Therapeutic challenges of kinase and phosphatase inhibition and use in anti-diabetic strategy

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Abstract
The development of kinase and phosphatase inhibitors as novel therapeutic agents has been stimulated by the discovery that most biological processes are controlled by the reversible phosphorylation of proteins. Most of the early results in this area were generated in oncology, at the same time as the human genome, with its 500+ kinases and 100+ phosphatases was deciphered. Because of this, we know a great deal about which processes signalling inhibitors interfere with, but little about the overall consequences. In this study, kinases will be briefly reviewed, followed by some of the early problems in developing kinase inhibitors, as biochemical reagents, and clinically active pharmaceuticals in oncology. The discussion will then switch to the potential role of kinases and phosphatases in controlling the disease process in Type II diabetes. Phosphatase inhibitors should augment insulin receptor tyrosine kinase signalling. Glycogen synthesis and glycogenolysis are phosphorylation dependent, and amenable to kinase inhibition, as are some nuclear hormone receptors, and these will be briefly discussed.

Introduction: what do kinases and phosphatases do?
There are several reasons why phosphorylation is a major method of control in biological systems, and a very attractive target for therapeutic intervention [1]. Phosphorylation is one of the most ubiquitous of biological processes, widely used in cellular control. The formation of simple monophosphate esters is close to energetically neutral, but the kinetics of both the forward and the backward reaction are exceptionally slow in the absence of catalysts. These reactions are very easy for enzymes to control, and are totally dependent on the activity of the enzymes [2]. Phosphorylation drastically alters a protein’s surface by adding negative charge, which can force major structural reorganizations, controlling the activation of enzymes or association with other proteins, for example by gaining binding sites for specific phosphoprotein recognition domains. Frequently activation and aggregation are combined, leading to very localized enzyme activity.

Large numbers of kinases are present just inside the cell membrane, where they turn external signals, such as hormones binding to cell surface receptors, into changes in phosphorylation status in the cell. As many kinases are activated by regulatory phosphorylation, this allows for cascades, whereby kinases activate one another sequentially. These cascades allow a single activating kinase to activate many downstream kinases, all phosphorylating the final target substrates, resulting in huge signal amplification [3]. As cells always contain active phosphatases, low-level phosphorylation signals tend to be squelched by the phosphatases, until they reach a high enough threshold to overcome the endogenous quenching. Thus, local phosphatase activity determines the intensity threshold for cellular response to phosphorylation, and activation of phosphatases is the usual method of terminating such signalling.

Why kinases attracted attention in oncology
Cancer researchers became interested in kinases because transformed cells have much higher levels of phosphoproteins than normal cells. Then it was discovered that the phorbol ester tumour promoters were activators of PKCs (protein kinase Cs), and two viral, oncogenic, tyrosine kinases, v-SRC and V-ErbB were identified as having originated in animals. RB a tumour suppressor, which is specifically inactivated by many viral oncogenes, was found to be inactivated normally during the cell cycle by phosphorylation. These facts pointed to kinase inhibitors as potential anticancer drugs, with the idea that tumours have overactive kinase cascades, allowing proliferation without external stimulus [4]. As the ‘war on cancer’ drove the initial explosion in molecular biology, most early discoveries were in proliferative pathways rather than for such things as glucose signalling.

Problems of kinase inhibition
There are probably more cellular binding sites for ATP than any other molecule, and most kinase inhibitors are competitive ligands for the kinase ATP binding site. This makes selectivity, and the proof that one has selectivity, very challenging problems. Two of the earliest kinase targets were the PKCs and the cellular equivalent of v-erbB, the EGFR
(epidermal growth factor receptor). Staurosporin, a low nanomolar PKC inhibitor with good cellular antiproliferative activity was quickly found, as were a series of micromolar inhibitors for EGFR. Within a few years second generation EGFR inhibitors had nanomolar enzyme and cellular inhibition. This initial success merely allowed many of the problems inherent in the approach to reveal themselves. PKC ended up with 11 different isoforms, often operating in opposition to one another, and as signalling pathways were elucidated, the roles of PKCs remained elusive. EGFR biology was kinder, with only two highly related isoforms, all at the beginning of a major proliferative pathway. However, selectivity proved to be as much of a problem as expected, and the early 1990s’ definition of a selective kinase inhibitor was one that had been tested in only one assay. A bigger problem was the poor in vivo activity in tumour models. Many of the early inhibitors had very poor physicochemical and pharmacokinetic properties, but pharmacodynamic assays revealed that even when the target was considerably inhibited in vivo, slowing of tumour growth was minimal. However, in the mid-1990s two kinase inhibitors with good in vivo activity were developed. One of them Iressa® is a 1 nM EGFR inhibitor, with excellent pharmacokinetic and pharmacodynamic properties, which has been shown to work very well in animal models, and in about 10% of patients with lung tumours. Recently, responders have been found to have activating mutations in the EGFR kinase domain, allowing for screening for probable clinical benefit [5]. The other successful inhibitor, Gleevec, offers a considerable contrast. CML (chronic myelogenous leukaemia) has a diagnostic chromosome translocation (Philadelphia chromosome) that fuses most of ABL (an antiproliferative tyrosine kinase) to BCR (breakpoint cluster region). The fusion protein BCR–ABL is a very active, proliferative, tyrosine kinase, and once one more mutation occurs patients develop the symptoms of CML. Gleevec is a 50 nM inhibitor of BCR–ABL with excellent pharmacokinetic and pharmacodynamic properties, which has been shown to work very well in animal models, and in about 10% of patients with lung tumours. Recently, responders have been found to have activating mutations in the EGFR kinase domain, allowing for screening for probable clinical benefit [5]. The other successful inhibitor, Gleevec, offers a considerable contrast. CML (chronic myelogenous leukaemia) has a diagnostic chromosome translocation (Philadelphia chromosome) that fuses most of ABL (an antiproliferative tyrosine kinase) to BCR (breakpoint cluster region). The fusion protein BCR–ABL is a very active, proliferative, tyrosine kinase, and once one more mutation occurs patients develop the symptoms of CML. Gleevec is a 50 nM inhibitor of BCR–ABL with excellent pharmacokinetics, but it has dramatic effects in CML. Nearly 90% of patients not yet in blast crisis respond to Gleevec, with dramatic drops in leukaemic cell counts, and a significant 2 year survival benefit [6].

Before extrapolating these results to other therapeutic areas one has to consider the unique difficulties involved in treating cancers. Cancer is a disease of genetic instability; consequently, in transformed cells, further mutations will occur frequently and stochastically, inducing many subclones with different driving mutations in the tumour. Thus, an agent that inhibits one pathway, even the original transforming pathway, may well affect only part of the tumour, leading to a limited, but meaningless response in the patient. Successful inhibition puts evolutionary pressure on tumour cells to mutate around the blockade, and thus gain a great selective advantage. Comparing this situation with a cell where insulin sensitivity has been improved by inhibition of PTP-1B (protein tyrosine phosphatase) the inhibited cell is at no selective disadvantage relative to a cell where a mutation reverses the inhibitory effect. The cell is not transformed, and is not mutating, so the biological basis for therapy is stable. Furthermore, we understand the biology of this system, whereas with tumours, we are usually guessing at which targets must be inhibited. Despite their oncology-derived reputation, kinase inhibitors are not inevitably cytotoxic or antiproliferative, but for a cancer patient to benefit from a treatment, the inhibitor needs these properties. Kinase or phosphatase inhibitors developed in cancer programmes have either cytotoxic or antiproliferative effects, not because this is inherent to the entire genus, but because this is what useful anticancer agents do, and the agents have been targeted accordingly. Kinases and phosphatases seem to turn up over and over again in multiple pathways, making them appear frighteningly pleiotropic. However, in patients, the toxicity seen is usually mechanistically predictable, and manageable.

### What is diabetes, and why does insulin have a central role in it?

Classical diabetes, (insulin-dependent) ‘diabetes mellitus’ or Type I diabetes, was usually seen in the young. The disease is inexorable, fatal in a few months, and is caused by an autoimmune destruction of the pancreatic islet β-cells. In the true foundation of all modern ideas of drug therapy, Banting and Best demonstrated that the loss of a pancreatic hormone insulin was responsible for the symptoms and pathology of the disease, and that the disease could be stopped in its tracks by insulin treatment. Some of the first people treated by insulin, who had life expectancies of a few months at the most, lived another 60 years. The last 50 years have seen the rise of a second form of diabetes, Type II or non-insulin-dependent diabetes mellitus. This occurred in elderly, usually overweight, people, who respond poorly to insulin. However, in the last 20 years Type II diabetes has been seen even in teenagers, mainly among the severely obese.

Glucose is absorbed through the gastrointestinal tract, and can be synthesized in the liver. It can be stored as glycogen in liver and muscle, and some other organs, and its main use in the body is as a fuel. Plasma glucose levels much below 100 mg/dl are classified as hypoglycaemia and above 180 mg/dl are defined as hyperglycaemia. Relatively mild hyperglycaemia does not appear to have much in the way of short-term consequences, but even without other co-morbidities, chronic hyperglycaemia leads to large increases in cardiovascular disease, nephropathy and neuropathies. Very high blood glucose levels on the other hand have acute consequences as the osmotic balance of the entire body is upset by the high osmolality of plasma containing greatly excessive glucose. Such high blood glucose levels normally only occur in the absence of insulin, and may be fatal.

When a meal or glucose release from the liver raises plasma glucose levels, the pancreas releases insulin, a hormone, with a very short half-life. The most metabolically active organ is the brain, accounting for one-sixth of all energy consumption in humans. Neuronal cells have lost all ability to metabolize fatty acids for energy and cannot store glucose as glycogen. Thus the brain must have a continuous supply of glucose to function. The liver produces glucose from glycogen or...
amino acids, and releases them into the circulation at times of low blood glucose to ensure a steady supply to the brain. But, if blood glucose levels fall too fast, the liver cannot compensate, the brain quickly runs out of energy, and unconsciousness or death rapidly ensue. Thus, severe hypoglycaemia has immediate, drastic consequences, so the system is primarily set up to avoid acute shortages, and only secondarily to prevent high glucose levels. This puts major constraints on diabetes treatments, and iatrogenic hypoglycaemic episodes are severe problems in diabetes management.

In Type II diabetes there is a slow rise in fasting glucose levels over several years. However, insulin levels rise more quickly than glucose levels, so the initial lesion is a failure of organs to take up glucose in response to insulin properly. As the disease progresses, the pancreas starts to fail, and at some point insulin output plunges. Now the patient presents with the classical symptoms of diabetes, but insulin rarely restores euglycaemia, and insulin sensitizers, such as glitazones, are required to make tissues respond properly to therapeutic insulin. Type II diabetes is usually a combination of overnutrition, combined with a genetic defect that allows the pancreas to fail.

The insulin receptor and insulin downstream signalling

Cells which utilize glucose on demand have insulin receptors on their surfaces [7]. The dimeric insulin receptor has an extracellular, high affinity, insulin binding site connected through the membrane to a cytosolic kinase, which is normally inactive, unable to bind ATP, but insulin binding forces the kinase domains to change conformations, allowing ATP to bind in the active site. The two kinases cross-phosphorylate their kinase domains, stabilizing the active conformation. They then recruit proteins by SH2 (Src homology 2) domain interactions, especially the large adapter protein, IRS-1, that has 16 phosphorylatable tyrosine residues. One of the major proteins recruited to IRS-1 by its SH2 domain is PI3K (phosphoinositide 3-kinase). PI3K converts phosphatidylinositol-4,5-bisphosphate into the corresponding phosphatidylinositol 3,4,5-trisphosphate, which recruits phosphoinositide-dependent kinase 1 and AKT (PKB) to the cell membrane via phosphatidylinositol 3,4,5-trisphosphate-recognizing PH domains. Activated AKT phosphorylates the insulin-sensitive glucose transporter GLUT4, allowing it to be transported to the cell surface, where it can actively scavenge glucose out of plasma and pull it into the cell. Although this mechanism connects the insulin receptor to GLUT4, it is not the whole story, as many other stimuli activate AKT, but none lead to GLUT4 mobilization. At the very least, insulin receptor must activate a second permissive pathway, and it is probable that AKT activation is more indirect.

Inhibition of any of these kinases would exacerbate insulin resistance but phosphatase inhibitors should keep this pathway stimulated by preventing the normal down-regulatory dephosphorylations. This ‘insulin-sensitizing’ system would only kick in after excess glucose causes insulin secretion, and although the signal is prolonged, it will decay once insulin is no longer available, reducing the chances of inducing hypoglycaemia. The primary phosphatase targeted, PTP-1B, dephosphorylates the insulin receptor itself [8]. Very elegant genetic experiments have implicated PTP-1B as the main insulin receptor phosphatase in vivo. PTP-1B also downregulates leptin signalling in the hypothalamic paraventricular nucleus, and PTP-1B knockout mice are very resistant to diabetes and obesity.

Perhaps unsurprisingly, downregulation of the IR/IRS-1 system is one of two obvious abnormalities seen in muscle of insulin resistant individuals. However, this lesion involves serine/threonine kinases, which phosphorylate IRS-1 on Ser307, reducing the efficiency of IRS-1 coupling to, and tyrosine phosphorylation by, the insulin receptor. PKCα, PKCβII, PKCθ, IKKβ, ROKα, p70S6k, p38, extracellular-signal-regulated kinase and c-Jun N-terminal kinase are all IRS-1/S/T-kinases which have been implicated in insulin resistance when activated by various pathways, and are targets for kinase-inhibitory insulin sensitizers [9].

The other major abnormality is the failure of muscle to produce sufficient glycogen. This reaction requires glucokinase, and GS (glucogen synthase). Conversion back into glucose requires phosphorylase and glucose-6-phosphatase. GS and phosphorylase are regulated oppositely by kinases, GS being inactivated by S/T phosphorylation (PKA, GS kinase 3β) and phosphorylase being activated by phosphorylase kinase [1,10]. When these kinases are active, glycogenesis is slowed, and glycolysis is accelerated. Insulin activates a phosphatase, localized on glycogen particles, which dephosphorylates both GS and phosphorylase, stimulating the flow of glucose into glycogen. Thus inhibition of PKA, phosphorylase kinase and GSK3β are potential anti-diabetic strategies.

References

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