Conference Report Editor: Jayne Carey
jayne.carey@elsevier.com

conference report

Rounding up the usual suspects: protein kinases as therapeutic targets

Stephen B. Shears, shears@niehs.nih.gov

Signal Transduction: Targets for Effective Therapeutics
Seaport Hotel, Boston, USA
November 8–9 2004

Organisers:
Cambridge Healthtech Institute

We are acquiring an exponentially increasing amount of information concerning specific molecular abnormalities that promote disease phenotypes. We are also witnessing some momentous technological advances that are modifying the strategies underpinning modern drug development. Many candidate drugs intervene in signal transduction events, that is, the intermolecular communication processes operating within regulatory networks that mediate the essential cell processes of growth, differentiation, and survival. It is thus a very useful exercise to gather together experts who are participating in the discovery of new signal transduction processes and add to that melting pot some of the champions of the latest technological advances that are currently driving pharmacological intervention in signaling pathways. This was just the mix of participants at a recent meeting organized by the Cambridge Healthtech Institute ('Signal Transduction: Targets for Effective Therapeutics', November 8–9 2004 at the Seaport Hotel, Boston).

Kinomics and chips

Protein kinases are the cornerstones of many cell signaling systems. As one of the chairmen (Amar Singh, Northwestern University) reminded us, the human genome encodes >500 protein kinases, so it is not surprising that these were the major focus of the conference. For example, Jos Joore (from Pepscan, based in the Netherlands) promoted the 'PepChip' peptide microarray as a new high-throughput screen for examining the specificity of kinase inhibitors, and for profiling kinase activities in cells (so-called 'kinomics'). Over 1100 different, short peptides containing consensus sites for phosphorylation by specific kinases are covalently coupled to a proprietary microarray surface. The peptides are currently all chosen from the freely-accessible Phospho.ELM database (, although Pepsan is working to increase the breadth of the array. A mere 50 µl of cell lysate from cells is supplemented with ^{32}P-ATP and added to the chip, and the resultant phosphorylation profile is assessed by an isotope imager. Several slides validating the procedure were presented, but the discussion following the presentation raised some concerns. For example, the peptides are linear; most are only nine residues long, and none are more than twelve, and so they do not include specificity determinants arising from secondary structure (Pepscan does intend to develop arrays with longer peptides). Additionally, the profiles can be rather sensitive to assay conditions, such as levels of ATP, Mg^{2+}, and Mn^{2+}. Furthermore, the detergents that are used to lyse the cells can disrupt protein–protein interactions that regulate many kinases activities. The latter point resurfaced later in the conference when Brian Wong (Rigel Pharmaceuticals, San Francisco) argued that *in vitro* assays frequently fail to satisfactorily imitate the intracellular milieu. This is why Rigel has developed a proprietary human mast cell assay to screen for drugs blocking release of inflammatory mediators. Rigel's first clinical candidate to emerge from this approach is an inhibitor of the Syk tyrosine kinase that can be administered intranasally to treat allergic rhinitis and asthma. Erik Bush (Myogen, Colorado, USA) also preferred the intact cell approach – in this case myocytes – in his company's quest to develop drugs that might prevent heart hypertrophy.

Lack of specificity

Joore drew attention to a problem with other high-throughput technologies – and kinase research in general: lack of specificity of some anti-phosphopeptide antibodies used to detect protein kinase activation. Anyone who, following a western blot, has failed to see the elusive single-band-on-a-gel (of the appropriate size!), can appreciate that particular headache. Antibody specificity is clearly a make-or-break issue for Li-Cor Bioscience's in-cell western assay, which was described by Michael Olive (Lincoln, Nebraska, USA). Here, cells in 96- or 384-well plates are fixed, permeabilized, and immunoblotted with an anti-phosphopeptide antibody against the target protein, together with an antibody that will record total amounts of either the same protein, or alternately, a housekeeping protein. Both signals can be

1359-6446/04/$ – see front matter PII: S1359-6446(04)03324-0

conference report

visualized simultaneously with secondary antibodies labeled with spectrally-distinct near-infrared dyes. The plates and the cells are near transparent to the infrared radiation, so this greatly increases signal-to-noise ratios. Nevertheless, Li-Cor itself, which also markets the imaging system for these in-cell assays, places a warning in its brochures that potential users should still employ traditional westerns blots to check antibody specificity.

DiscoveRx (Fremont, California, USA) claims to have developed proprietary anti-phosphopeptide antibodies with high specificity, in order to screen kinase activation using their enzyme fragment complementation technique, which was described by Richard Eglen. This is a fluorescence based β-galactosidase reporter assay, with a twist. A 4 kDa fragment is removed from the β-galactosidase, inactivating it. The fragment can be chemically coupled to a kinase peptide substrate. The non-phosphorylated conjugate can complement the inactive β-galactosidase, but binding by anti-phosphopeptide antibodies sterically impedes this complementation. Thus, as the target peptide becomes increasingly phosphorylated by a cell lysate, the β-galactosidase signal is progressively reduced.

Off-target effects

A crucial issue is to minimize off-target effects of candidate drugs. Bert Klebl (Axxima Pharmaceuticals, Germany) demonstrated how immobilized kinase inhibitors were being used by his company as affinity chromatography tools. Most notably, this had uncovered several hitherto unsuspected off-target hits from the supposedly selective p38 inhibitor, SB 203580. Axxima exploits this technique for its compound optimization program. Paul Steed (Amphora Corporation, Research Triangle Park, North Carolina, USA) proposed that specificity could be greatly improved by his company's two-dimensional approach to drug development: the simultaneous screening of a chemical library against an array of potential targets. Some recent developments in microfluidics and robotics has enabled Amphora to make rapid progress in developing new inhibitors of Akt and the p38 MAP kinase cascades that are extremely specific and highly potent (single-digit nanomolar affinity). Another area of research in which technological advances are accelerating is mass spectroscopy (MS). However, Julian Whitelegge (UCLA) pointed out that new technology is outstripping computational capacity; the constriction in the pipeline is now the rate at which the data can be analyzed. Whitelegge also noted that there was no single 'magic-bullet' MS technique. A complementary suite of MS techniques is generally required.

Upstream of kinase activation itself is the binding of an extracellular ligand to a transmembrane receptor, which frequently drives receptor–receptor association. Indeed, these are initial crucial steps for activation of many signaling pathways. Image cross correlation spectroscopy (presented by Anja Nohe, University of Maine in Orono, USA) is a relatively new technique that can visualize these intermolecular interactions on the surface of live cells. This methodology combines high-magnification confocal signals from two non-identical fluorophores used to label a pair of proteins. The latter might, for example, comprise a receptor and a marker for caveolae (microdomains in the plasma membrane that act as portals for receptor endocytosis). Each of the fluorophores distributes in a punctate pattern across the cell surface, but the analytical software is trained to acknowledge only when the two different signals coincide, that is, when the two proteins co-localize. Moreover, the amplitude of this cross-correlation signal is directly proportional to the concentration of double-labeled molecules, enabling quantification of cluster densities and sizes. Nohe showed us how this technique had provided new information on the functionally important interaction between caveolin-1β and receptors for bone morphogenetic protein.

Receptor endocytosis

Two talks highlighted the importance of receptor endocytosis in signal transduction and therapeutics. John O'Bryan (National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA) demonstrated that the intersectin scaffolding protein regulates trafficking and signaling of epidermal growth factor receptor/tyrosine kinase (EGFR). David DeGraff and colleagues (AstraZeneca) have been pursuing recent revelations in the scientific literature that certain mutations in EGFR render it especially sensitive to Gefitinib, a drug which successfully treats only a subset of non-small-cell lung cancers. DeGraff proposed that these mutations inhibit receptor endocytosis, making EGFR more accessible to the drug. Kyle Sloop (Eli Lilley) also focused in his talk on receptors. He described how his company had achieved some success in using antisense phosphorothioate oligonucleotides against the glucagon receptor to improve glucose tolerance in diabetic rodents. However, it is notable that Isis Pharmaceuticals (Carlsbad, California, USA) is to-date the only company to have succeeded in getting an antisense drug licensed, namely, Vitravene for the treatment of AIDS-associated retinitis, although Isis does has other antisense products in clinical trials. Some other companies believe the future is brighter for a competing gene-silencing technology, namely, RNA interference. It would have been useful to have had a presentation from a representative of this field.

A novel signaling paradigm involving protein kinases was also unveiled. Wei Duan (National University of Singapore) presented data in support of his hypothesis that cell signaling by PRK1/PKN, a member of the protein kinase C superfamily, was facilitated by a specialized subpool that was tightly integrated into the plasma membrane. This integration is apparently essential for PRK1 activation by the Rho GTPase. Duan proposed that this information could push the goal of pharmacological intervention in this signaling process in the direction of lipophilic molecules.

Haunting issues

The naysayers have argued that high intracellular ATP concentrations would preclude the use of competitive inhibitors of ATP binding by protein kinases, or that specific inhibitors could never be developed because of conservation in the ATP binding pocket, or that kinase inhibition would have numerous detrimental effects upon normal cells. Some of these issues still haunt us. However, the different presentations at this conference more than justified that protein kinases can be effective therapeutic targets.

feature

Yet, perhaps it was too narrow a selection for a conference topic. A wider and more diverse audience would have been attracted by the addition of, for example, a dedicated session on ion channels (a disclaimer: this is a topic relevant to the author's own research). Ion channels are, after all, the most rapidly acting of signaling entities, and with over 400 known genes, they are running a close second to the protein kinases. New opportunities for automated high-throughput screening of ion channels are also emerging, and both the advantages and disadvantages of these techniques deserve dissemination to a wider audience. The advent of diagnosis and targeted chemotherapy by nanotechnology was another missing topic that could have been useful to include in this conference. Hopefully, the organisers will be willing to revisit this important subject of signal transduction as targets of drug development in a more expansive manner. Although the conference was definitely rewarding, it was ultimately a frustratingly short day-and-a-half.

Stephen B. Shears
Inositol Signaling Section,
Laboratory of Signal Transduction,
National Institute of Environmental Health Sciences,
Research Triangle Park,
NC 27709, USA
e-mail: shears@niehs.nih.gov

feature

Osteolytic bone diseases: physiological analogues of bone resorption effectors as alternative therapeutic tools

Dominique Heymann, Yannick Fortun, Francoise Rédini and **Marc Padrines,** EA3822; INSERM ERI 7, Nantes, France

The treatment of osteolytic diseases has relied predominantly on the use of bisphosphonates. Although the efficacy of bisphosphonate treatment in inhibiting bone resorption has been clearly demonstrated, several secondary and undesirable side-effects have been also reported. In this context, alternative treatments to bisphosphonate therapy, based on the knowledge of osteoclast biology, have been proposed. Bone resorption is tightly regulated by numerous factors including hormones, cytokines and integrins. Among these cytokines, the OPG–RANK–RANKL molecular triad, three members of the TNF cytokine/receptor family, is key in regulating osteoclast activities. Similarly, αvβ3 integrin allows the binding of osteoclasts on calcified tissues. Thus, cytokines, their signaling and integrins represent new targets to treat osteolytic diseases and here we describe new alternate strategies for the treatment of osteolysis.

Bone is a specialized connective tissue formed by mineralization of an organic matrix that confers its elastic and strength properties. Bone remodelling allows the skeleton to adapt to mechanical constraints and maintains phosphocalcic homeostasis through coordinated phases of formation and resorption. Thus, bone remodelling involves the synthesis of organic matrix by osteoblasts and bone resorption by osteoclasts. The equilibrium between osteoblastic and osteoclastic activities is tightly regulated by numerous extracellular molecules (cytokines, hormones and vitamins), which interact with membranous and/or nuclear receptors, thus inducing intracellular signalling. Among these molecules, the Tumor Necrosis Factor (TNF) and TNF-receptor family is particularly implicated [1]. Any disturbance in the equilibrium of osteoblast–osteoclast activities leads to the development of bone pathologies. Thus, increased osteoclast activity is observed in many osteopathic disorders, including postmenopausal osteoporosis, Paget's disease, primary bone tumors, lytic bone metastases, multiple myeloma, rheumatoid arthritis or aseptic prosthesis loosening, leading to increased bone resorption, hypercalcemia and a loss of bone mass [2,3]. In some rare cases, tumour development leads to osteoformation without osteolysis, as in some forms of osteosarcoma or osteoblastic metastasis predominating in patients with prostatic adenocarcinoma. In most cases, the skeletal manifestation of malignancy is focal osteolysis. This imbalance in favour of bone resorption can result from the acquisition of new cellular properties by bone cells: increase in the proteolytic activity, alteration in local or humoral factors expression. Tumour products can either stimulate

1359-6446/04/$ – see front matter PII: S1359-6446(04)03265-9