Protein Kinases: Therapeutic Opportunities

The latest trends in protein kinase and phosphatase research were described in a one-day symposium 'Protein kinases: therapeutic opportunities' organised by the Society for Medicines Research. The meeting was held on 25 March 1999 at Pfizer's UK research complex in Sandwich, Kent. The seven speakers from academia and industry provided a clear view of the role of kinases and phosphatases in cell signalling and disease, followed by discussion on the therapeutic opportunities presented by the increasing implication of abnormal phosphorylation in diseases and the development of small molecules to treat these. Abnormal phosphorylation has been implicated in a wide range of diseases and more than 20 hereditary disorders have been shown to be caused by mutations in kinase and phosphatase genes. Thus specific small molecule intervention in the phosphorylation processes involved in cell signalling offers widespread opportunities for novel therapy in cancer, immunology, inflammation and neurodegeneration.

The day began with an excellent overview on the role that phosphorylation and dephosphorylation play in the body by **Prof Sir Philip Cohen (Dundee, Scotland).** Reversible phosphorylation of proteins catalysed by protein kinases and protein phosphatases regulates almost all aspects of cell life. Scaling up from yeast, which contains 106 kinases and 31 phosphatases, it is estimated that there are 2000 kinases and between 300 and 500 phosphatases in the human genome. A third of all proteins can be regulated by reversible covalent phosphorylation, and so each kinase must phosphorylate 15–20 proteins, and the average phosphatase dephosphorylates approximately 50 proteins. Specific inhibitors of protein and lipid kinases and phosphatases are required to dissect the pathways involved in signal transduction, and this is a major challenge. However some do exist; for example, the immunosuppressant cyclosporin A binds to cyclophilin and is a specific inhibitor of the phosphatase calcineurin (calcium-dependent protein phosphatase 2B) in T-cells, preventing IL-2 production and blocking T-cell proliferation. This evidence encouraged the further search for specific inhibitors of kinases and phosphatases.

One of the most studied kinase families is that containing the membrane-bound tyrosine kinases (TKs) VEGF, EGF, PDGF and NGF, which are overexpressed in certain cancers. SU101 (structure 1) (Leflunomide), currently being used in phase III studies, is one such inhibitor This compound is known for its immunosuppressant effects in rheumatoid arthritis, but it is also very potent in the suppression of PDGF-expressing tumours. Specific inhibitors of VEGF and FGF have found a use in angiogenesis and thus may be of benefit in a wide range of diseases, especially where tumour cell growth is dependent upon new blood vessel generation. Several compounds are in development, such as SU5416, PD173074 and SU5402 (structure 2).

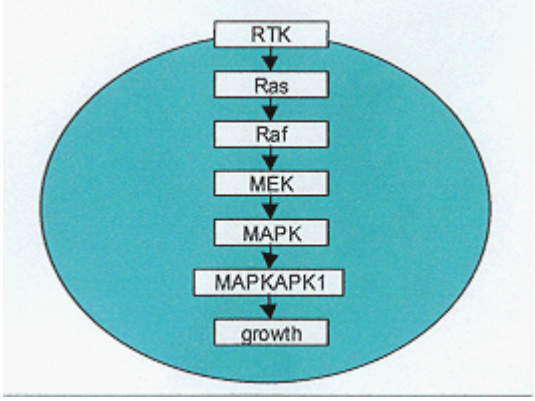


Figure 1

Overproduction of Ras occurs in over 30% of cancers and this pathway, which leads on to the mitogen-activated protein (MAP) kinase system discussed below, is of central importance to cell growth and differentiation (Figure 1). Compounds such as PD98059 (structure 3) and U0126 work downstream from the membrane TKs by binding to MAP kinase kinase-1 (MEK) to prevent phosphorylation of the serine residue by Raf. Here these compounds stop one kinase activating another. Targets further downstream interfere with the cell cycle pathway itself, and insofar as such compounds block cellular proliferation they may have applications in cancer. Purvalanol B (an analogue of olomucine) does not distinguish between the cyclin dependent kinases but is very selective over other (non-cyclin dependent) kinases.

In hypertension it has been shown that Ca^{2+} sensitisation of smooth muscle is mediated by a Rho-associated protein kinase (RhoK1/p160Rhok). A Yoshitomi compound Y27632 (structure 4) is a potent inhibitor of this kinase and is very selective *in vitro*. In inflammation, the pyridinyl imidazole SB-203580 (structure 5) blocks the production of TNF by inhibiting stress-activated protein kinase 2 (SAPK2, or p38) and is effective in chronic inflammatory disease models. Despite a close similarity between SAPK3 (p38γ) and SAPK2 (p38α/β), SB-203580 only inhibits SAPK2.

Since the majority of compounds are competitive with ATP, their inhibitory potency is very dependent on the ATP concentration: it is crucial to both measure and quote this factor alongside IC_{50} values. As the ATP binding sites of kinases are highly conserved, the observation of selectivity among some of the above compounds is aiding understanding of the mode of action. Analysis of co-crystal structures helps to explain this; contacts outside the ATP binding pocket determine the specificity. A study of the interactions between SB-203580 and amino acid residues on the kinase have revealed that the fluoryl benzyl group interacts with a residue outside of the ATP binding pocket (even though the drug is competitive for ATP). Site-directed mutation studies of SAPK2, 3 and 4 have shown the critical nature of. Thr106, whereas most kinases have

methionine/glutamine in this position which prevents access by SB-203580 to the ATP pocket. There are 12 other kinases with Thr106 (e.g. TGFb, Lck, Raf) and all are sensitive to SB-203580. Raf operates via a novel feedback loop to reactivate itself, making it a poor drug target as reported activators may actually be inhibitors.

To evaluate a compound's specificity would require a very large panel of kinases for screening; compounds such as SB-205380 with a known structural requirement can make use of sequence data rather than assays. It is particularly important that compounds used to determine the site of action should have known selectivity. For example, although Ro-318220 (structure 6) is used widely as a 'specific' PKC inhibitor, it actually also inhibits kinases from a range of families, including MAPKAP kinase-1 and p70 S6 kinase. Novel approaches are being used to elucidate *in vivo* selectivity, one example being the use of stable cell lines producing drug insensitive forms. The growing list of compounds showing specificity for individual kinases will allow clearer dissection of signalling pathways.

Phosphatases

The second talk given by **Dr Nick Tonks (Cold Spring Harbor, USA and CEPTYR, USA)** was an exciting update on the potential for protein tyrosine phosphatases (PTPs) as targets for drug discovery. PTPs have been shown to act both positively and negatively in the regulation of cellular signalling. They are a broad family of enzymes that will rival protein tyrosine kinases in structural diversity and complexity. Genome sequencing efforts suggest that there will be about 300 PTPs in total, of which approximately 100 have already been identified. Differences in the regulatory targeting sequences distinguish the different PTPs, of which there are primarily three types.

Major progress has been made in determining the crystal structures of PTPs, resulting in detailed mechanistic information. The phosphate group on a tyrosine residue of the target substrate interacts with the signature motif in the active site of the PTP. Substrate binding induces a large conformational change; Cys216Ala and Asp181Ala mutations have decreased activity 1000-fold despite retaining the ability to bind substrate. Experiments with TCPTP and PTP1B have shown that these phosphatases recognise discrete substrates. The structure of a PTP1B-peptide substrate complex also allowed the development of an assay which has revealed that these enzymes display high substrate specificity *in vivo.* This suggests that inhibition of a defined PTP by a drug will only interfere with selected signalling events in cells.

PTEN is a PTP which has been identified as the tumour suppressor on 10q23 and is deleted or mutated in a large number of glioblastoma, endometrial and prostate cancers. PTEN contains the signature motif and is a dual-specificity phosphatase that prefers highly acidic substrates. It has recently been shown that PTEN selectively dephosphorylates the 3 position of PIP_3. Phosphatidyl inositol phosphates are physiological substrates for PTPs. PTPs act both positively and negatively and have diverse roles in a wide range of physiological diseases. At CEPTYR they have isolated a

number of small molecule leads representing structurally distinct pharmacophores, with application to diabetes, obesity, immunosuppression and cancer.

The last session of the morning focussed on the physiological role of tyrosine phosphorylation in vascular smooth muscle function. **Dr Alun Hughes (Imperial College, UK)** first gave an overview of smooth muscle cell functions. Vascular smooth muscle cells (VSMCs) serve two roles in blood vessels and have distinct phenotypes; differentiated VSMCs (contractile) are specialised for sustained force production, regulate vascular tone and have prominent microfilaments and contractile machinery. Dedifferentiated VSMCs (synthetic) participate in the response to injury, are involved in atheroma formation and re-stenosis following surgery, and have well-defined Golgi.

Tyrosine phosphorylation is a key intracellular event in the proliferation and migration of VSMCs in response to injury. This response is very well characterised in VSMCs, with medial smooth muscle cell proliferation being the first wave, smooth muscle cell migration the second wave and intimal smooth muscle cell proliferation being the third wave. There is good evidence that the first wave mediators are bFGF-receptor TK together with thrombin and angiotensin activating a wide range of TKs subsequent to receptor activation. The second wave mediators are PDGF via receptor TK together with osteopontin and integrin, which activate other TKs such as src. The third wave is again mediated by PDGF-receptor TK, angiotensin-activating src, fyn and jak, and TGF-b receptor TK. Response to injury can be shown to be attenuated by the TK inhibitor AG-17, which reduced neointima formation following injury. New selective TK inhibitors may open up this area and help determine appropriate points of intervention.

The contraction of smooth muscle cells may also be determined by the degree and/or site of tyrosine phosphorylation within the cell. Growth factors such as PDGF induce a rise in intracellular Ca^{2+} and contraction in isolated blood vessels as a result of activation of voltage-operated calcium channels. Dr Hughes presented evidence correlating the activity of a range of TK inhibitors with the opening of voltage-operated calcium channels, and in most cases the inhibitory potency of s-src corresponded with the blockade of calcium channel opening. The non-receptor TK pp60c-src is present in high amounts in smooth muscle and is a likely candidate for the endogenous TK. In addition, contractile agonists (e.g. 5HT, noradrenaline and angiotensin) also induce tyrosine phosphorylation and the functional responses can be inhibited by the TK inhibitor tyrophostin. Growth factors induce contraction of isolated blood vessels via activation of calcium channels. There is evidence for src kinase and MAP kinases playing an important role in the regulation of Ca^{2+} entry and contraction in differentiated VMSCs, supporting the potential use of novel selective kinase inhibitors in a wide range of vascular diseases.

Kinase Inhibitors in Cancer Therapy

The afternoon session began with two presentations on the use of protein kinase inhibitors in cancer therapy. Currently available cytotoxic treatments for cancer are limited by toxicity and efficacy. Kinase inhibitors offer potentially improved anti-cancer therapeutics, particularly as signalling targets have proven an effective strategy for

hormonal tumours and a range of targets have been identified. The first speaker, **Dr Judith Sebolt-Leopold (Parke Davis, USA),** gave evidence for specific inhibitors of the MAP kinase pathway blocking growth of murine and human tumours. MEK is a dual-specificity kinase, activated by phosphorylation on S218/S222. A new class of MEK inhibitors, (4-iodo phenylamino)benzoic acids and their corresponding hydroxamic derivatives, have high potency and selectivity for MEK-1. Inhibition of MEK activity has been shown to induce G1 block and thus impair cell cycle progression. PD184352 (structure 7) is a potent inhibitor of MEK *in vitro* (IC_{50} 0.6 nM) with high selectivity compared with a wide range of other kinases. It is not an ATP-competitive inhibitor, nor competitive for the MAPK site on MEK, similar to PD98059 (structure 3). However, when MEK-1 mutants were made with glutamate or aspartate substituting for the two serines, PD184352 inhibited their activity while PD98059 did not.

When a panel of murine and human carcinoma cell lines were studied, diverse responses were seen with PD184352. From experiments using PD184352 as a tool to probe basal MAPK levels it was suggested that cells which do not have high levels of active MAPK do not rely on this pathway for proliferation. High levels of MAPK activity, however, may predict susceptibility of tumour cell lines to PD184352.

Colon scC26 cells are very sensitive to PD184352 *in vivo* (IC_{50} *ca* 300 nM), while PC3 (prostate) cells are not sensitive to PD184352, suggesting that proliferation in this cell line is not dependent on MAPK. Oral dosing studies (48–400 mg b.i.d.) in the C26 tumour model showed total suppression of MEK up to 9–12 h post-dose with no toxicity after 14 days' dosing. Total inhibition of tumour growth was seen from ~120 mg and the animals were not immunosuppressed. Efficacy correlated with blockade of MAPK activity in excised tumours. This series of compounds appears promising as non-cytotoxic anticancer agents in selected tumours. However, the selectivity for MEK-1 over other MEKs was raised during questions from the floor. The suggestion that the resistance to these compounds in some cells could be due to the mix of MEK-1 and MEK-2 rather than just MEK-1 is currently being investigated.

Dr Andy Barker (Zeneca) talked about the epidermal growth factor receptor (EGFR), which is highly expressed in a range of cancers, particularly non-small cell lung, colorectal, gastric, pancreatic and ovarian types. Aberrant expression of receptor kinase subtypes is common in a range of solid tumours of epithelial origin. Zeneca's programme started from a high throughput screen based on a radiolabelled *in vitro* assay, from which a lead quinazoline structure was identified. Initial changes in the 6- and 7- positions led to structure 8 (R^6 = MeO, R^7 = MeO) with a kinase IC_{50} of 5 nM, but problems of hydroxylation at the benzylic methyl group compromised *in vivo* activity. Changing the aniline group to that shown in structure 9, while keeping the same R^6 and R^7 groups eliminated this problem and preserved *in vitro* activity, but poor solubility and logP again reduced activity in the A431 xenograft model. Replacing the 6-MeO group with a water-soluble morpholine side chain resulted in ZD1839, with a kinase IC_{50} of 23 nM and reasonable *in vivo* activity in the xenograft model. This compound is now in full development, and clinical data are due to be released later in the year.

A key aspect of the biology of kinases in inflammation was then dealt with by **Dr Catherine Regnier (INSERM, France)**, whose presentation referred to the research conducted at Tularik in California, USA. This work concerned the activation of the NF-RB protein by I-RB. This signalling pathway has been under intensive investigation because of its central importance in the cellular activation caused by IL-1 and TNF. NF-RB is usually inactivated in the cytosol by binding to I-RB, but upon release from this protein translocates to the nucleus and activates cytokine transcription. Breakdown of the NF-RB/I-RB complex is facilitated by phosphorylation of I-RB, a process that involves a specific protein kinase. Dr Regnier's talk focused on the identification of this kinase using molecular biological techniques and *in vitro* experiments. Initial attention centred around a so-called conserved helix-loop-helix ubiquitous kinase (CHUK), which when overexpressed in HeLa cells induced NF-RB activation. CHUK was known to mediate in the NF-RB pathway downstream of two other proteins known as NIK (NF-RB inducing kinase) and TRAF. A series of elegant experiments showed that CHUK interacts with, and phosphorylates at the serine residues 32 and 36, theI form of I-RB, and was renamed IKKI. Subsequently, IKKI has also been shown to phosphorylate the ß form of I-RB known as I-RBß, and another kinase has been identified, called IKKß, which is selective in the phosphorylation of I-RBß. IKKI and IKKß can form homo- and heterodimers, the latter being preferred. NIK stimulates phosphorylation of both IKKI and IKKß, the former predominating. *In vivo,* a complex of IKKI and IKKß, together with NIK, NF-RB and relA, is likely to exist. High throughput screening is underway to identify inhibitors of IKKI and IKKß, and IKK-knock out animals have been produced in collaboration with Roche Bioscience. During the discussion that followed this talk the limitations of the purely *in vitro* methods that led to these discoveries were highlighted, and it was acknowledged that confirmation of this model would require the discovery of selective inhibitors of these various kinases, so that their importance in a whole organism could be ascertained. Work on such compounds is underway at Tularik, and leads have been identified.

The final talk of the day discussed the design of novel inhibitors of kinases through molecular modelling. **Dr Martin Noble (Oxford University, UK)** concentrated on the modelling of cyclin-dependent kinases involved in cellular proliferation. Cyclin-dependent kinase activity is modulated by a number of mechanisms (Figure 2), all of which are reversible. For example, the activity of the transcription factor E2F-1 is modulated through binding to DP-1, which is phosphorylated by CDK-4 and CDK-6, and the protein RB which also binds to the complex.

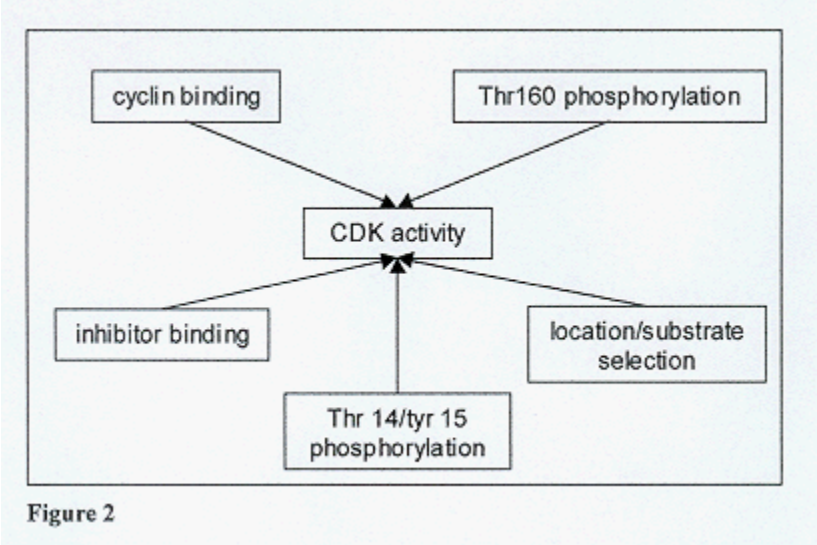
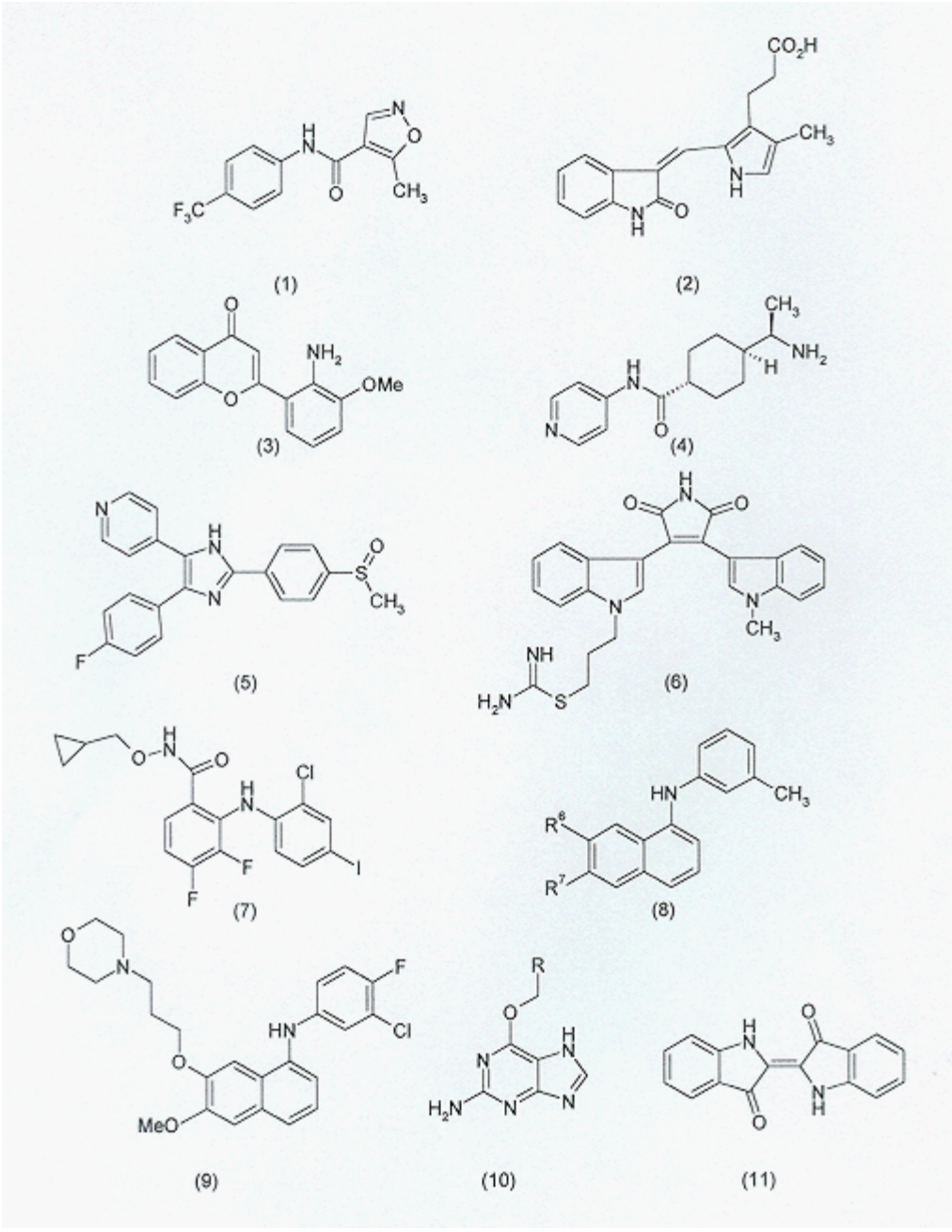


Figure 2

CDK-2 is a well-characterised kinase which is inhibited by stautosporine. X-ray crystal structure analysis shows that the binding of stautosporine extends well into the kinase active site, and suggests that after binding the conformation of the protein changes radically from a flat to a heavily folded shape. One point to consider regarding specificity was that the lysine 89 residue that lies near the active ATP binding domain varies quite considerably among kinases. Dr Noble's talk examined in some detail the binding of purine-based CDK inhibitors such as structure 10, in which the R group included cycloalkyl and branched alkyl chains. The hydrophobic interactions of such groups with the site otherwise occupied by the ribose group of ATP suggests that direct chemical similarity with the sugar moiety is not essential for activity.

Another interesting insight into the mechanism of action of these enzymes came from studying a Chinese herbal remedy for leukaemia. Qing Dai derives much of its activity from indirubin (structure 11), which is a structural analogue of indigo. Indirubin is an inhibitor of CDK-2, for which cyclin A is a substrate, with an IC_{50} of 2.2 T M.

In conclusion, this was a successful meeting that drew a deservedly large audience of about 150 to see an internationally renowned group of speakers present contemporary findings in an intensively investigated area. Although still at an early stage, and clearly of enormous complexity, there is great scope for therapeutic innovation and real drug discovery from this area.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