) as PKC- $\alpha$  inhibitors.

### Substitution of the lactam moiety

Various modifications of the lactam moiety have been reported: 7-*O*-alkyl analogs of UCN-01 have been prepared as selective PKC inhibitors, while the imide nitrogen has been replaced with hydrazine, or has substituted with alkyl groups (Takahashi et al., 1990). As noted above, *N*-alkylation of the lactam abolishes activity against most protein kinases. However, such a substitution does not affect potency against PGP, and *N*-benzyl and *N*-methylpyridyl substituted analogs of midostaurin have been proposed as selective PGP inhibitors (Wang et al., 2003).

### Modification of the sugar moiety

Additional analogs have been prepared by synthetic or biosynthetic approaches, replacing the daunomycin with other sugars bearing

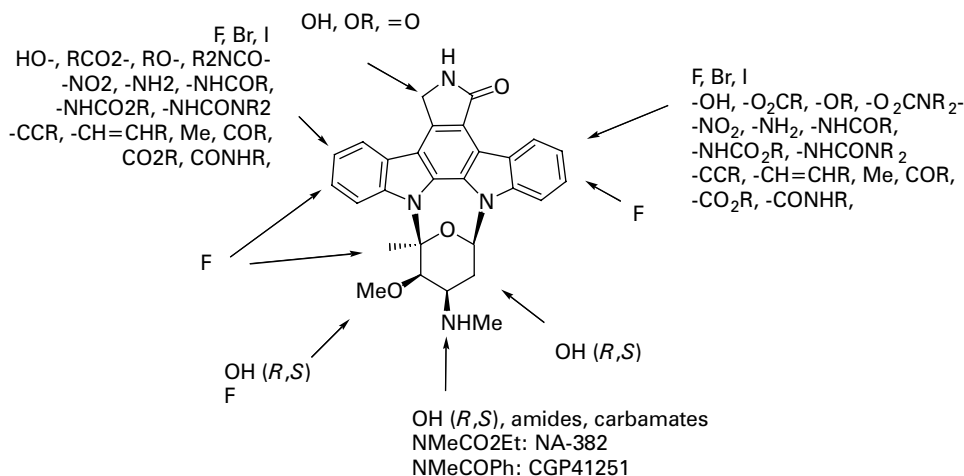


Figure 3. Synthetic analogs of staurosporine.

hydroxyl, ketone, or oxime functionalities (Saulnier et al., 2002; Salas et al., 2005; Cai & Ouyang, 2001; Li et al., 2000; Yamada et al., 1995; Ootsuka et al., 1993). These analogs target PKC, topoisomerase I, myosin light chain kinase, or antiangiogenic activity. The sugar moiety of STP has also been replaced with carbocyclic (Moffat et al., 2005; Monse et al., 2004; Riley & Simpkins, 1999) and heterocyclic (Shankar et al., 1994; Vice et al., 1994) analogs as well as regioisomeric forms, in which the nitrogens are bound to the C1' and C2', C1' and C4', or the C1' and C6' carbons of the sugar (**Figure 4**) (Saulnier et al., 2002; Saulnier et al., 2005a; Nichols & Simpkins, 2004; Marminon et al., 2002a; Anizon et al., 1998; Ruediger et al., 2002). Analogs in which the sugar has been replaced with a tether have been prepared by Vice et al. (1996; 1994).

## INDOLOCARBAZOLE MONOGLYCOSIDES

### Sugar-modified analogs

The indolocarbazole monoglycosides are analogs of rebeccamycin, and the synthesis of analogs has been reviewed regularly by Prudhomme (2004; 1997; (et al., 1999); 2000; and 2003). Bis-dechlororebeccamycin is an inhibitor of topoisomerase, but not of PKC, while STP inhibits protein kinases, but not topoisomerase. The dramatic differences in the biological activity of arcyriaflavin, rebeccamycin, staurosporine, and K252 point to the essential role of the sugar in

controlling the selectivity of these compounds towards the various targets.

A systematic investigation of the role of the sugar on the biological activity of the indolocarbazole mono-*N*-glycosides has been performed by Anizon et al. (1997; 2003), Ohkubo et al. (2000), Faul et al. (2004), and Zhang et al. (2005). Varying the sugar leads alternatively to inhibition of topoisomerase I, protein kinase C, or cdk4, as well as varying levels of cytotoxicity. No simple relationship can be made either to the sugar or to its anomeric configuration (Prudhomme, 2004; Prudhomme, 2003). For example, the  $\beta$ -glucosyl and  $\beta$ -galactosyl indolocarbazoles are more active than the corresponding  $\alpha$ -glycosides against topoisomerase I, while the  $\alpha$ -mannopyranoside is more active than the  $\beta$ -glycoside; in the allopyranoside series, the two configurations are equally potent.

### Conformation of the indolocarbazole *N*-glycoside

The complex structure-activity relationship between the sugar and the biological activity against protein kinases and topoisomerase I indicate that the specific three-dimensional arrangement provided by rotation around the *N*-glycoside bond is responsible for the selectivity profiles. The conformation of indolocarbazole *N*-glycosides has been investigated by Gilbert et al. (1999). They found equilibrium between two alternative conformations of the *N*-glycoside, one

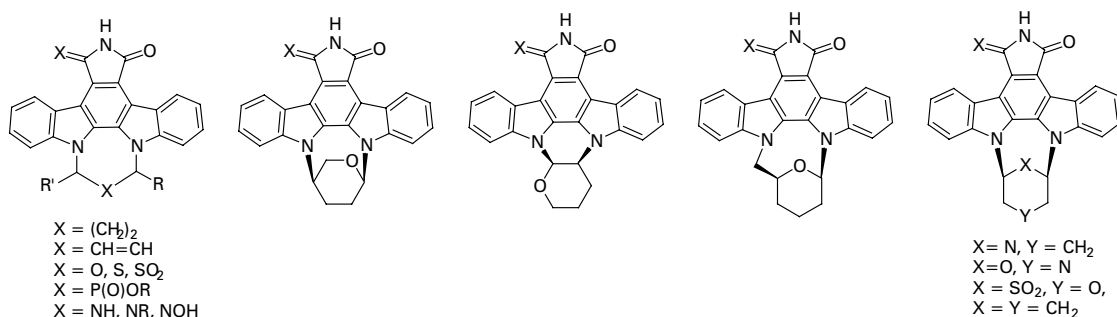


Figure 4. Bridged indolocarbazoles

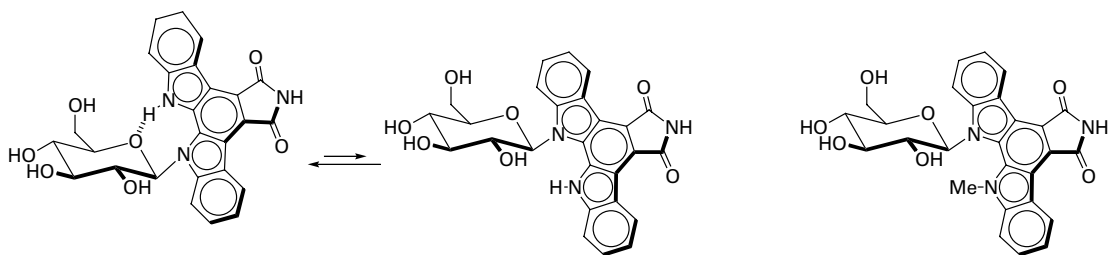


Figure 5. Conformation of the indolocarbazole N-glycoside

of which is stabilized by a hydrogen bond between the pyranose oxygen and the indolocarbazole NH. They have recently shown that the corresponding *N*-methyl compound, which favors the alternative conformation, is less active against topoisomerase I, which suggests that the hydrogen-bonded conformation is the active one in this system (Facompre et al., 2003).

### Modification of the imide

In contrast to protein kinases, substitution of the imide nitrogen of rebeccamycin is not deleterious to activity against topoisomerase I (Pereira et al., 1996). The diethylaminoethyl-substituted analog of rebeccamycin, BMY-27557-14, is currently in clinical trials. Replacing the imide nitrogen with a hydrazine is also a favorable modification. The 2-[1,3-dihydroxypropyl] substituted hydrazine structure has been found to be particularly favorable (Denny, 2004).

Interestingly, reduction of the imide to a lactam induces significant activity against PKC (Anizon et al., 1998; Moreau et al., 1998). This is noteworthy, as the maleimides and lactams are usually equally potent against kinases, and it is therefore difficult to invoke an unfavorable interaction of the maleimide of rebeccamycin in PKC. The origin of this effect remains to be elucidated.

### Substitution of the indole

NB-502 is a natural product 1,11-dihydroxyl analog of rebeccamycin, having a formyl hydrazine substituent at the imide nitrogen, and lacking the glucose 4' methyl group. Ohkubo et al. (1999) have systematically varied the position of the indole OH groups. Optimization of the substituent led to Edotecarin (Denny, 2004). The 3,9-dibromo analog was investigated by Moreau et al. (1999), and found to improve potency

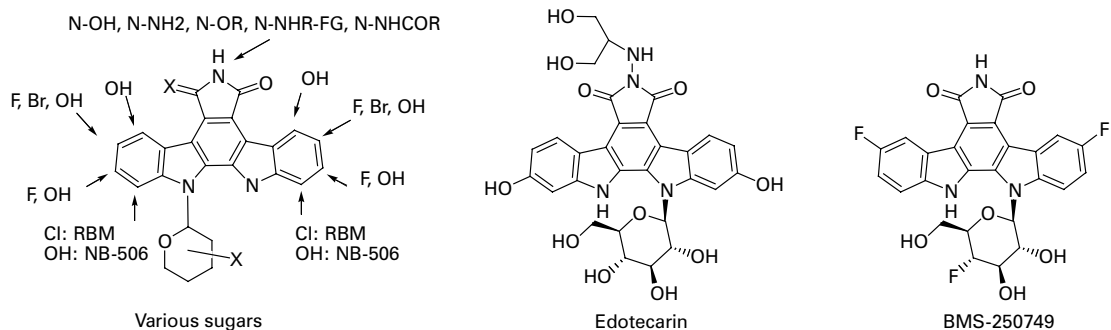


Figure 6. Rebeccamycin analogs



Table 1. Residual activity in presence of 1  $\mu$ M Go6976, as a % of control. 17 other kinases tested, residual activity 33%–100% (Goekjian & Jirousek, 2001; Davies et al., 2000).

Inhibitor	PKC-a	PKD1	MAPKAP -K1b	MSK1	PHK	CHK1	S6K1
Go6976	7%	18%	5%	2%	1%	3%	12%
IC(50)	2 nM			10 nM			

Table 2. Inhibition constants for selected indolocarbazoles shown in Figure 7 against cyclin-dependent kinases (Zhu et al., 2003a; Sanchez-Martinez et al., 2003).

Inhibitor	Cyclin D1-cdk4	Cyclin E-cdk2	Cyclin B-cdk1	CamK II	PKA
A	0.076 $\mu$ M	0.52 $\mu$ M	2.1 $\mu$ M	12.4 $\mu$ M	>20 $\mu$ M
B	0.042 $\mu$ M	0.144 $\mu$ M	-	>2 $\mu$ M	>2 $\mu$ M
C	0.05 $\mu$ M	0.16 $\mu$ M	-	-	0.12 $\mu$ M

CHK1 (**Table 1**). Indolocarbazole compounds have been targeted towards cdk's (**Table 2**) (Zhu et al., 2003a; Al-Awar et al., 2001), viral pUL97 (Slater et al., 2001; Zimmermann et al., 2000), HIV-1 integrase (Yan et al., 2005), PDGF auto-phosphorylation (Uings et al., 1998), or topoisomerase I (Moreau et al., 2001).

## BISINDOLYLMALEIMIDES

The bisindolylmaleimides (BIM), e.g. arcyriarubin A, result from the deletion of the central indolocarbazole bond. This provides considerable conformational flexibility, and these compounds have been shown to bind to the ATP-binding site in a highly canted conformation (Messerschmidt et al., 2005; Gassel et al., 2004; Komander et al., 2004). The BIM were first prepared by Sarstedt and Winterfelt (1983), then others, as intermediates for the synthesis of the indolocarbazole aglycons (Brenner et al., 1988; Bergman & Pelcman, 1987; Joyce et al., 1987; Kaneko et al., 1985). Toullec et al. (1991) and Davis et al. (1992a; 1992b) later identified potent and selective PKC inhibitors based on these compounds, including GF109203X, Ro 31-8220, and others shown in **Figure 8** (Wilkinson et al., 1993). These compounds have found extensive

use in biological investigations of signal transduction pathways, although the biological effects of the BIM can be mediated through both PKC and non-PKC pathways (Han et al., 2000). A comprehensive study of the activity of a selection of these compounds against a panel of kinases by Davies et al. (1992a; 1992 b) showed that these were also active against MAPKAP-K1b, MSK1, S6K1, and GSK3 and weakly active or inactive against 18 other kinases. A recent comparison between bisindolylmaleimides and indolocarbazoles demonstrate that the BIM system is indeed significantly less potent against cyclin dependent kinases (Sanchez-Martinez et al., 2003).

Similar substitutions to those investigated for the indolocarbazoles have been performed on the indole, maleimide, and indole nitrogens (**Figure 8**), and will not be detailed here. Xie et al. (1996) have prepared BIMs substituted with lexitropsin oligopeptides which bind the DNA minor groove, in order to target topoisomerase I. Sasaki et al. (1998) have prepared inhibitors of cdc2 kinase based on a multiple pseudosubstrate construct bearing a BIM bound to an oligopeptide pseudosubstrate via an oligoethylene glycol chain. BIMs also exhibit antagonist activity against the nicotinic cholinergic receptor (Mahata et al., 2002). In addition, two bisindolylmaleimides are

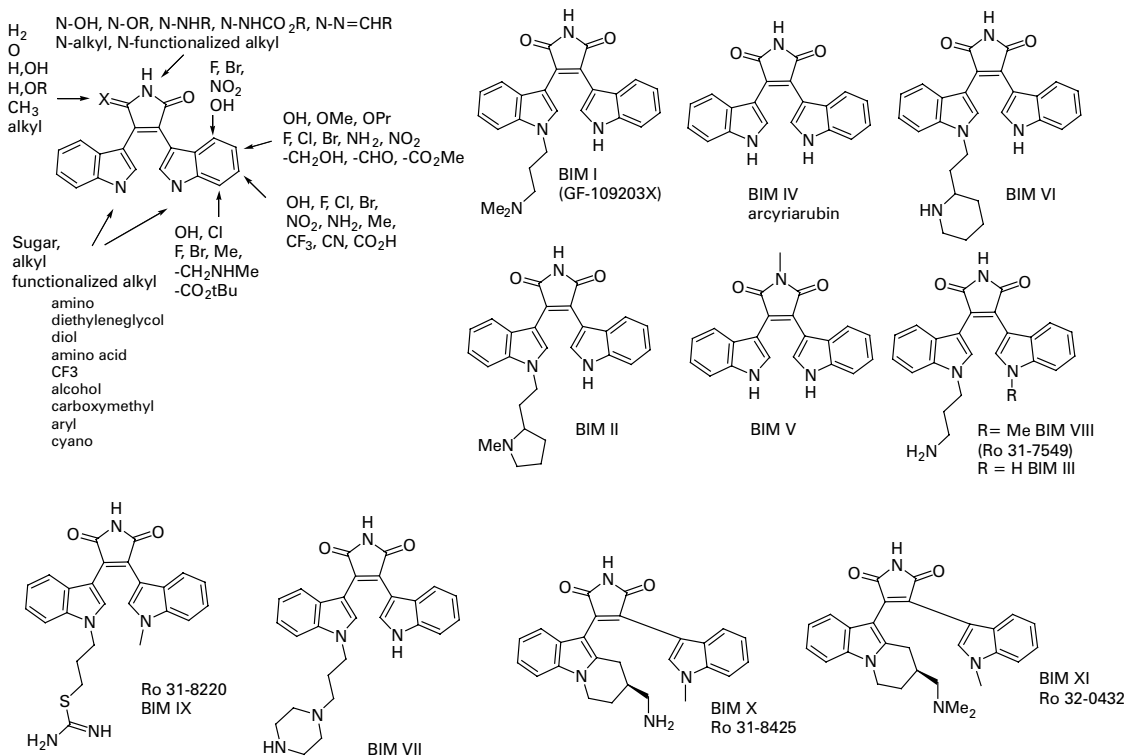


Figure 8. Substituted bisindolylmaleimides

currently undergoing clinical trials, Enzastaurin (LY317615.HCl), a PKC- $\beta$  selective inhibitor (Pearce & Miller, 2005; Faul et al., 2003), and Ro 31-7453 (Dupont et al., 2004; Salazar et al., 2004).

### MACROCYCLIC BISINDOLYLMALEIMIDES

The macrocyclic bisindolylmaleimides are formed by connecting a tether between the indole nitrogens of the bisindolylmaleimide, in an effort

to constrain the conformation of the flexible pharmacophore. Indeed, the presence of a 14-membered macrocycle eliminates the major "syn" conformation observed in the bound and unbound form of the BIMs, in favor of a pseudo-C<sub>2</sub> symmetric "anti" conformation, in which the two C<sub>2</sub> indole hydrogens point to opposite faces of the maleimide (Komander et al., 2004; Bartlett et al., 2005). Ruboxistaurin (LY333531, **Figure 9**) is a highly selective inhibitor for the  $\beta$ -isoform of

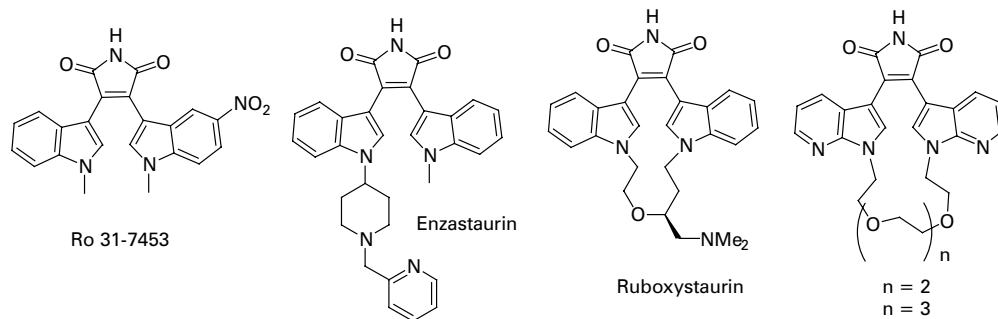


Figure 9. Selective bisindolylmaleimides and macrocyclic bisindolylmaleimides.

protein kinase C over other isoforms and other kinases (**Table 3**) (Faul et al., 2003; Jirousek et al., 1996). One observes a significant degree of selectivity for PKC- $\beta$  over other isoforms, as well as over other kinases. In a comprehensive screen, Fabian et al. (2005) have shown submicromolar binding to 9 kinases, low micromolar binding to 16 kinases, and >5 micromolar binding to 94 out of 119 kinases (**Table 4**). Significant binding is observed only to PIM1 (55 nM) and to mouse STK3 (90 nM). In a separate screen of 29 kinases, some inhibition was found to PDK1, MSK1, MAPKAP-K1a, and S6K1 (10–20% residual activity at 10  $\mu$ M) (Komander et al., 2004). However, the concentration tested is >1000 times the IC50 against PKC- $\beta$ . Ruboxistaurin is currently in Phase III clinical trials in Europe, and is recruiting for Phase III clinical trials in the US and Canada for diabetic retinopathy, and for Phase II for diabetic macular edema (<http://clinicaltrials.gov>; Vinik, 2005; He & King, 2005; Vinik et al., 2005; Aiello et al., 2005).

Kuo et al. (2003) have recently reported a series of macrocyclic bisindolylmaleimides and their aza analogs bearing an 8-, 11-, 14-, or 17-

atom oligoethyleneglycol linker (16-, 19-, 21-, or 24-membered macrocycle, **Figure 9**). The 19- and 21-membered macrocyclic bisindolylmaleimides are potent inhibitors of GSK-3 $\beta$  (22 nM) with only weak activity against PKC- $\gamma$ . However, the 19-membered macrocyclic bisindolylmaleimide maintains a strong activity against PKC- $\beta$ II (6 nM) and PKC- $\theta$  (65 nM). The corresponding monoaza bisindolylmaleimide with a 19-membered macrocycle is more selective for GSK-3 $\beta$ , while the 19-membered bis(7-azaindolyl)maleimide is >30 fold selective for GSK-3 $\beta$  over PKC- $\beta$ . Finally, the 21-membered bis(7-azaindolyl)maleimide is a potent inhibitor of GSK-3 $\beta$  (48 nM), but is inactive against PKC- $\beta$ II (IC50>10 $\mu$ M). A screen against a panel of 50 kinases shows that the 21-membered ring compound does not inhibit any of the kinases at a concentration of 10  $\mu$ M (residual activity >61%). It is interesting to note that ruboxistaurin is quite selective for PKC- $\beta$  over GSK-3b (31% residual activity at 10  $\mu$ M ruboxistaurin) (Komander et al., 2004), indicating that orthogonal selectivities can be achieved within the macrocyclic bisindolylmaleimides.

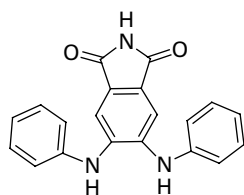
*Table 3. Inhibition constants ( $\mu$ M) of PKC isoforms by ruboxistaurin, enzastaurin, and staurosporine (Faul et al., 2003).*

Inhibitor	$\alpha$	$\beta$ 1	$\beta$ 2	$\gamma$	$\delta$	$\epsilon$	$\zeta$	$\eta$	PKA	CamK	CKII	srcTK
ruboxistaurin	0.36	0.005	0.006	0.3	0.25	0.6	>100	0.052	>100	6.2	>100	>100
enzastaurin	0.8	0.03	0.03	2	1	0.3	8	0.4	>100	10	>100	>100
staurosporine	0.045	0.023	0.019	0.11	0.028	0.018	>1.5	0.005	0.10	0.004	14	0.001

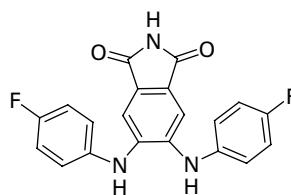
*Table 4. Binding constants ( $\mu$ M) to protein kinases for ruboxistaurin and staurosporine (Fabian et al., 2005).*

Inhibitor	AAK1	FLT3	KIT	PIM1	PIM2	SLK	STK18	STK3_m	STK4	16 kinases	94 kinases
ruboxistaurin	0.96	0.27	0.38	0.055	0.13	0.94	0.37	0.09	0.22	>1 $\mu$ M	>5 $\mu$ M
staurosporine	0.0013	0.0018	0.1	0.015	0.004	.00002	.00075	.00015	0.0002	a	a

a. staurosporine: >5  $\mu$ M: 11 kinases; <1  $\mu$ M: 108 kinases



DAPH 1 (CGP52411)



DAPH 2 (CGP 53353)

Figure 10. Dianilinophthalimides.

## DIANILINOPHTHALIMIDES

An alternative bond deletion consists of removing the indole C-C bonds, leading to a dianilinophthalimide that maintains the basic pharmacophore, but allows greater conformational flexibility and a less conjugated system. Trinks et al. (1994) have prepared the dianilinophthalimides, e.g. DAPH1 (CGP52411) and substituted analogs, as potent and selective inhibitors of the EGF receptor (Trinks et al., 1994; Weidner et al., 1999). The dianilinophthalimides adopt a non-planar, unsymmetrical propeller-shaped conformation (Trinks et al., 1994), and a modeling study suggests that these compounds bind as ATP analogs with one of the aniline rings in the ribose-binding site (Furet et al., 1995). The bis-(4'-fluoroaniline) analog DAPH 2 (CGP 53353)

has been shown to inhibit PKC- $\beta$ 2 and EGF at equal potency, and to have viable antitumor activity in live mice (Dinney et al., 1997; Buchdunger et al., 1994; Buchdunger et al., 1995). More recently, Blanchard et al. (2004) have shown that the DAPH1 inhibits the formation of fibrils of the A $\beta$ 1-42 peptide implicated in Alzheimer's disease.

## 4-ARYL-SUBSTITUTED CARBAZOLES

Deletion of one of the indole nitrogens leads to the 4-aryl pyrrolo[3,4-c]carbazole system (Figure 11). The binding motif of the indolocarbazoles and bisindolylmaleimides demonstrate that the two indole rings are in non-equivalent environments, and that considerable

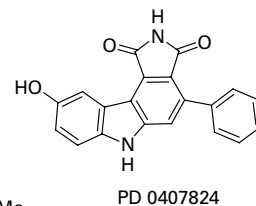
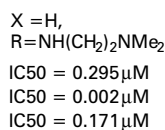
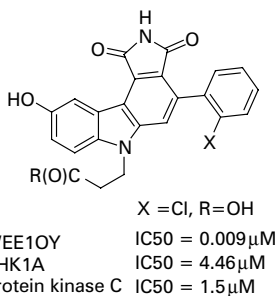
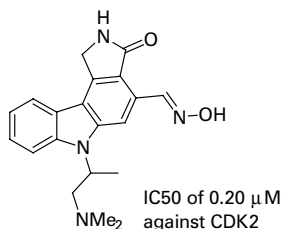
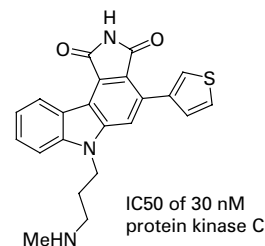
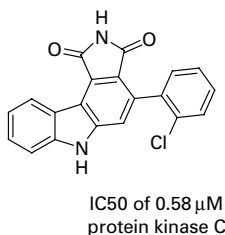
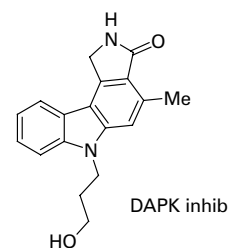


Figure 11. 4-Substituted pyrrolocarbazoles.

conformational flexibility and steric freedom is available for one of the indole systems. The 4-arylpyrrolocarbazoles were initially prepared by Kleinschroth et al. (1990) as PKC inhibitors, in particular bearing a 4-(2'-chlorophenyl) ring. A variety of new synthetic approaches to these compounds have been reported (McCort et al., 1999; Adeva et al., 2000; Adeva et al., 2000a; Adeva et al., 2000b). Heteroaryl-substituted analogs were subsequently prepared by Broka (1996), and were found to be more potent against PKC. 4-arylcarbazoles have also been reported as checkpoint kinase Wee 1 and CHK1 inhibitors by Booth et al. (2003), while the cytotoxicity of the tetrahydrocarbazole compounds has been studied by Caballero et al. (2003). The 4-oxime-substituted compounds were prepared by Kanai et al. (2003) as cdk2 inhibitors. The 4-methylpyrrolocarbazoles have been recently prepared by Teruta et al. (2004) as inhibitors of death-associated protein kinase (DAPK).

A study of the crystal structure of the complex of PD 0407824 with Wee1 kinase was recently reported by Squire et al. (2005). The compound binds as an ATP analog, with the imide ring forming the two conserved hydrogen bonds to the hinge backbone. The 9-hydroxy group forms an additional hydrogen bond to the backbone, while the 4-phenyl group is twisted relative to the carbazole ring, and fits into a well-defined hydrophobic pocket.

## ATOM-SUBSTITUTED ANALOGS OF INDOLOCARBAZOLES AND BISINDOLYLMALEIMIDES

### Indenocarbazoles

The analogs of indolocarbazoles, in which one of the indole nitrogens was replaced with a carbon, have been reported by the Hudkins group (1996; 2000) as PKC, VEGFR2 and trkA inhibitors (**Figure 12**). Underiner et al. (2002) and Singh et al. (1999) have prepared indenocarbazole analogs of the STP ring system, which show activity against trkA and VEGF-R2 (KDR), but weak activity against PKC and PDGFR. A parallel synthesis approach to these compounds was reported by Tripathy et al. (2002). An optimized indenopyrrolocarbazole, CEP-5214 and its prodrug form CEP-7055 have been reported by Gingrich et al. (2003) as a potent inhibitor of VEGFR3 (Flt-4), VEGFR2 (KDR), VEGFR1 (Flt-1), and Flt-3 with IC(50)s of 4 nM, 8 nM, 16 nM, and 1.6 nM, respectively. CEP-5214 was weakly active or inactive against a panel of 12 other kinases.

### Azaindolocarbazoles

The 7-aza analogs of the indolocarbazole system were first prepared by Slater et al. (1995) and again by Routier et al. (2002a; 2002b) as topoisomerase inhibitors (**Figure 13**). A series of glycosylated azaindolocarbazoles, analogs of

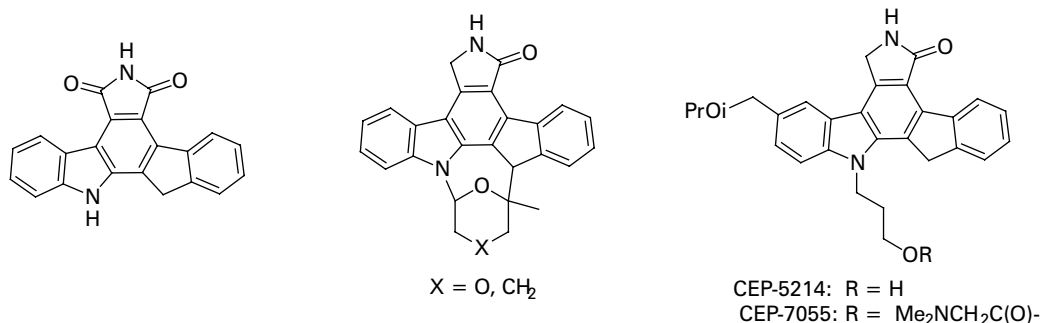


Figure 12. Indeno[a]carbazoles.

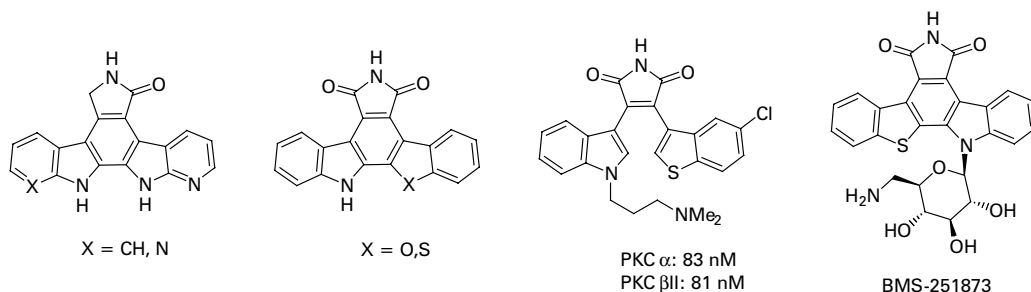


Figure 13. Nitrogen, oxygen, and sulfur atom-substituted indolocarbazoles.

rebeccamycin, have been prepared by Marminon et al. (2003a), Prudhomme et al. (2003) and Messaoudi et al. (2005). The 7,10-diazaindolocarbazole analog was also reported by Marminon et al. (2003b), while C1'-C2'-bridged analogs were recently reported by Messaoudi et al. (2005). The azaindolocarbazole compounds exhibit a distinct activity profile from rebeccamycin, although the targets are not yet identified. The aza analogs of the bisindolylmaleimides were first prepared by Davis et al. (1992) as PKC inhibitors, while their macrocyclic homologs, GSK-3b inhibitors prepared by Kuo et al. (2003), are discussed above.

#### Benzofuro- and benzothienocarbazoles

Analogues of the indolocarbazoles, in which one of the nitrogens has been replaced with a sulfur or an oxygen, have been reported by Hudkins et al. (2001), Ma et al. (2001), and Sanchez-Martinez et al. (2003a) (Figure 13). Sanchez-Martinez et al. (2003b) found out that the benzothienocarbazole was slightly less potent against cyclinD1-cdk4 than the corresponding indolocarbazole compound. A series of 3-(indolyl)4-(benzothienyl)maleimides were prepared by Zhang et al. (2003) as PKC and GSK-3b inhibitors. Glycosylated benzothieno-

carbazoles and benzofuranocarbazoles have been reported by Balasubramanian et al. (2004/2005) as topoisomerase I inhibitors. The benzothienocarbazole was found to be optimal, and the 3,9-difluoro compound, BMS-251873, shows in vivo efficacy and is proposed as a potential clinical candidate.

### RING-MODIFIED ANALOGS OF INDOLOCARBAZOLES AND BISINDOLYLMALEIMIDES

#### Inverted indoles

##### Indolo[1,2-a]- $\beta$ -carboline

Indolocarbazoles having an alternative fusion of the indole ring have also been prepared. The antitumor natural product BE-54017 has an indolo[1,2-a] $\beta$ -carboline ring system (Nakase et al., 2000). Analogous indolo[1,2-a] $\beta$ -carbolines have been prepared by Jaquith et al. (2001). A deletion of the [1,2-a] indole C-C bond leads to the 5-aryl pyrrolo[3',4':5,6]pyrido[3,4-b]indole-1,3(2H,3aH)-diones, prepared by Teller et al. (2000) as selective PDGF receptor inhibitors. The 8,9-dimethoxy-5(2-methylphenyl)- $\beta$ -carboline compound, although slightly less active in

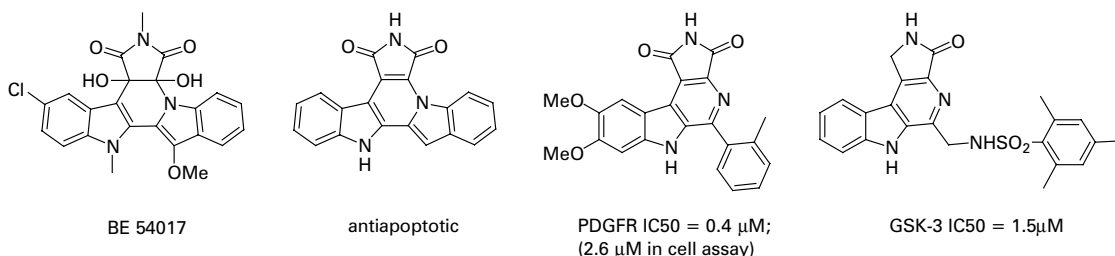


Figure 14.  $\beta$ -Carboline analogs of indolocarbazoles.

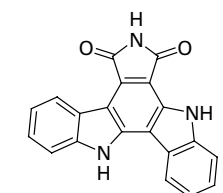
isolated PDGFR than the 9-methoxy-5-(2-methoxyphenyl) analog, shows better cellular activity and better selectivity. Mahboobi et al. (1999a) reported that the 9-methoxy-5-phenyl analog is an inhibitor of both topoisomerase I and II. 5-alkylsulfonamide analogs have been studied by Laronze et al. (2005), and were shown to be micromolar inhibitors of GSK-3. A stereo-controlled strategy for the synthesis of the corresponding reduced 5-phenyltetrahydrocarbolines has been recently reported by Dardennes et al. (2005). Finally, a series of spirocyclic dihydrocarbolines were prepared by Mahboobi et al. (1999b) as tyrosine kinase inhibitors.

#### Indolo[3,2-a]carbazoles

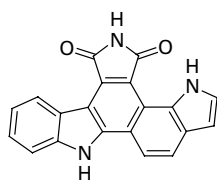
The isomeric fusion of the indolocarbazole, in which the indole nitrogens are meta rather than ortho on the carbazole benzenoid ring, provide a different shape to the planar indolocarbazole ring system. These compounds have been prepared by Henon et al. (2005), Janosik et al. (1999), Bergman et al. (1999) and Fonseca et al. (1995). However, no biological data was provided for these compounds.

#### Indolo[6,7-a]carbazoles

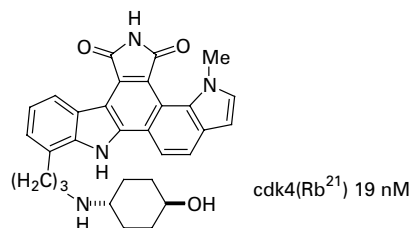
The indolocarbazoles, in which one of the indoles is fused via the benzenoid ring, have also been investigated (**Figure 15**). A series of indolo[6,7-a]carbazoles have been investigated by Engler et al. (2003), Zhu et al. (2004) and Faul et al. (2004) as cdk4 inhibitors. Optimization for the selectivity and solubility provided the 7-(3-alkylaminopropyl)-3-*N*-methyl analogs, which were selective for cdk4 over a panel of 44 other kinases.



indolo[3,2-a]carbazoles



indolo[6,7-a]carbazoles



cdk4(Rb<sup>21</sup>) 19 nM

Figure 15. [3,2-*a*] and [6,7-*a*] isomers of indolocarbazoles.

## **Arylcarbazoles**

### Benzo[*a*]carbazoles

A series of substituted 3,4-phenylindolylmaleimides and benzo[*a*]carbazoles have recently been reported by Peifer et al. (2006) as potent VEGFR2 inhibitors. In particular, the 3-indolyl-4-(trimethoxyphenyl)maleimide was a potent and selective inhibitor of VEGFR2, while the corresponding trimethoxybenzo[*a*]carbazole was significantly less active. The cytotoxicity of a series of benzo[*a*]carbazoles and their activity against cyclin-dependent kinases, GSK3, and topoisomerase I have recently been investigated by Routier et al. (2006). The 2-dimethylaminoethyl-10-hydroxybenzo[*a*]pyrrolo[3,4-*c*]carbazole compound showed the strongest cytotoxicity, although it is inactive against cdk2, GSK-3, and topoisomerase I, and the target of this compound remains to be identified. Benzo[*a*]pyrrolo[3,4-*c*]carbazole-1,3-dione was similarly found to be inactive against PARP-1, a non-kinase target, but the fused cyclopentane compound was found to be a nanomolar PARP-1 inhibitor by Tao et al. (2006).

### Naphtho[*a*]carbazoles

The indolocarbazole analogs replacing the indole ring with a naphthalene were investigated as cdk4 inhibitors by Sanchez-Martinez et al. (2003b) and against cdk2, gsk-3, and topoisomerase I by Routier et al. (2005; 2001). There are three regioisomers, the "up-angular" naphtho[2,1-*a*]carbazole, the "down-angular" naphtho[1,2-*a*]carbazole, and the "linear" naphtho[2,3-*a*]carbazole. The naphtho[2,1-*a*]carbazole isomer was significantly more active against cdk4 (45 nM) than either the

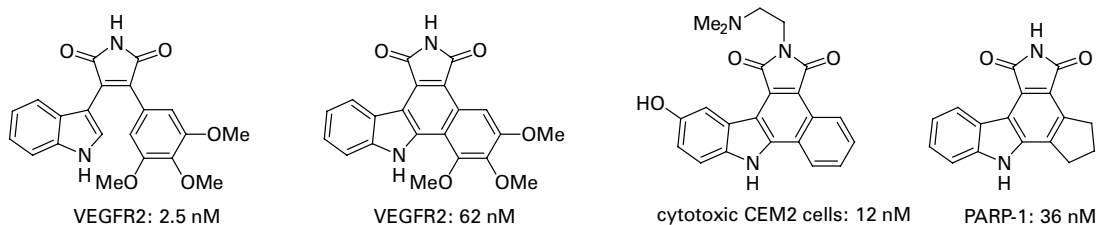


Figure 16. Indolylphenylmaleimides and benzo[a]carbazoles.

benzo[a]carbazole (260 nM), naphtho[1,2-a]carbazole (430 nM) or naphtho[2,3-a]carbazole (inactive) compounds (Sanchez-Martinez et al., 2003b). This compound was selective for cdk4 over a panel of 7 kinases. Routier et al. (2005) found that the linear naphtho[2,3-a]carbazole isomer bearing dimethylaminoethyl and hydroxyl substituents was highly cytotoxic against L1210 cells, although inactive against cdk5, gsk-3, or topoisomerase I.

#### Benzothiophene- and quinoline-fused carbazoles

A "down-angular" benzothieno[4,5-a]carbazole compound and a family of "up-angular" indazolo[5,4-a]carbazoles have been prepared by Hudkins et al. (2001) (Figure 17). The quinolino[a]carbazoles have been reported by Zhu et al. (2003b). The activity of five "up-angular" quinolino[a]- or isoquinolino[a]carbazole isomers was investigated against cyclin D1-cdk4. The isoquinolino[6,5-a]carbazole compound was found to be the most active among the "up-angular" isomers, with an IC<sub>50</sub> of 69 nM, while the "down-angular" quinolino[4,3-a]carbazole and the "linear" quinolino[3,2-a]carbazole compounds were inactive.

### Heteroarylcarbazoles

#### 5-Membered ring heterocycles

Didemnimide C is an 3-[1-N-methyl-1H-imidazol-5-yl]-4-[indol-3-yl]maleimide natural product, in which the indole ring of arcyrarubin A is replaced by an imidazole ring (Terpin et al., 1998). The imidazo[a]carbazoles granulatumide ([4,5-a] isomer) and isogranulatumide ([1,5-a]b-carboline isomer) were subsequently isolated in 1998 (Berlinck et al., 1998), while the remaining isomers were prepared by Andersen et al. (1999). Isogranulatumide has recently been shown by Jiang et al. (2004) to be an inhibitor of Chk1 kinase (IC<sub>50</sub> = 0.1 μM), while Sanchez-Martinez et al. (2003b) found out that synthetic granulatumide was weakly active against cyclin D1-cdk4 (IC<sub>50</sub> = 0.74 μM).

Analogues of isogranulatumide, in which the indole has been replaced with a 7-azaindole, as well as those in which the imidazole has been replaced with a pyrrole and by a maleimide, have been prepared by Hugon et al. (2003a; 2003b; 2003c). The later compounds exhibit cytotoxic activity against L1210 murine leukemia cell lines (Prudhomme et al., 2004a; Prudhomme et al.,

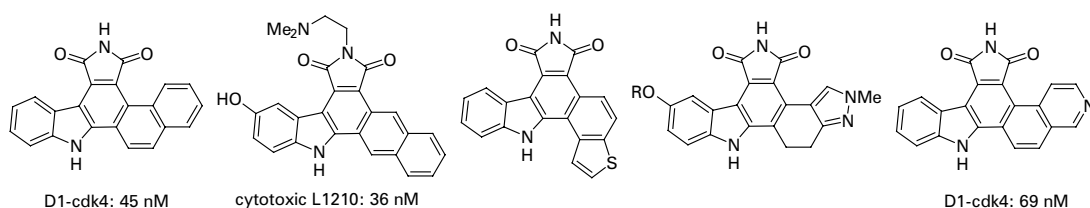


Figure 17. Carbazoles fused to bicyclic aromatics via the carbocyclic ring.

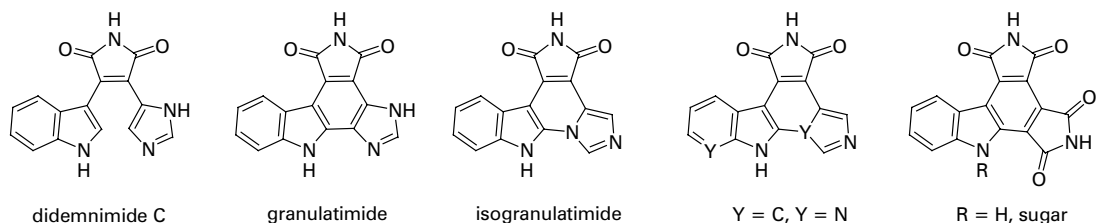


Figure 18. Granulatimide, isogranulatimide, and analogs.

2004b). Glycosylated analogs of the bis-maleimidocarbazoles have recently been prepared in the same group by Henon et al. (2005; 2006). Their biological activities are under investigation. The dihydrofuro[3,2-a]pyrrolo[3,4-c]carbazole-1,3-dione analog was prepared by Tao et al. (2006) as a PARP-1 inhibitor, but was found to be inactive.

#### 6-Membered ring heterocycles

The pyrido[4,3-a]carbazole and tetrahydropyrido[4,3-a]carbazole analogs of the indolocarbazoles were prepared by Ator et al. (2001) as potential inhibitors of VEGFR2, PARP-1, and MLK3 (**Figure 19**). The 3-methyl pyridocarbazole analog, for example, inhibits VEGFR2 with an IC<sub>50</sub> of 94 nM. In an interesting recent development, Williams et al. (2005) have shown that a ruthenium complex of the pyrido[2,3-a]carbazole analog was a selective, sub-nanomolar inhibitor of GSK-3 $\alpha$  and GSK-3 $\beta$ , effective in intervening in a wnt-mediated signal

transduction pathway in a cellular system. The quinoline analogs, studied by Zhu et al. (2003), are discussed above, while a benzopyran-fused analog has been prepared by Hudkins et al. (2001).

#### RING-EXPANDED ANALOGS

Arcyroxepin A is a ring-expanded oxepine analog of arcyriaflavin A, isolated from *Arcyria denudata* by Steglich et al. (1980), along with arcyriaflavin B and arcyriarubin B (**Figure 19**). Mahboobi et al. (1999c) have reported the synthesis of the carbon analogs of Arcyroxepin, in which the central ring of the indolocarbazole structure was expanded with a methylene group, along with its glycosylated forms. This provides added flexibility and an interesting three-dimensional structure to the molecule, as well as altering the electronic environment of the conjugated system (Mahboobi et al., 1999d).

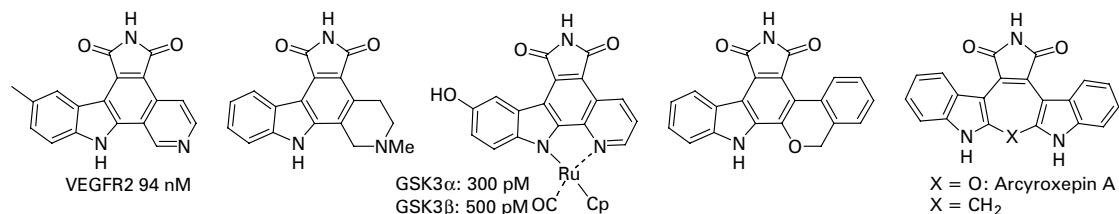


Figure 19. Carbazoles fused to six-membered heterocycles and ring-expanded indolocarbazole analogs.

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## PERSPECTIVES

The concept of an analog is per se subjective, and the lines that have been drawn here are necessarily arbitrary, as many other structures could be considered as analogs of the indolocarbazole alkaloids. Nonetheless, the great variety of structures that have evolved from STP show that this compound has been a tremendous source of inspiration to medicinal and synthetic chemists. It has been a productive lead compound, not just for PKC, but also for GSK-3 $\beta$ , checkpoint kinases, cyclin dependent kinases, flt-3, DAPK, EGFR, VEGFR, PDGFR, and PIM1, as well as non-kinase targets such as topoisomerase I, PARP-1, and p-glycoprotein. Exquisite selectivity has been achieved based on this promiscuous inhibitor, either by inducing a conformational twist within the indolocarbazole system, or by judicious choice of substituents, which can provide either a specific three-dimensional structure, or specific interactions with the target protein. This effort is far from exhausted, as many novel structures and kinase targets remain to be studied. Our own efforts in this area focus on the C1' position of rebeccamycin and staurosporine: evaluation of steric, conformational, and electronic factors in a critical region of the ATP-binding site (Belhadj & Goekjian, 2005).

A second fundamental issue that has emerged within this class of inhibitors has been the structural and conformational basis for selective inhibition of the ATP-binding site of protein kinases. Each class of inhibitor provides a unique conformational profile, which exploits subtle

differences in the ATP-binding sites of the various kinases. Our own studies focus on the macrocyclic bisindolylmaleimides, which adopt fundamentally different conformations as a function of the macrocyclic tether (Goekjian et al., 1999a; Goekjian et al., 1999b; Goekjian et al., 1998; Goekjian et al., 1999c; Blackenbiller et al., 1998; Stroble et al., 1998). Bartlett et al. (2005) have recently published an extensive conformational study of the MBIM, and the implication for kinase selectivity. They conclude that the pseudo-C2 symmetrical "anti" conformation binds to AGC group protein kinases, while GSK-3 $\beta$  binds to the highly canted "syn" conformation. Our interests focus on the selectivity shown by ruboxistaurin between PKC- $\alpha$  and PKC- $\beta$ . Our studies indicate that two conformations are present in the 14-membered ring macrocyclic bisindolylmaleimides. Does ruboxistaurin bind to PKC- $\beta$  in a different conformation than to PKC- $\alpha$ , or does PKC- $\beta$  have a higher affinity than PKC- $\alpha$  for the same "anti" conformer of ruboxistaurin? An understanding of the structural basis for isoform-selectivity of ruboxistaurin will aid in the design of new kinase inhibitors.

The original "hit" in the search for kinase inhibitors, staurosporine, has played a special role in the now vast domain of ATP-competitive kinase inhibitors. The questions above are only two of the important issues that have evolved from this field, and ever more opportunities arise for fundamental, medicinal, clinical and therapeutic research in this area. Some of these questions will be addressed elsewhere in this issue.

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# PROTEIN KINASE RESEARCH IN THE UNIVERSITY OF HELSINKI

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## PROTEIN KINASE C

Protein kinase C (PKC) is a family of isoenzymes, which play a major role in signal transduction of normal cells (Nishizuka, 1995), and may be involved in the transformation of normal cells into malignant ones (Bredel and Pollack, 1997). Newly synthesized PKC is a water-soluble cytoplasmic protein with no kinase activity. Upon activation by endogenous second messengers, such as diacylglycerol and  $\text{Ca}^{2+}$  (the classical isoenzymes) and by exogenous compounds, such as phorbol esters, they undergo a large conformational change, translocate to cell membranes and become enzymatically active and phosphorylate their specific substrate proteins. In experimental settings a commonly used activator of PKC is the tumour promoting reagent phorbol ester that exhibits very high affinity for several PKC isoenzymes (Ron and Kazanietz, 1999).

There are currently only a small number of clinically used drugs, whose mode of action may include the inhibition of protein kinase C. Antiestrogens, tamoxifen and probably also

toremifen, inhibit protein kinase C when they are administered at high doses. In the treatment of malignant gliomas, tamoxifen has shown some efficacy and this effect has been suggested to be due to inhibition of PKC (Bradshaw et al., 1993). However, their action is non-specific, and there are considerable risks for the occurrence of side effects.

During the past 25 years several biotechnological and pharmaceutical companies have made substantial efforts to develop drugs that specifically affect protein kinase C and its isoenzymes. Biomedical research has revealed that PKC plays an important role in various mechanisms of inflammation. For example, protein kinase is a very important regulator in interleukin-2 mediated activation of T cells. In addition to the inflammatory diseases, the PKC inhibitors have a large potential in the treatment of diabetic complications, various types of cancer, neurological diseases, as well as cardiac and circulatory diseases.

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## PKC STUDIES IN THE UNIVERSITY OF HELSINKI

The Helsinki PKC group has adopted a drug discovery-oriented approach to study PKC as a drug target. We use molecular modelling to design new compounds and screen natural products showing activity on PKC from the Nordic biosphere. Based on these pieces of information, our aim is to synthesize compounds with high affinity and specificity to the phorbol ester-binding site of either PKC $\alpha$  or PKC $\delta$ . The synthesized compounds that activate, inhibit or otherwise modulate PKC $\alpha$  or PKC $\delta$  are studied in bioactivity screens and cell cultures.

There are seven University of Helsinki groups that participate in the "Protein Kinases – Novel Drug Targets of Post-genomic Era" project. Five of the groups are affiliated within the Faculty of Pharmacy and two of the groups are from the Faculty of Agriculture and Forestry. In the Faculty of Pharmacy, medicinal chemistry-related activities are carried out in Prof. Jari Yli-Kauhaluoma's laboratory. Graduate students Olli Aitio, M. Sc. and Gustav Boije af Gennäs, M. Sc. are studying the molecular modelling of protein kinase C and the organic synthesis of the PKC modulating agents, respectively. In the Prof. Raimo Tuominen's group, Dr. Elina E. Ekokoski and graduate student Virpi Talman, M. Sc., are in charge of carrying out the *in vitro* pharmacological characterization of the new PKC-targeted compounds. They employ the phorbol-binding and phosphoryl transfer activity assays to show the binding affinity and enzymatic effects of the compounds. Moreover, they are currently developing cell-based assay systems to see whole cell effects (e. g. cell division) of the prepared compounds. They also study the effect of the synthesized ligands on the translocation of PKC $\alpha$  and PKC $\delta$  from cytosol to the cellular membranes upon activation. This translocation can be observed in real time by means of confocal microscopy when the fusion proteins PKC-GFP are used as reporters (**Figure 2**). Docent Päivi Tammela's group is responsible for developing high-throughput assay systems for evaluating PKC-binding agents. Docent Moshe Finel's molecular biology laboratory produces the required recombinant proteins for binding and activity assays. Finally, Prof. Jouni Hirvonen's laboratory focuses on the transport of the most

interesting PKC-modulating compounds in the Caco-2 model and evaluates the pharmacokinetic properties of the compounds.

The groups from the Faculty of Agriculture and Forestry (Department of Applied Chemistry and Microbiology) investigate the cyanobacteria and fungi obtained from the Nordic biosphere. Prof. Kaarina Sivonen's and Prof. Annele Hatakka's laboratories study cyanobacterial and fungal extracts as sources of PKC-modulating agents. Bioassays have been carried out in collaboration with Prof. Pia Vuorela (Åbo Akademi University, Finland) and Prof. Stein Ove Døskeland (University of Bergen, Norway). The fungi are studied by graduate student Andres Perez, M. Sc., and cyanobacteria by laboratory technician Matti Wahlsten. Docent Jouni Jokela carries out the characterization of new compounds and is responsible for the development of PKC assay based on LC-MS.

## MEDICINAL CHEMISTRY

The medicinal chemistry-related studies are aimed at developing isoenzyme specific inhibitors for PKC $\alpha$  and PKC $\delta$ . Molecular modelling and the design of new selective and specific PKC $\alpha$  and PKC $\delta$  inhibitors are based on the previously published high resolution X-ray crystal structure of PKC $\delta$  with phorbol-13-acetate. In addition, recently published X-ray crystal structure of the catalytic domain of PKC $\theta$  has been used to design PKC $\alpha$  and PKC $\delta$  binding compounds.

The phorbol ester-binding moiety of PKC has been mapped for the optimal binding area and geometry of various functional groups. Subsequently, the Available Chemicals Directory database of more than one million commercially available compounds has been screened. Next, the molecules found from the screening were docked onto the binding moieties of PKC $\alpha$  and PKC $\delta$ . The best ligands were selected as templates for further synthetic elaboration to improve their binding and inhibitory properties. These synthetic compounds have been prepared by using methods of modern organic chemistry, such as solid-phase organic synthesis and microwave-assisted synthesis.

Some of the PKC-binding compounds synthesized in the medicinal chemistry group have shown promising results in various

apoptosis studies in our and Prof. Janet Lord's (University of Birmingham, United Kingdom) laboratories (Galkin et al., accepted for publication). These compounds selectively induce apoptosis in leukaemia cells, such as HL-60, AML and CLL, but not in fibroblasts. The optimisation of these ligands that displace the bound phorbol ester will be carried out in Prof. Janet Lord's and Prof. Johann Hofmann (Universität Innsbruck, Austria) groups. Finally, the synthesis of a compound library consisting of bistratene A derivatives will be carried out in collaboration with Prof. Peter Goekjian's and Prof. Olivier Piva's groups, both affiliated with the CNRS-Université Claude Bernard in Lyon, France.

Finally, we have studied in collaboration with the Technical Research Centre of Finland the use of a highly ubiquitous natural product *betulin*, lupene-3 $\beta$ ,28-diol, as a starting point in our synthesis of protein kinase C modulating agents (**Figure 1**). These studies are funded by the National Technology Agency and the Academy of Finland. Birch bark is a rich source of betulin, as it contains approximately 40 percent of this pentacyclic triterpene. There is a surprising link between the birch trees and protein kinases. It has been demonstrated recently that betulin possesses significant anti-inflammatory activity that is mediated by inhibition of PKC (Huguet et al., 2000). Also, a close derivative of betulin, *betulinic acid*, induces programmed cell death (apoptosis) in human melanoma cells, and this process seems to involve mitogen-activated protein kinase activation (Tan et al., 2003).

## MOLECULAR PHARMACOLOGY AND CELL BIOLOGY

The central objective of the molecular pharmacology research of the Faculty of Pharmacy is to collect experimental evidence for the specificity and efficacy of the newly synthesized compounds. For these experiments Docent Moshe Finel's molecular biology laboratory expresses functional recombinant human PKC $\alpha$  and PKC $\delta$  isoenzymes in insect cell cultures. The Finel group has also made efforts to set up a bacterial expression system in *E. coli*. Fluorescence-based imaging of PKC activation in living cells has been studied in Prof. Raimo Tuominen's laboratory. This approach is important for studies, where compounds targeting the regulatory domain are in focus. In principle, translocation should be inhibited by compounds that bind to the phorbol ester-binding site (**Figure 2**). Biological screening of the synthetic compounds or the natural products has been carried out *in vitro* using [ $^3$ H]phorbol 12,13-dibutyrate binding and phosphoryl transfer activity assays. As PKC enzyme specimen, partially purified rat brain homogenate, or recombinant constructs of PKC $\alpha$  or PKC $\delta$  (regulatory domain, C1 domains and whole enzyme) have been used.

Currently, we are setting up a cell-based assay to test the effect of the PKC-modulating compounds on living mammalian cells. The assay will be based either on PKC-mediated cell proliferation or on PKC-mediated changes on cell

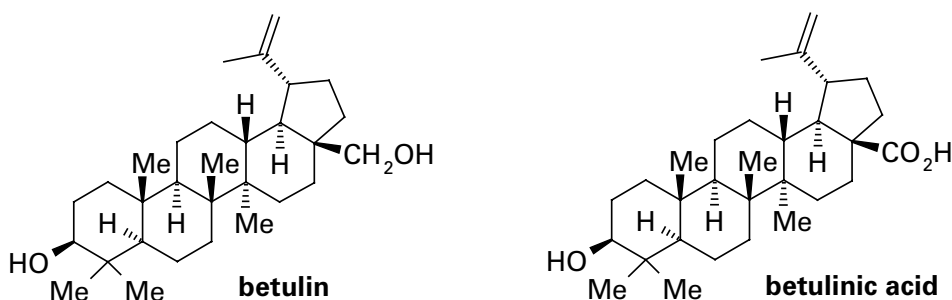


Figure 1. Chemical structures of betulin and betulinic acid.

morphology. In addition, we will continue the use of GFP-tagged PKC-constructs in translocation experiments in living cells. Also the FRET-constructs of PKC $\alpha$  and PKC $\delta$  made so far will be tested and validated for possible use in determining PKC activity in intact cells.

## HIGH THROUGHPUT SCREENING AND PHARMACOKINETIC PROPERTIES

In the Tammela's HTS and Hirvonen's eADME groups Caco-2 primary permeability assays of protein kinase active ligands have been developed, and they are presently validating the automated 96-well format assay. In addition, they have developed primary toxicity assays automated in 96-well formats. Four cell lines and lactate dehydrogenase (LDH) leakage assay, WST-1 assay and/or intracellular ATP level detection can be used depending on the actual ligands to be assayed.

The HTS and eADME groups aim also at developing a transporter MDR-1 assay. Efflux

proteins act as membrane transporters, transporting compounds (e.g. drugs and their metabolites) from the cells. Since cancer cells often overexpress the efflux protein MDR-1 to cause resistance against anti-cancer agents, the measurements for MDR-1 inhibiting activity will be used to evaluate further potential of ligands developed/isolated in this project.

## CYANOBACTERIA AND FUNGI AS SOURCES OF BIOACTIVE COMPOUNDS

Cyanobacteria (blue-green algae) have been identified as one of the most promising groups of organisms for isolation of new bioactive natural products (Burja et al., 2001). In earlier studies, cyanobacteria have been found to produce a wide variety of toxins including the protein phosphatase inhibitors (microcystins, nodularin) and protein kinase C activators (lyngbyatoxin) (Sivonen and Jones, 1999). The production of bioactive pharmacologically interesting compounds of fungi (including basidiomycetous

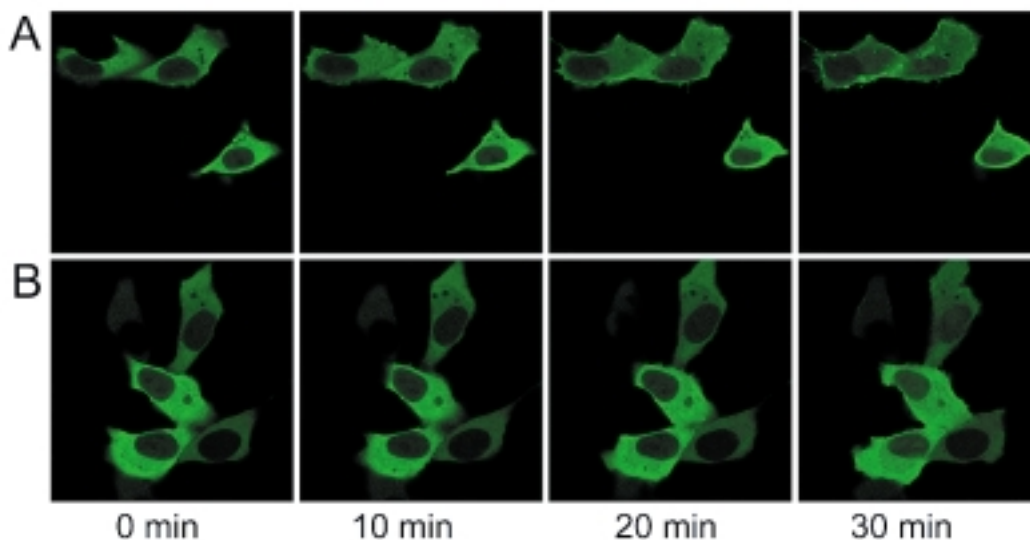


Figure 2. TRA-22 inhibits phorbol ester induced translocation of GFP-tagged PKC $\alpha$ .  
A) HeLa cells were treated with 100 nM PMA (phorbol 12-myristate 13-acetate) and confocal microscopic images were captured every 30 seconds.  
B) The cells were pretreated with 20  $\mu$ M TRA-22 for 10 minutes before PMA-treatment.

polypore fungi) has been well established (Zjawiony, 2004). For example, an antitumor medicine in Japan originates from a white-rot fungus (Ng, 1998) and triterpene-containing extracts from *Ganoderma lucidum* suppress PKC (Lin et al., 2003). At the Department of Applied Chemistry and Microbiology, research groups studying cyanobacteria and fungi have established large culture collections of these organisms. Cyanobacteria culture collection contains 1,000 strains mostly isolated from the Finnish Lakes and the Baltic Sea (both pelagic and benthic environments). The collection of fungi (600 strains) consists mostly of wood rotting fungi isolated from the Finnish old forests. These microbial collections are used in the project as sources of new bioactive compounds (apoptosis modifying and protein kinase modulating compounds). Cyanobacteria were studied earlier in the MAST EU-project and were found to contain many interesting bioactivities (Surakka et al., 2005; Herfindal et al., 2005; Selheim et al., 2005).

In the present project, cyanobacteria and fungi have been grown, extracted and activities studied by radioactive PKC assay. The laborious use of the radioactive method led the researchers to develop a new high throughput assay based on multi-well plates handled by a robot and measured by LC-MS to detect PKC inhibitors/

promoters. Known PKC inhibitors, such as staurosporine, quercetin, myricetin, and kaempferol were used as model compounds in the development of this system. The LC-MS analyzes hundred of samples within a few hours and will be used to look for the cyanobacterial and fungal extracts and fractions.

We mass cultivated several cyanobacteria showing interesting bioactivities in bioassays (**Figure 3**). From one of these (*Nostoc* strain) an antiapoptotic compound was isolated and purified and its structure was solved based on mass spectrometry (electrospray ionization, MS/MS fragmentation) and NMR (1D and 2D  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and  $^{15}\text{N-NMR}$ ). This research confirmed that the antiapoptotic compound belonged to a group of cyclic peptides. The antiapoptotic activity was investigated in the Døskeland laboratory.

Several cyanobacterial strains were found in collaboration with Prof. Døskeland to contain interesting bioactivities (e.g. selectively killing leukaemia cells) and will be pursued further in the project. In addition, potential new PKC modulating activities found in the new bioassay will be studied further. The mass cultivation of the certain strains is under progress, and the isolation and purification of the compounds and their structural elucidation will be undertaken.



Figure 3. Mass cultures of cyanobacteria containing interesting compounds for structure elucidation and detailed analysis of bioactivity.

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# PROTEIN KINASES: POTENTIAL TARGETS FOR NEW DRUGS AGAINST PARASITIC PROTOZOA

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## THERAPEUTIC INDICATIONS, AREAS TO BE CONSIDERED

Many human diseases in tropical and subtropical regions are caused by single cell eukaryotic parasites. These diseases include household names such as malaria, sleeping sickness and river blindness, as well as less well-known diseases such as leishmaniasis.

Much of the population at risk lives in developing countries. Hundred's of millions are infected and ten's of millions die or are unable to carry out their daily labours. Unfortunately, and despite increasing international focus on these diseases, treatments of parasitic diseases are costly and/or exhibit clinical side effects and/or are becoming ineffective due to increasing resistance to existing treatments. Research, both in public and private institutions, needs to be stepped up to find effective prophylactic and therapeutic regimens for these diseases.

This review summarizes current knowledge of the use of protein kinase inhibitors for the treatment of leishmaniasis and human African trypanosomiasis (sleeping sickness) in the human population, diseases that are uncontrolled in poor areas of the world.

## DESCRIPTION OF DISEASES, CONDITIONS AND THEIR PREVALENCE

### Leishmaniasis

Leishmaniasis is a spectrum of parasitic diseases, caused by species and subspecies belonging to genus *Leishmania*. The disease is usually transmitted through the bite of some 30 species of Phlebotomine sandflies. These parasites exist in two forms: extracellular promastigotes in the female sandfly vector and as intracellular amastigotes in the macrophages of the mammalian host. The latter stage of the parasite is responsible for all the diseases observed and is the target for potential new drugs ([www.who.int/tdr/diseases/leish/default.htm](http://www.who.int/tdr/diseases/leish/default.htm); Desjeux, 2004). Potential hosts include humans and/or dogs depending on the parasite species. Leishmaniasis has been designated a neglected and emerging disease by the WHO.

There are three main forms of leishmaniasis in humans:

- Visceral (VL)
- Cutaneous (CL) and
- Mucocutaneous (MCL)

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There are approximately 12 million cases of leishmaniasis worldwide, which are found primarily in tropical and subtropical regions spanning 88 countries worldwide. About 2 million new cases appear every year (1.5 million CL and 0.5 million VL) with an annual death of about 60,000 people. It is estimated 350 million people are at risk of contracting the disease.

VL occurs primarily on the Indian subcontinent and in Sudan. However, a number of cases have been reported in Southern Europe, primarily in those countries bordering the Mediterranean Sea (Spain, Portugal, Italy, France, Malta and Greece). Co-infections with HIV have also been reported and have changed the epidemiology of the disease in some countries (Desjeux and Alvar, 2003). CL is also present around the Mediterranean basin, primarily in Greece, Turkey, Lebanon, Syria, Israel, Egypt, North Africa and the Middle East. However, 90% of the CL cases occur in Iran, Afghanistan, Saudi Arabia, Brazil and Peru.

### **Human African trypanosomiasis**

This disease, also known as the Sleeping Sickness, is found in Africa restricted to areas inhabited by the tsetse fly (*Glossina*), where parasitic protozoa (trypanosomes) are transmitted to people through the bite of the fly. The flies live primarily where moisture is found, such as pools of water or shaded vegetation. The epidemiology of the disease depends on the interaction of humans, tsetse flies, trypanosomes and animals such as cows, sheep and goats. Epidemiological observations indicate a correlation between tsetse fly numbers and rainfall of the previous month. The presence of tsetse flies does not necessarily imply that the human disease is present in the area.

Sleeping sickness exists as two forms, each caused by a separate species of parasite. The chronic form is caused by *Trypanosoma brucei gambiense*, present in western and central Africa. The acute form is caused by *Trypanosoma brucei rhodesiense* and is present in eastern and southern Africa. Both forms can be deadly if untreated, however the rate of disease progression differs between the two forms. In the acute form symptoms appear early, central

nervous system (CNS) invasion is rapid, and death occurs in weeks to a few months. The chronic form of African sleeping sickness progresses more slowly and symptoms may not appear for months or years until the disease is quite advanced. As in the acute form, the parasites eventually invade the CNS resulting in death if untreated (Kiminyo, 2002).

It is estimated that over 50 million people, primarily adults, are at risk. There are over 300,000 new cases reported each year, with an upward trend. Nearly 50,000 people die of the disease each year (WHO World Health Report 2004-[www.who.int/whr/2004/en/](http://www.who.int/whr/2004/en/) and [www.who.int/mediacentre/factsheets/fs259/en/index](http://www.who.int/mediacentre/factsheets/fs259/en/index)). African trypanosomiasis has also been designated a neglected and emerging disease by the WHO. It is a new epidemic.

### **SHORT HISTORICAL PERSPECTIVE ON THE DIAGNOSIS, PREVENTION AND TREATMENTS**

#### **Leishmaniasis**

Of the three types of diseases, visceral (VL) and cutaneous (CL) leishmaniasis are the focus of most studies. VL, also known as kala azar in India, is caused by *L. donovani* or *L. infantum*, and is the most dangerous type of leishmaniasis. If untreated, it generally results in death primarily through its effects on spleen and liver with associated weight loss, fever and anaemia. *L. infantum* also causes fatal canine VL in the Mediterranean region and Brazil. CL is caused primarily by *L. major*, *L. tropica* and *L. aethiopica* in the Old World, and the *L. mexicana* and *L. braziliensis* complexes in the New World. CL produces skin ulcers on exposed parts of the body, where an infected sand fly vector fed, leaving the victim scared and potentially disabled. The third form of disease, MCL, also known in South America as Espundia, is primarily caused by *L. aethiopica* and *L. b. braziliensis*. This disease is typified by lesions in the nose, mouth and throat leading to extensive destruction of the mucous membranes. Misdiagnosis and delays in treatment result in severe disfigurement of the oral-nasal and pharyngeal region leading to

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hideous mutilation and suffering for the life of the patient (Berman, 2003; [www.who.int/tdr/diseases/leish/default.htm](http://www.who.int/tdr/diseases/leish/default.htm)).

### **Details of any existing diagnosis, prevention or treatment methods**

The main drugs currently in use to treat leishmaniasis were introduced 50–60 years ago, and the therapeutic arsenal is rather limited (Croft and Coombs, 2003; Murray, 2004; Singh and Sivakumar, 2004). Current drugs to treat VL require long courses, parenteral administration and hospitalization, and are generally expensive (see **Table 1**). Drugs based on pentavalent antimony (Pentostam, Glucantime and generic sodium stibogluconate), the first line of defense, are toxic and increasing resistance (>90%) has led to the abandonment of this treatment in regions where disease incidence is high, such as Bihar, India (Bryceson, 2001). Second-line drugs, such as pentamidine and amphotericin B, are highly effective, but their use is limited by toxicity and availability in some areas. Use of pentamidine has been discontinued in some areas of India due to increasing parasite resistance. Three lipid-associated formulations of amphotericin B (Ambisome, Abelcet and Amphotec) have been used to treat VL. The efficacy of these formulations is good and side effects low, however prohibitively high cost has prevented their extensive use.

Few new drugs are in the pipeline for treatment of VL (Guerin et al., 2002). These include Miltefosine, recently registered in India, Paromomycin and Sitamaquine. Miltefosine is the first oral drug for VL. This drug has mild, primarily GI tract related side effects. Its major drawback is contraindication in females of childbearing age unless contraception is used. Long drug half-life in patients and a narrow therapeutic index suggest that parasite resistance may rapidly appear if Miltefosine is used alone over long-periods. Paromomycin has shown high efficacy when given parenterally for 21 days. The traditional formulation of the drug was discontinued, but the drug was rescued by the International Dispensary Association and WHO/TDR, and is presently undergoing phase III trials

in India. Finally, Sitamaquine an oral 8-aminoquinoline has shown promising results, but little information on toxicity or efficacy is available and development has been slow. Phase III trials for this drug have been initiated (Murray, 2004; Singh and Sivakumar, 2004).

Many of the same drugs used to treat VL are also in use for CL and MCL (Blum et al., 2004). Both systematic and local treatments are used depending on the clinical presentation and species of *Leishmania*. Pentavalent antimony and pentamidine are routinely used for systematic therapy with the same complications described above for VL. Limited tests on additional drugs (amphotericin B and lipid formulations, allopurinol, Miltefosine, itraconazole and fluconazole) have been carried out, however extensive data on toxicity, formulation or dosage is lacking. Local treatments consist of either intralesional infiltration with pentavalent antimony or topical ointments containing paromomycin. However, considerable variations in patient responses to local and systematic treatments have been observed depending on the *Leishmania* species. Systematic, not local treatment is recommended in patients infected with *Leishmania* species that potentially causes MCL.

HIV co-infected patients in Europe and elsewhere, regardless of the drug used, tend to respond slowly to treatment and have high relapse rates (~60%). In some cases drug toxicity is exacerbated. Preliminary studies indicate that Miltefosine may be useful for maintenance therapy. Long-term treatment of these patients with a limited drug arsenal may promote the development of parasite resistance (**Table 1**).

Current treatments of leishmaniasis are not satisfactory: either because of rapidly acquired resistance to the available drugs, their side effects, and/or their expense (Berman, 2003; Croft and Coombs, 2003). A great need exists for new drugs to treat Leishmaniasis. Most existing primary drugs are toxic and require parenteral administration and hospitalization; several show signs increasing parasite resistance or are expensive. Treatment under difficult field conditions, poor compliance, HIV co-infections and anthroponotic transmission promote the development of secondary resistance.

## Trypanosomiasis

As already eluded to, human African trypanosomiasis has two forms: West African Sleeping Sickness, caused by *Trypanosoma brucei gambiense* (*T. b. gambiense*) and East African Sleeping Sickness, caused by *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*). Local symptoms may be evident within few days at the site of the tsetse fly bite. In the former disease, the affected persons may be infected for extended periods (even years) without showing any overt signs of disease. The chronic form is usually well advanced by the time typical symptoms are observed. Initial symptoms are headaches, fever, itching, extreme fatigue, and aching of muscles and joints, etc. If the disease is diagnosed early enough, cure rates are high. In the second neurological phase, parasites cross the blood-brain barrier and attack the central nervous system (CNS). This is associated with confusion, lack of coordinated movement, seizures, disturbance of the sleep cycle, loss of weight, etc. When treated after the onset of the second phase, the neurological disorder is permanent. In the latter case, it is not known how trypanosomes enter the CNS. If left untreated, disease symptoms become worse and the patient eventually dies within months to years. In the acute form similar symptoms are seen, but disease progress is much more rapid, and if

untreated, results in death within a few weeks to months (Legros et al., 2001; Kiminyo, 2002; Stich et al., 2002). The disease is characterized by the multiplication of the protozoa in the blood and lymph of the infected person. The protozoa can be transmitted through blood. They can also pass from the mother to the fetus, leading to abortion (Kiminio, 2002; Stich et al., 2002).

Potential for epidemics in many parts of Africa exists due to the lack of appropriate tsetse fly control programs, disease surveillance, and difficulties in diagnosing the disease before the parasites cross the blood-brain barrier and cause damage to the CNS. This is especially the case with *T. b. gambiense*, where the symptoms may not be apparent until it is already too late.

Targets for the drugs are trypanosomes. There are a number of drugs available to treat the disease. As WHO concludes, the drugs are old, difficult to administer and not always successful ([www.who.int/mediacentre/factsheets/fs259/en/index](http://www.who.int/mediacentre/factsheets/fs259/en/index)). The drugs are divided into two groups (**Table 2**): those that treat the disease during the early phases and drugs used to treat the disease during the later manifestation phase.

Because of the difficulties in screening the populations at risk, new drug treatment strategies should emphasize targeting trypanosomes after they have entered the brain. Therefore, new drugs must be able to cross the blood-brain barrier to reach the parasites.

Table 1. Overview of drugs used for the treatment of Cutaneous and Visceral Leishmaniasis.

	Side effects	Comments
Impavido (miltefosine)	not with HIV, reproductive toxicity	
Pentostam (pentavalent antimonials),	toxicity	resistance, expensive
Stibanate, (pentavalent antimonials),	toxicity	resistance, expensive
Sodium Stibogluconate(pentavalent antimonials)	toxicity	resistance, expensive
Glucantime (pentavalent antimonials),	toxicity	resistance, expensive
Fungizone (amphotericin B)	toxicity, intravenous	
Ambisome (amphotericin B-liposomal)		very expensive
Amphocil (amphotericin B-colloidal)		optimum doses not clear
Pentamidine (amphotericin B-lipid complex)		resistance

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## CURRENT PRIORITY AREAS TO BE CONSIDERED

In this section we will concentrate only on aspects related to drug development and testing. Important areas, such as diagnostics and vaccine development, will not be addressed in this review. It is important to note that health care givers in the field must accurately, rapidly and reliably identify these diseases and decide on an appropriate drug treatment to be administered to the patient. Physicians and laboratory technicians working in many endemic regions suffer from the lack of inexpensive, sensitive and specific methods for early diagnosis of leishmaniasis or human African trypanosomiasis.

### Drug development

At present, for both leishmaniasis and human African trypanosomiasis, the precise drug targets are still unknown. Thus, there is a need to emphasize a more systematic approach to identify the possible targets. One approach for treating these diseases is by targeting cell cycle dependent kinases (CDKs) of the parasites.

Correct coordination of events during the cell-cycle division cycle is necessary for the survival and growth of eukaryotic cells. These events are controlled at specific cell cycle check-points. The

progression through the cell cycle requires the activation and inactivation of CDKs. In humans this process has been extensively studied, and the different CDKs (CDK1, 2, 4 and 6) and the cyclin subsets (cyclin A, B, D and E) with which they interact have been identified. Additional CDKs have been shown to have essential functions in apoptosis and transcription (Vermeulen et al., 2003). However, gene expression in *Leishmania* and Trypanosomes is regulated in large part post-transcriptionally, thus it is unlikely that CDK homologues play any significant role in this process.

CDKs are involved in the regulation of the cell cycle progression by activation and deactivation of proteins through protein phosphorylation. Thus, control over CDKs can decide the life and death of a cell. Among the best known inhibitors of the CDKs are purine and pyrimidine derivatives, to which compounds such as Olomocine and Roscovitine belong. Their mode of action is not yet fully understood but, due to their similarity in structure to ATP, they may act as competitive inhibitors for the protein kinase ATP binding pocket. However, from data available with Cdk2, they do not appear to bind to the protein kinases in a manner similar to the ATP (Fischer, 2003; Fisher and Gianella-Borradori, 2003, 2005). Additional heterocyclic compounds can also act as CDK inhibitors, and examples include oxindoles, aminothiazoles, anillinoquinazolines,

Table 2. Overview of drugs for the treatment of trypanosomiasis.

	Side effects	Comments
<b>Early phase</b>		
Suramine	Digestive track problems	Discovered in 1921
(used against <i>T.b. rhodesiense</i> )		
Pentamidine	Well tolerated	Discovered in 1941
(used against <i>T.b. gambiense</i> )		
<b>Late phase</b>		
Melarsoprol (contains arsenic)	Severe allergic reaction	Discovered in 1949, resistance
(used against <i>T.b. gambiense</i>	(3–10% death rate)	
and <i>rhodesiense</i> )		
Eflornithine		Discovered in 1988, expensive,
(used against <i>T.b. gambiense</i> )		difficult to apply

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pyridopyrimidines, flavonoids (flavopiridol) and staurosporines (Knockaert et al., 2002a). Among other small molecular weight compounds, paullones are strong CDK inhibitors (Schultz et al., 1999).

It should be stressed that CDK inhibitors are not necessarily selective for specific CDKs, and may for example inhibit protein kinases other than CDKs. Indeed, CDKs are just one possibility to kill the parasites. For example enzymes in the glycolytic, trypanothine and thioredoxin pathways may be possible drug targets.

### **Drug testing and resistance**

In both leishmaniasis and human African trypanosomiasis, only a limited number of drugs are in the pipeline to treat the diseases. There are a number of problems to be overcome. First, in many instances, the short- and long-term effectiveness of the compounds still needs to be clearly established (including the number of relapsing patients). Second, synergism between drugs needs to be determined. Third, more extensive testing of drugs on late stages of the disease needs to be carried out. Finally, studies need to be carried out to establish the optimal conditions to reduce the development of resistance to the applied drugs (Legros et al., 2001, Docampo and Moreno, 2002)

### **CURRENT KNOWLEDGE OF USING KINASE INHIBITORS TO COMBAT LEISHMANIASIS AND HUMAN AFRICAN TRYPANOSOMIASIS**

Correct coordination of events during cell cycle division is necessary for the survival and growth of eukaryotic cells. Cell cycle has been investigated in trypanosomes and involves the coordination of the G<sub>1</sub>, S, M and G<sub>2</sub> stages for both nuclear, as well as mitochondrial DNA. The latter DNA in trypanosomatids is present as an interlocking network in a single structure, the kinetoplast, at the base of the flagellum (Shlomai, 2004). The chronology of genome replication for the nucleus and kinetoplast is not absolutely synchronized, and the DNA synthesis in the latter

organelle starts prior to the nuclear S phase. In addition, the kinetoplast completes segregation prior to nuclear mitosis. Furthermore, parasite cytokinesis in trypanosomes does not appear to depend on either nuclear S or M phases suggesting that the cell cycle check-points in these parasites are different from normal eukaryotic cells (McKean, 2003). Less is known about cell cycle and replication in *Leishmania*, although it is probably similar to *T. brucei*. Differences between human and parasite cell cycle co-ordination suggests that the CDKs regulating cell division in the parasites may be potential targets for new drugs

Eleven CDK homologues, cdc2 related kinases (CRKs), have been identified in *T. brucei* and *L. major* (Parsons et al., 2005). *Leishmania* CRK1 and 3 were shown to be essential for parasite growth (Mottram et al., 1996; Grant et al., 1998; Wang et al., 1998; Hassan et al., 2001), and the latter gene was able to rescue *Schizosaccharomyces pombe cdc2<sup>ts</sup>* mutants (Wang et al., 1998). Incubation of promastigotes with flavopiridol (a general cdc2 inhibitor) caused cell cycle arrest and accumulation of the cells at the G<sub>2</sub> or G<sub>2</sub>/M phases (Hassan et al., 2001). More recently, RNA interference (RNAi) knockdown studies of different cyclin and CRK genes in *T. brucei* have shown that three (CRK1, CRK2 and CRK3) orthologues are essential for cell division in the blood stream form. Using this technique, CRK1 and CRK2 were shown to control the G<sub>1</sub>/S passage, while CRK3 arrested cell division at the G<sub>2</sub>/M transition (Li and Wang, 2003; Tu and Wang, 2004). Knockdown of these CRK genes or their cyclin pairs resulted in accumulation of cells at the G<sub>1</sub> or G<sub>2</sub>/M phase. The CRK3 depleted blood stream trypomastigotes, and unlike the insect procyclic stage, failed to enter cytokinesis and cell division. In the blood stream stage, the nucleus and kinetoplast DNA continued to divide, and the cells accumulated multiple nuclei and kinetoplasts. Therefore, it was concluded that the absence of cytokinesis and cell division doesn't prevent the cells from entering G<sub>1</sub> and S phases (Li and Wang, 2003; Tu and Wang, 2004).

The genomic sequences for *Leishmania*, *T. brucei* and *T. cruzi* were published recently (Berriman et al., 2005; El-Sayed et al., 2005; Ivans et al., 2005). All three parasites belong to the same family, the Trypanosomatidae, and share many

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orthologous clusters of protein coding genes. However, they also have in common many unique or unusual biological and genetic mechanisms that are not found or are poorly represented in other eukaryotes, including glycosomes, polycistronic transcription, trans-splicing and kinetoplast DNA. Mining the genomes of these parasites for proteins with unusual or different properties will promote the identification of new leads for drug discovery and development.

### **KNOWLEDGE GENERATED, OPPORTUNITIES, USE AND IMPACT IN THE MEDICAL FIELD**

Sequencing of parasite and human genomes has provided unique opportunities for the analysis and comparison of parasite and human kinomes (Manning et al., 2002; Ward et al., 2004; Parsons et al., 2005). The TriTryp kinome (*T. brucei*, *T. cruzi* and *Leishmania*) was recently published (Parsons et al., 2006). A total of 68% of the typical eukaryotic protein kinases in these parasites are found in orthologous gene clusters. Interestingly, only a few protein kinase genes appear to be specific to each species, *T. brucei* (~2%), *T. cruzi* (~6%), and *Leishmania* (~10%). Analysis of the three kinomes highlighted some unusual characteristics of the trypanosome/*Leishmania* protein kinase families. Two of the six major protein kinase families, the receptor-linked tyrosine (TK) and tyrosine-like kinases (TLKs), are missing from the TriTryp kinome. However, tyrosine phosphorylation and tyrosine protein phosphatases have been reported in these parasites (Parsons et al., 1994; Parsons et al., 1995; Furuya et al., 1998; Salotra et al., 2000). This is probably due to the members of the dual specificity kinase (STE) family, which are capable of phosphorylating both serine/threonine and tyrosine residues. Members of the STE family appear to be over represented in these parasites when compared to the human kinome (Parsons et al., 2006). Interestingly, the CMGC is also over represented in the parasite kinomes compared to the humans. This may be correlated with the complex parasite life cycles, or changing, harsh environmental conditions (pH, temperature, osmolarity, host proteins, nutrients, etc.)

encountered by these parasites. The remaining two families, CAMK and ACG, appear to be poorly represented compared to the human kinome (Parsons et al., 2006).

Availability of the TriTryp kinome will facilitate identification of important parasite protein kinases. Further investigations of these enzymes by various molecular biological and proteomic techniques, molecular modelling of inhibitor - protein kinase interactions, reverse genetic engineering of parasites, and RNAi will allow the rapid evaluation of the target function(s) and their critical role in parasite survival. This will facilitate the selection of essential parasite protein kinases prior to drug development and optimization.

### **DRUG DEVELOPMENT STRATEGIES**

Research on the development of new protein kinase inhibitors for these neglected diseases is accelerating with the publication of the various parasite genomes, and increased cooperation between pharmaceutical companies, public institutions, universities and research institutions (Troullier et al., 2002; Davis et al., 2004). New methods to identify and screen potential compounds are being developed.

#### **Screening using live parasites**

African trypanosomes pass through several developmentally regulated stages during their life cycle in the Tsetse fly vector and mammalian host. The extracellular blood stream trypomastigote stage that is responsible for disease can be readily cultured *in vitro* and be used to screen different compounds for activity. Many methods have been used to assay potential compounds, including counting parasites (manual or automated), incorporation of radioactive nucleotides, enzymatic activity - colorimetric assays (reduction of MTT and other similar compounds to the purple formazan salt by active mitochondrial enzymes), or fluorescent assays such as Alamar Blue (Raz et al., 1997). Similar assays have been used to screen synthetic or natural products for activity against the *Leishmania* promastigotes, the extracellular stage of the parasite. This stage of the parasite was widely used in the past for

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screening, because it can be grown easily in culture. However, promastigotes reside in the sandfly vector; are briefly found in hosts and are not responsible for human or animal diseases that are caused by the intracellular amastigote stage. Stage specific differences in metabolism and protein expression have been observed between the two parasite stages. Compounds active on promastigotes may show no or large differences in activity when tested on the intracellular stage that causes leishmaniasis and visa versa (Serenó and Lemesre, 1997; Callahan et al., 1997). Differences in ED<sub>50</sub> between species of *Leishmania* are also observed.

Alternatively, chemical libraries can be screened directly on macrophages (differentiated and non-differentiated macrophage cell lines, peritoneal and bone-marrow derived mouse macrophages, or human monocyte derived macrophages) infected with parasites. Macrophages are infected using various conditions, parasite to macrophage ratio, time period and stage. Excess parasites are removed, and the infected macrophages are incubated with or without the chemicals for 2-3 days. The macrophages are Giemsa stained, and the percent of infected macrophages, as well as the number of parasites/infected macrophage, are enumerated using a light microscope, and compared to untreated control macrophages. This procedure is very time consuming and is not readily adaptable to high throughput (HTP) assays of large chemical libraries. Recently, methods to culture axenic amastigotes in the absence of macrophages for several *Leishmania* species have been developed (Debrabant et al., 2004). The properties of axenic amastigotes more accurately reflex those found using intracellular amastigotes, and can be used for accurate and reliable primary drug screening (Serenó and Lemesre, 1997; Callahan et al., 1997).

Production of genetically engineered parasites that express various reporter genes (red fluorescent protein dsRed, green fluorescent protein GFP,  $\beta$ -galactoside and luciferase) should streamline this process even further (Misslitz et al., 2000; Okuno et al., 2003; Lang et al., 2005; Sundar et al., 2005). Optimally, mutant *Leishmania* can be used to develop simple HTP assays for screening chemical libraries directly on infected macrophages. Fluorescent parasites

have been used to screen compounds by fluorescent activated cell sorting (FACS), microplate readers or fluorescent microscopes, while luciferase or  $\beta$ -galactoside expressing parasites have been used to screen the potential compounds using microplate readers or ELISA readers (Plock et al., 2001; Okuno et al., 2003; Lang et al., 2005; Sundar et al., 2005). Procedures using transgenic parasites need to be optimized and standardized in order to accurately measure parasite and host cell viability. In addition, it is important to use recently isolated parasite strains and frequently thaw new stabilates so parasite virulence and other properties don't change with time in culture. Afterwards, these HTP assays can be employed for drug discovery, in effect measuring parasite responses to new, hopefully effective compounds. Using such *in vitro* systems, drug development can be significantly accelerated increasing the number of compounds screened for activity on the intracellular stage of the parasite. Active compounds identified by these procedures are then subjected to several rounds of structure-activity analysis, synthesis and screening on infected macrophages prior to testing in animal models.

### **Recombinant enzymes**

Reagents have been produced against substrates and protein kinases of higher eukaryotes. Unfortunately, many of these fail to react with the parasite homologues making it difficult to elucidate the signalling pathways and protein kinases involved. However, completion of the *L. major* and *T. brucei* genome has facilitated the identification protein kinase genes of interest. These genes can be expressed as active recombinant proteins in either *E. coli*, baculovirus or other systems for further characterization, and be used in HTP screening of chemical libraries. These compounds need to be tested using the live parasites, as already described, in order to confirm their activity and lack of toxicity for host cells. Employing this approach, recombinant *L. mexicana* CRK3 was used to screen 634 antimitotic compounds from different sources. Approximately 6.6% of the compounds inhibited CRK3 by >90%, of these 59% showed good activity on infected macrophages or were not toxic to the host cells.

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Six compounds inhibited amastigotes in infected macrophages at  $ED_{50}$  <6 micromolar range (Grant et al., 2004).

Identification of the inhibitor's target is important for understanding the drug's mechanism of action. This can facilitate further optimization of the inhibitor's chemical functional groups via molecular modelling using existing protein kinase structures and information on interactions between ATP, the chemical inhibitors and the active site of homologous protein kinases. Affinity chromatography can be valuable in identifying the target enzyme a specific inhibitor or family of compounds (Knockaert et al., 2002). In this case, an inhibitor is covalently coupled to a solid support, and cell lysates are incubated with the resin. Bound proteins are eluted, digested and the peptides sequenced. The amino acid sequences are compared to those found in the parasite databank. This technique has been used to identify the targets of several paullone and purvalanol inhibitors of Trypanosomatidae (Knockaert et al., 2000; Knockaert et al., 2002). These compounds are promising inhibitors of CDKs and glycogen synthetase kinase-3 (GSK-3) in cancer cells (Schultz et al., 1999; Gray et al., 1999; Leost et al., 2000). Interestingly, while the covalently bound purvalanol B purified several members from the CMGC group, including CDK1 from higher eukaryotes, CK1 belonging to the OPK group II was the primary protein kinase purified from *L. mexicana* (Knockaert et al., 2000). Similarly, GSK-3 was purified from higher eukaryotes on the gwennpaullone affinity resin together with the mitochondrial malate dehydrogenase (MDH). MDH binds apparently to the affinity resin via the NAD/NADH binding site. When *L. mexicana* lysates were used, MDH was the major protein purified. Paullones were shown to inhibit mitochondrial MDH, as well as *L. mexicana* promastigotes at low micromolar concentrations. Together these results suggest that caution should be employed when extrapolating mechanisms of action and targets for protein kinase inhibitors from higher eukaryotes to parasites.

Finally, genetic or functional knockouts of specific protein kinases, using either homologous recombination (*Leishmania* and *T. brucei*) or RNAi (*T. brucei*), enzyme over expression, and

utilization of analog-sensitive mutants can be employed in order to obtain a better understanding of the function and roles of specific protein kinases targeted by the inhibitors. Commercial development of new drugs is to a large degree dependent on public-private funding, as private companies find it difficult, without risk reducing measures, to embark alone to develop drugs for markets that may not be financially feasible.

## CONCLUSIONS

Leishmaniasis and human African trypanosomiasis are widespread diseases in subtropical and tropical regions that cause extensive morbidity and mortality among populations living primarily in countries with limited resources. New drugs for the safe, effective, cheap and easy oral treatment of leishmaniasis and human African trypanosomiasis are urgently needed to prevent death or long-term disability. Few new drugs are in the pipeline, and most of these will take several years to receive approval and registration. Availability of several cheap and safe oral drugs will promote the use of combination therapy and prevent the development of drug resistance.

The urgency of developing effective drugs to combat leishmaniasis and human African trypanosomiasis in a most appropriate and effective manner has been internationally recognised. The projects are primarily internationally and publically driven, as pharmaceutical companies are unlikely to dedicate their resources to combat a neglected rare disease that is unlikely to bring sufficient financial profits (Troullier et al., 2002; Davis et al., 2004). One example is the Drugs for Neglected Diseases initiative (DNDi, [www.dndi.org](http://www.dndi.org)), which was formed by five public institutions (Oswaldo Cruz Foundation, Indian Council for Medical Research, Kenya Medical Research Institute, Ministry of health of Malaysia and Pasteur Institute of France), Medecins sans Frontieres and the UNDP/WORLD BANK/WHO's Tropical Diseases Research Programme. This organization includes leishmaniasis and human African trypanosomiasis among their target diseases.

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# ”PROTEIN KINASE RESEARCH” -TEEMANUMERON ARTIKKELIEN SUOMENKIELISET YHTEENVEDOT

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## PROTEIINIKINAASI C ISOENTSYYMIT – KOHDEPROTEIINEJA UUSILLE HOITOMUODOILLE

### YHTEENVETO

Proteiinikinaasi C (PKC) perhe koostuu seriini/treoniinispesifisistä proteiinikinaaseista. PKC isoentsyymit voidaan luokitella kolmeen ryhmään:

- 1) konventionaaliset PKC:t (cPKC)  $\alpha$ ,  $\beta$ I,  $\beta$ II ja  $\gamma$
- 2) uudet PKC:t (nPKC)  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\eta$ /L ja  $\mu$  sekä
- 3) atyyppiset PKC:t (aPKC)  $\lambda$ / $\iota$  ja  $\zeta$ .

PKC isoentsyymit ovat tärkeässä osassa signaalin kuljetusreiteissä osallistuen viestinkuljetukseen synapseissa, ionivirtojen aktivoinnissa, erityksessä, solun jakaantumisessa, solukierron kontrolloimisessa, erilaistumisessa ja kasvainsojien jakaantumisessa. PKC-perheestä on tulossa kasvavan kiinnostuksen kohde etsittäessä uusia hoitomuotoja useisiin eri sairauksiin. Tässä katsauksessa esitellään eri PKC isoentsyymien toimintaa ja mahdollisia keinoja vaikuttaa niihin.

PKC $\alpha$ -spesifinen antisense-oligonukleotidi apriinokarseeni (ISIS 3521) ei osoittautunut hyödylliseksi syöpälääkkeenä. PKC $\beta$ -inhibiittori rubokstauriini-mesyalaatti (LY333531) on faasi III:sen

kliinisissä tutkimuksissa ja se hidastaa diabeettisen retinopatian, nefropatian ja neuropatian oireita. Kokeellinen syöpälääke AD 198 vaikuttaa aktiivisella PKC $\delta$ -isoentsyymillä. Muille PKC-isoentsyymeille ei ole vielä olemassa spesifejä pienen molekyylikoon omaavia inhibiittoreita tai aktivaattoreita. Tällaiset modulaattorit olisivat tärkeitä PKC isoentsyymien toiminnan vaikutusten selvittämisessä. Samoin niiden avulla voitaisiin tutkia, olisivatko PKC isoentsyymit sopivia kohdemolekyyliä esim. syöpäsairauksien tai diabeteksen hoitomuotoja kehitettäessä.

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# SYÖPÄÄ AIHEUTTAVAT TYROSIINIKINAASIT: BCR-ABL MALLI

## YHTEENVETO

Tyrosiinikinaaseja (TK) ja niiden sukulaisproteiineja tiedetään olevan yli 100 erilaista. TK:t osallistuvat solunulkoisten ärsykkeiden välittymiseen solun sisään sekä useiden elintärkeiden prosessien säätelyyn, kuten solun jakautumiseen ja solu-kuolemaan. Tyrosiinikinaasit voivat itse muuntua syöpää aiheuttaviksi eli onkogeenisiksi. Toistaiseksi on löydetty seitsemän onkogeenisistä tyrosiinikinaasia.

Tässä artikkelissa esitellään BCR-ABL tyrosiinikinaasimalli, jonka selvittäminen auttoi kehittämään onnistuneen syöpähoidon. Kroonisessa myelooisessa leukemiassa esiintyvä ns. Ph-kromosomilöydös johtuu lähekkäin sijaitsevien kromosomien 9 ja 22 sisältämien BCR ja ABL geenien muodostamasta hybridistä. BCR-ABL-hybridi osoittautui lisätutkimuksissa erinomaiseksi lääkevaikutuksen kohdemolekyyliksi. 1980-luvun lopulla kehitettiin imatinibimesylaatti, joka esti BCR-ABL hybridin kinaasiaktiivisuutta ja siten pysäytti leukeemisten solujen kasvun ja aiheutti nii-

den solukuolemaa *in vitro* ja *in vivo*. Imatinibia on testattu kliinisissä kokeissa 8 vuoden ajan Ph+-leukemiapotilailla. Artikkelissa kuvataan näistä tutkimuksista saatuja rohkaisevia tuloksia. Kirjoittajat esittelevät BCR-ABL hybridin ja imatinibin myös rakenteena, jolle voidaan perustaa uusien syöpäterapioiden jatkokehittelyt.

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# PROTEIINIKINAASIT LEUKEMIATERAPIAN KOHDEMOLEKYYLEINÄ

## YHTEENVETO

Leukemiassa verisolujen ja niiden esiasteiden normaali tasapaino solun jakautumisen, erilaisumisen ja solukuoleman välillä on häiriintynyt. Tämä johtaa kypsyttömiä prekursorisolujen määrän kumuloitumiseen luuytimessä, joka puolestaan hidastaa normaaliin esiastesolujen kasvua ja kehittymistä. Seurauksena ovat leukemiassa tyypilliset löydökset, kuten neutropenia, trombosytopenia ja anemia. Leukemian neljä päätyyppiä ovat akuutti lymfoplastinen leukemia (ALL), akuutti myeloinen leukemia (AML), krooninen lymfosyyttinen leukemia (CLL) ja krooninen myeloinen leukemia (CML).

Leukemia esiintyy yleensä vanhemmalla iällä, tosin ALL esiintyy useimmiten lapsilla. Vaikka ALL on lähes parannettavissa (80–85 % remissio lapsilla), aikuisiän leukemian ennuste on edelleen huono. Uusia leukemian hoitomuotoja tarvitaan siten kipeästi. Uusien hoitojen kehittäminen tähtää joko solujen erilaistumisen pysähtymisen estoon tai leukemisten solujen kasvun estoon tai niiden kuoleman edistämiseen. Useat nykyisin kemoterapiassa käytetyt yhdisteet eivät ole riittävän spesifisiä, joten ne vahingoittavat myös normaaleja soluja.

Imatiniibin, lyhytketjuisen proteiinikinaasi-inhibiittorin, hyvä menestys CML:n hoidossa on avannut uusia näköaloja kohdennettuun leukemiaterapiaan. Tässä katsauksessa kirjoittajat esittelevät uusia yhdisteitä, joiden kohdeproteiineina toimivat erilaiset proteiinikinaasit. Eräät proteiinikinaasit saattavat muuttua itse onkogeenisiksi, toisaalta proteiinikinaasien aktivointi tai inhibointi on osoittautunut leukemisten solujen kuolemaa edistäväksi. Katsauksessa esitellään BCR-ABL tyrosiinikinaasiin, FLT-3 tyrosiinikinaasiin sekä Jak-tyrosiinikinaasiperheeseen vaikuttavia yhdisteitä, kuten myös seriini/treoniinikinaaseja (PKC, Ras-Raf-Mek-Erk-signaalireitin kinaasit) inhiboivia tai aktivoivia yhdisteitä. Näistä yhdisteistä tulee todennäköisesti lupaava "toisen aallon" leukemiaterapia-perhe.

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# STAUROSPORIINI JOHTOMOLEKYYLINÄ: KATSAUS INDOLO- KARBATSOLIALKALOIDIEN INSPIROIMIIN KINAASI- INHIBIITTOREIHIN

## YHTEENVETO

Staurosporiini on yksi monista varhaisimmista proteiinikinaasin ATP-sitoutumiskohtaan kohdistetuista proteiinikinaasi-inhibiittoreista. Staurosporiini on rakenteena innoittanut kehittelemään laajan valikoiman selektiivisiä kinaasi-, topoisomeraasi- ja p-glykoproteiini-inhibiittoreita.

Tässä katsauksessa esitellään alkuperäisestä staurosporiini-rakenteesta muunneltuja inhibiittoreita. Näitä ovat staurosporiinin johdannaiset, indolokarbatsoli-monoglykosidit, substituoidut indolokarbatsolit, bisindolyylimaleimidit, makrosykliset bisindolyylimaleimidit, dianiliiniftalimit, 4-aryylikarbatsolit, heteroaryl(o)karbatsolit ja aryl(o)karbatsolit, sekä atsaïndolokarbatsolit. Selektiivisiä ja voimakkaita inhibiittoreita on kehitetty seuraaviin kohdeproteiineihin: PKC, GSK-3 $\beta$ , myosiinin kevyen ketjun kinaasi, "checkpoint" kinaasit, sykliinistä riippuvaiset kinaasit, flt-3, DAPK, EGFR, VEGFR, PDGFR ja PIM1, kuten myös eikinaasi kohdeproteiineihin, esim. topoisomeraasi I, PARP-1 ja p-glykoproteiini, jotka perustuvat alkuperäiseen indolokarbatsolijohdantomolekyyliin.

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# PROTEIINIKINAASITUTKIMUSTA HELSINGIN YLIOPISTOSSA

## YHTEENVETO

Proteiinikinaasi C:n (PKC) isoentsyymit ovat hyvin merkittäviä signaalinvälityksessä ja solujen muuttumisessa pahanlaatuisiksi. Erilaisten aktivaattoreiden vaikutuksesta proteiinikinaasi C:n isoentsyymien konformaatio muuttuu huomattavasti, ne sitoutuvat membraaniin, muuttuvat entsyymaattisesti aktiivisiksi ja fosforyloivat kohdeproteiininsa. PKC-isoentsyymien yleisaktivaattorina käytetään forboliesteriä, joka sitoutuu niihin hyvin voimakkaasti.

Tämän tutkimushankkeen keskeisinä tavoitteina on etsiä, tunnistaa, suunnitella ja valmistaa spesifisiä ja affiniteetiltaan hyvin sitoutuvia yhdisteitä, jotka estäisivät esimerkiksi PKC-alfan tai PKC-deltan aktivoitumisen forboliesterin vaikutuksesta. Tällaisia yhdisteitä suunnitellaan tietokoneavusteisesti ja valmistetaan synteettisesti hyödyntämällä esimerkiksi kombinatoriaalisien kemian menetelmiä. Toisaalta näitä yhdisteitä voidaan etsiä ja tunnistaa luonnosta. Tässä hankkeessa niitä etsitään pääasiassa kotimaisista sinileivistä, sienistä ja kasveista. PKC:n modulaattoreiden ja inhibiittoreiden käyttö erilaisten sairauksien hoidossa olisi poikkeuksellisen laajaa käsittäen muun

muassa autoimmuunitauteja (nivelreuma, punahukka), sydän- ja verisuonisairauksia (verenpaine-tauti) ja syöpäsairauksia (pahanlaatuinen gliooma). Tässä katsauksessa esittelemme EU:n komission rahoittamassa "Protein kinases – Novel drug targets of post-genomic era" -projektissa mukana olevat Helsingin yliopiston tutkimushankkeet.

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# PROTEIINIKINAASIT LÄÄKEVAIKUTUKSEN KOHTEINA UUSILLE ALKUELÄINTAUTIEN HOITON SOVELTUVILLE LÄÄKEAINEILLE

## YHTEENVETO

Leishmaniaasit ja afrikkalaiset trypanosomiaasit ovat subtrooppisilla ja trooppisilla alueilla laajalle levinneitä tauteja, jotka aiheuttavat laajaa sairastuvuutta ja kuolleisuutta kehittyvien maiden väestössä. Uusia lääkkeitä, jotka olisivat tehokkaita, turvallisia, halpoja ja helposti suun kautta annosteltavissa, tarvitaan kiireisesti näiden tautien hoitoon estämään kuolemia tai pitkäaikaisia vammautumisia. Käytössä olevat lääkitykset ovat kehitetty 1950–60-luvulla, ne ovat vaikeasti annosteltavia, usein tehottomia ja niillä on paljon sivuvaikutuksia. Kehitteillä on ainoastaan muutamia uusia lääkkeitä ja niiden käyttööotto tulee kestämään vielä useita vuosia. Useiden halpojen ja turvallisten suun kautta annosteltavien lääkkeitä saatuus parantaisi yhdistelmähoitojen mahdollistumista ja siten voitaisiin estää myös resistenssin kehittymistä.

Lääkekehityksen kannalta ensimmäinen vaihe leishmaniaasien ja afrikkalaisen trypanosomiaasin tutkimisessa on täsmällisten lääkkeitä kohteiden määrittäminen. Eräs vartenotettava vaihtoehto ovat solukierrosta riippuvaiset kinaasit (cell cycle dependent kinases, CDKs). Eroavaisuudet ihmisen ja parasiittien solukierroksen säätelyssä ja niihin liittyvissä CDK-altyypeissä antavat uusia mahdollisuuksia lääkkeitä kehitykselle.

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