

# KINASES AND DRUG DISCOVERY

## *protein kinases as a validated drug target*

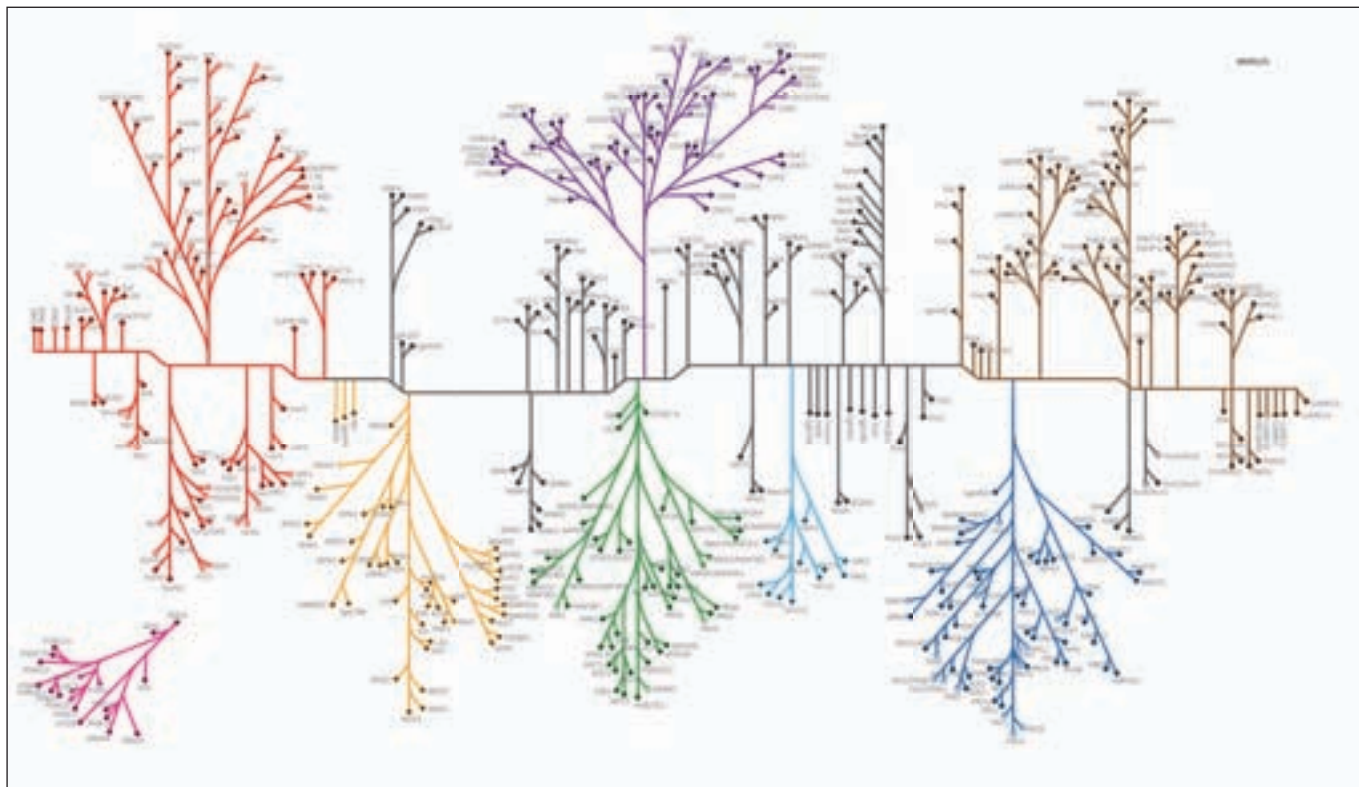
The role of protein and lipid kinases in cellular physiology and normal and abnormal growth has been well appreciated and has been the focus of intensive research in both academic arenas and pharmaceutical industries. The payoff for the pharmaceutical industries has been substantial.

According to the latest figures, there are more than 30 small molecule drugs amassing more than 30 billion dollars annually, with more than 150 in preclinical trials waiting FDA approval<sup>1</sup>. Furthermore, several monoclonal antibodies targeting receptor tyrosine kinases have made tremendous penetration into the cancer immunotherapy market such as Herceptin, Bevacizumab and Cetuximab, etc. The latest figure on the commercial impact of total sales of kinase-related drugs is estimated at \$240 billion during 2011-15 (supporting continuation of targeting kinases for the development of new drugs not only in cancer but also in many other indications<sup>1</sup>).

It is noteworthy that the majority of clinical trials target only 43 protein kinases and about 50% of these inhibitors target kinases which already have approved drugs. The full potential of the human kinome as a source of new drugs has not been fully exploited since more than 100 kinases have unknown function and 50% of all kinases are largely uncharacterised. Thus the search continues for identifying the functions of those kinases, developing modulators to control their activi-

ties and improve human health. Furthermore, most of the approved kinase targeting drugs are active against more than one type of cancer, and only a few have been approved for non-oncological indications. The latter include the first approval of a kinase inhibitor Fasudil, ROCK1/2 inhibitor, in 1995 for cerebral vasospasm and pulmonary arterial hypertension; and the recent approval of JAK3 inhibitor tofacitinib for rheumatoid arthritis; mTOR inhibitor sirolimus for organ rejection and PDGFR, VEGFR and FGFR inhibitor nintedanib for idiopathic pulmonary fibrosis<sup>2,3</sup>. Although many of the cancer-driving kinases are of protein tyrosine kinases class which includes receptor (EGFR, HER2, c-Kit, MET, PDGFR, VEGFR) and non-receptor kinases (BCR-Abl, Src, JAK), other kinases that do not belong to this group of kinases (serine/threonine kinases, STK) have been directly recognised as cancer drivers as well, such as BRAF, MEK, ERK, CDKs and lipid kinases such as class I PI3 kinases. The tremendous appeal for kinases as drug targets for almost all large and small pharmaceutical companies stems from the fact that these enzymes are involved in a wide range of

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Human Protein Kinase Families (Kinome) showing the various classes of kinases

pathological conditions such as cancer, inflammatory diseases, diabetes, infectious diseases, cardiovascular disorders, cell growth and the substantial revenue they generate<sup>2</sup>.

**Protein kinases as a one of the largest mammalian gene families**

The human kinome contains 518 eukaryotic protein kinases (ePK) genes that are sub-divided into seven families of typical and seven families of atypical protein kinases<sup>4,5</sup> comprising 1.7-2.5% of genes in eukaryotic genomes<sup>6</sup>. Most of ePK belong to a single superfamily, containing a conserved ePK catalytic domain that consists mainly of two subdomains (loops) with an ATP-binding pocket situated in between. The majority (67%) of the kinases belong to the Serine/threonine protein kinases (STK), and 17% belongs to tyrosine protein kinases (TPK and the remainder is called atypical protein kinases (aPKs) which lack sequence similarity to the ePK catalytic domain but are known to have catalytic functional activity)<sup>6</sup>. Although only a minor number of substrates are phosphorylated by TPKs, the importance of tyrosine phosphorylation is demonstrated by the many gain of function (GOF) and/or loss of function (LOF) mutations that are found in TPKs. The TPKs family of kinases comprises two main classes (receptor TPKs (58

members) and non-receptor TPKs (32 members). In contrast, the STPKs are a more heterogeneous class of enzymes and are divided into seven main families of typical ePKs, including the TKL (the Tyrosine Kinase Like group closely-related to the TPKs), the CMGC, AGC, CAMK, STE20, MAP3Ks, MAP4Ks and the CK1. Most of the atypical ePKs are STPKs based on substrate phosphorylated residues and their mode of regulation<sup>2,4,5</sup>. Another class of kinases called pseudokinases represent about 10% of the kinome, they have either weak activity or are presumed to be inactive. These pseudo-kinases are evenly distributed over the human kinome and indirectly or directly play a role in cellular regulatory functions as well as tumorigenesis such as ErbB3, a member of the EGFR family of receptor kinases. Although pseudokinases lack at least one of three motifs in the catalytic domain that are essential for catalysis, they can bind ATP and appear to have important regulatory functions<sup>7,8</sup>. Another important set of kinases that play essential roles in the eukaryotic signalling and which share the protein kinase-like (PKL) fold with the ePKs include the phosphatidylinositol (PI) kinases and related protein kinases<sup>9</sup>. The PI3Ks, which phosphorylate PI together with the atypical STPK mTOR, have been implicated in cancer and immunological disorders

and represent a rich source for drug development<sup>2,9</sup>. This was successfully rewarded by the recent FDA approval of the first PI3k delta targeting drug for follicular lymphoma and chronic lymphocytic leukemia (CLL).

The physiological activation of kinases occurs in many different ways and their mechanisms of activation have been summarised recently<sup>4</sup>. Kinases are organised in cascades, which are typically initiated by various receptors including receptor and non-receptor TPKs or STPKs, which further pass their signals through various downstream effectors such as the PI3K/mTOR, the RAS-RAF-MAPK, etc). Besides phosphorylating the hydroxyl groups of their substrate proteins and lipids, protein kinases also utilise non-catalytic functions for scaffolding, relocation, allosteric effects, subcellular targeting and DNA binding, as well as protein-protein interactions<sup>10</sup>. Abnormal hyperactivity can also occur via activating mutations, chromosomal rearrangements and/or gene amplification, or GOFs of the protein. It is noteworthy that one-third of all protein targets under investigation in the pharmaceutical industry are protein or PI3K kinases<sup>2,3,11</sup>.

### Methods used to study kinase effect and develop inhibitors

The most important step in drug discovery is the identification and validation of the target as the true direct driver, or in association with other proteins may cause an altered pathological phenotype. Many failures during the drug discovery process are ascribed mainly to a wrong target, or that the animal model does not mimic the exact phenotype in humans. Other factors such as physicochemical properties, poor absorption, distribution, metabolism and excretion characteristics and *in vivo* toxicological outcomes also impact the attrition rate of drug candidates<sup>12</sup>. In order to validate kinases as true targets, it is necessary to link the specific molecular target (kinase) to the *in vivo* model or diseases condition. Target validation is usually done through several strategies such as knock-out (K/O) of the enzyme either genetically or using RNA interference, or by pharmacological means if possible. Following target validation, a screening phase ensues using a library of diverse complexity and high throughput platforms to generate hit compounds that are capable of inhibiting the kinase under investigation. Screening protocols follow either activity-based assays<sup>13-16</sup>, ligand binding assays and, more recently, cell-based assays<sup>8</sup>. Activity-based assays are used to monitor the phosphorylation of substrates (proteins, peptides or lipids) and identify those compounds with

enough inhibitory activity that warrant pursuing for confirmation as true hits and transitioned to lead optimisation where the IC<sub>50</sub> of the resultant hit compounds are refined to achieve the highest inhibition possible at the lowest concentration possible. Such assays include radioactivity-based assays<sup>17</sup>, bioluminescent-based assays<sup>13-15</sup>, or fluorescent-based assays<sup>16</sup>. Other approaches to compound screening such as binding assays can also be used to identify compounds with highest binding affinity. Usually this step is followed by validating the hits as true hits (eliminating positive and negative false hits) using orthogonal assay<sup>13</sup> before transitioning to the next phase which tests the specificity of the hits towards the kinase target for minimal or no effect on other kinases in the kinome. This step, known as profiling of the compounds towards other probable or related proteins, usually other kinases, is critical. The purpose of profiling is to improve the safety profile of the compound that will be moving forward into the next phases of drug development. In some cases, it is desirable to profile the compounds against several other classes of enzymes or proteins to ensure the cleanliness of the compound from off-target effect. Profiling can be carried out against a small set of closely-related kinases (eg, tyrosine kinases class for a tyrosine kinase target), large set of related kinases (receptor and non-receptor tyrosine kinases), or an expanded set of the kinome depending on specificity and cost factors<sup>18</sup>. It is desirable to have one platform that can be used for assay development, screening and profiling so to minimise the cost and ensure easy interpretation of the data. This was demonstrated recently by using the ADP Glo platform which is able to screen and profile not only protein kinases, but also other classes of kinases such as lipid kinases and Glucokinases<sup>13-15</sup>. This enables the simultaneous profiling of diverse sets of kinases using only one platform.

Since most causes of tumour relapse is due to mutation of the kinases, translocation of the enzyme or the emergence of an alternative route for tumour signalling, a comparative analysis of mutations has to be considered. Thus, reliance totally on biochemical assays of quantifying kinase activity may not be advisable since they might not give an accurate measure of functional implications *in vivo*. Comparing mutational strengths in cultured cell-based assays may provide the cellular context, but does not provide the mixture of endogenous growth factors that mimic normal cellular environments and thus do not monitor the development in time and space. This makes it difficult to predict the

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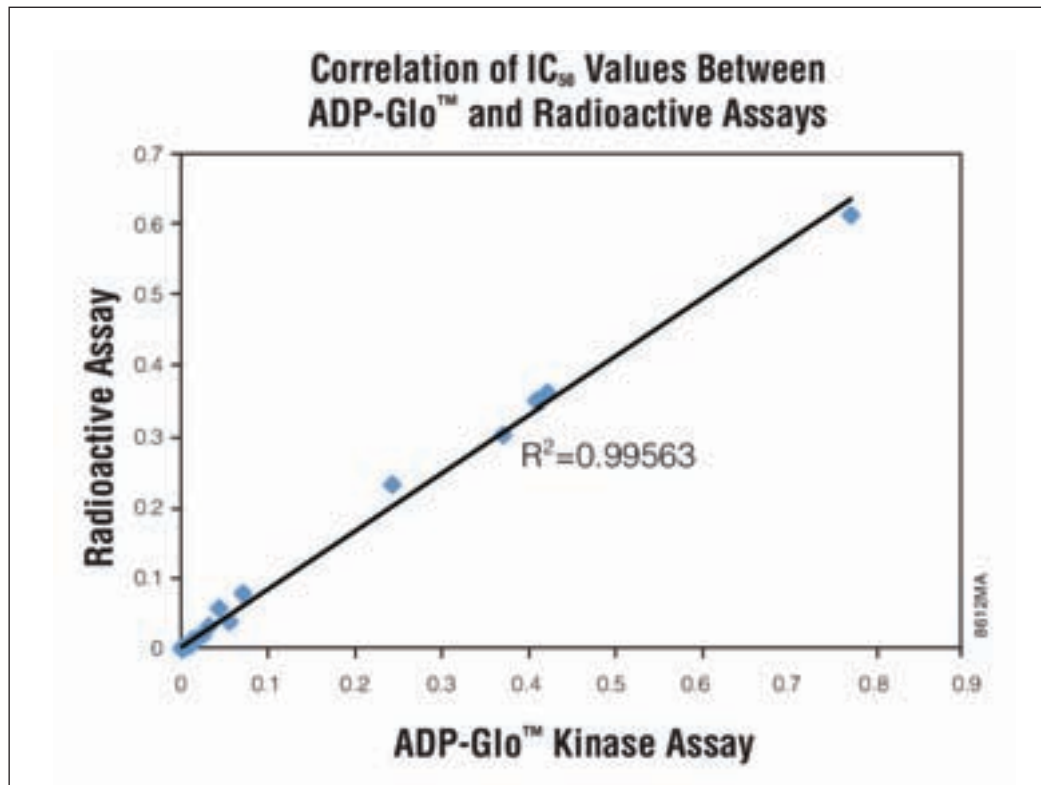
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Comparison of IC<sub>50</sub> generated with bioluminescent kinase assay and radioactivity-based assay, identical data are shown

effect of specific mutations on the phenotype of the developing organism. Novel approaches has been tried to overcome these limitations, such as the use of mRNA microinjection into zebra fish to assess mutation strength in a developing organism with intact organ structure and normal physiological levels of growth factors<sup>19</sup>. Others used a modified cell-based screening and demonstrated a unique capacity to identify novel kinase inhibitors that target the relevant conformation of a protein in its endogenous environment<sup>8</sup>.

**Current and future kinase targeted drugs in the clinic**

Drugs that are approved for kinases comprise small molecule class and protein therapeutics that target receptor tyrosine kinases such as EGFR, HER2 and VEGFR. Small molecule drugs offer several advantages over recombinant protein therapeutics. These include oral availability, greater penetration into tumour microenvironment for solid tumours, ability to cross blood-brain barrier for CNS-related maladies, ability to reach multiple points of intervention (intracellular and extracellular targets), relative ease of formulation and controlling dosages based on pharmacokinetic analysis. Other advantages include cost, storage

and ease of delivery. In contrast, protein therapies target only extracellular and membrane bound targets, are costly, require infusion or injections and are usually refrigerated. However, they provide exquisite target selectivity and relatively low side-effects. Because of their clinically-proven efficacy, protein therapeutics will continue to provide successful drugs and with improved manufacturing cost may become competitive to small molecule drugs<sup>20</sup>.

It is worth noting that all of the approved and clinically-advanced kinase inhibitors with a few exceptions, such as rapalogs and trametinib, are Type I inhibitors directed towards the ATP binding site. Some, such as dasatinib, target the ATP binding site (Type I), while imatinib (Type II inhibitor) targets the ATP binding site and adjacent hydrophobic pocket conferring better selectivity<sup>21,22</sup>. Due to the appearance of multiple mutations during the course of the disease such as CML, several generations of the drug have to be developed (nilotinib, dasatinib, bosutinib and ponatinib) to address the newly-generated mutations which cause resistance to the first-generation drug imatinib. Similarly, sorafenib, sunitinib, everolimus, temsirolimus, axitinib or pazopanib are indicated for various stages of renal cell can-



cer. For Non-Small Cell Lung Carcinomas (NSCLC), ceritinib, crizotinib and alectinib are approved for the treatment of NSCLC with ALK translocations, while gefitinib, erlotinib and afatinib are indicated for NSCLC with activated EGFR. The BRaf mutation (V600E) in metastatic melanomas responds to vemurafenib or dabrafenib in combination with trametinib to overcome MEK, while the acquired PLX8394 resistance occurs via EGFR-mediated RAS-mTOR signalling and is prevented by upfront combination therapy with PLX8394 and either an EGFR or mTOR inhibitor. Thus, a biological rationale and potential multiple therapy strategy may aid the development of PLX8394 in lung cancer patients who are resistant to PLX8934<sup>23</sup>. Because of the promiscuity of inhibitors targeting the conserved ATP binding site, there is an increasing interest in identifying inhibitors that do not compete with ATP (Type I and Type II). Kinase inhibitors with outstanding selectivity are likely to become important not only for minimising side-

effects but also to better understand the on-and off-target pharmacology of kinase inhibitors<sup>21,22</sup>. The approval of irreversible kinase inhibitors, such as BTK inhibitor ibrutinib for CLL indications, opened the door to new types of kinase inhibitors that are more selective and have longer pharmacokinetics than the ATP targeting Type I and II inhibitors<sup>21</sup>.

While the mutational status of kinases may be associated with various cancer conditions, the identification and validation of the driver kinase(s) in these diseases by genome-wide screening for kinase amplifications, translocations and/or mutations, as well as studying the multiple mechanisms of resistance, is an area of intense research to improve the efficacy of these targeted therapies. It is realised that treatment with a single kinase inhibitor alone may not be sufficient to improve patient survival due to the appearance of novel mutations and/or the appearance of new signalling mechanism that allow tumour escape. That may require a regimen where the inhibitor can be given

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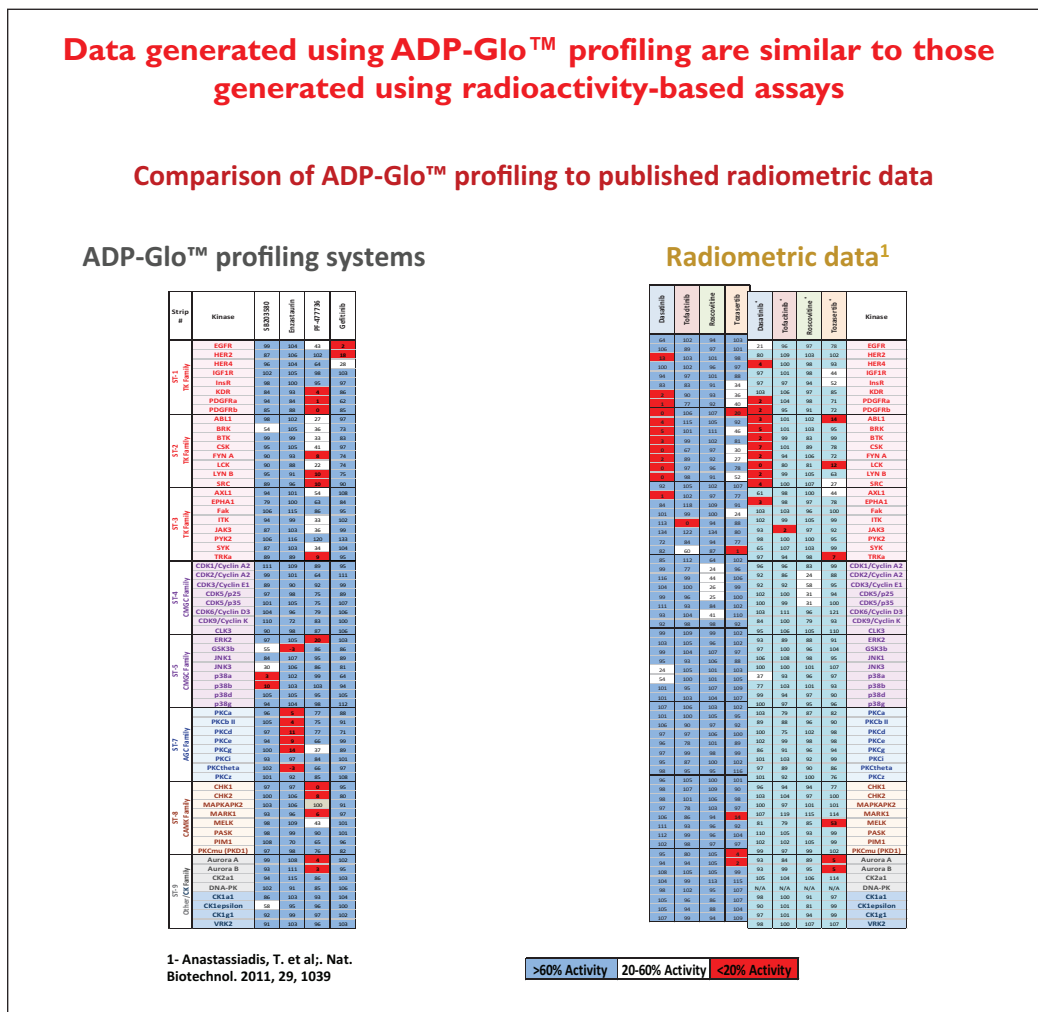
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Kinase profiling using bioluminescent kinase assay and radioactivity-based assay, similar results are shown

in combination with a chemotherapeutic agent, or with another kinase inhibitor that targets a different pathway<sup>24,25</sup>.

Recent studies have reported an association between PD-L1 expression and mutant EGFR mediated signalling which drives increased PD-L1 expression. A blockade of PD1 improved survival of mice in EGFR-driven murine lung tumors. Thus future treatment may include a combination of anti-PD-L1 and EGFR inhibitor as both might be more effective in NSCLC tumors with EGFR mutations and PD-L1 overexpression<sup>26</sup>.

As mentioned earlier, immunotherapy targeting receptor tyrosine kinases have shown great success with the approval of several monoclonal antibody therapeutics for several oncological indications. Most recently, immunotherapy via vaccination, with a growth factor or growth factor-derived peptide which targets its receptor tyrosine kinase, has been explored on targeted vaccination against

bevacizumab binding site on VEGF elicited efficient antitumor activity<sup>27</sup>.

A future approach that has received great appreciation in cancer is the concept of synthetic/sickness lethality (SSL). This concept states that when two genes have a SSL relationship, inhibition of either gene alone does not cause loss of viability/sickness, but simultaneous inhibition of both genes results in reduced cell viability or an impairment of cellular health/fitness<sup>28</sup>.

### Conclusion

Future cancer treatments of various types of tumours and the drugs administered to each patient will experience significant change. Treatment will be tailored to each individual and for a select tumour with a specific mutation at certain stage of the diseases, ie, personalised medicine<sup>29</sup>. As sequencing becomes cheaper and more readily available, this individualised, personalised

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approach will be the normal practice in the clinic. This, in fact, has been realised already for a number of therapeutics targeting the protein products of specific genes that are altered in human cancer. This has been illustrated by the administration of different drugs (imatinib, nilotinib, dasatinib, bosutinib and ponatinib) targeting chronic myeloid leukaemia carrying BCR-ABL fusion depending on the type of mutation present. Another example illustrating the value of gene sequencing is that the type of mutation in the same target (EGFR) have tremendous impact on tumour response. So, mutations such as exon 19 deletion and L858R mutations show dramatic tumour responses and favourable clinical outcomes with gefitinib or erlotinib therapy (24,25). In contrast, the NSCLC tumors carrying T790M mutation which accounts for one half of acquired resistance or KRAS mutation that makes EGFR irrelevant and thus does not respond to cetuximab treatment.

Thus, personalised therapies that target specific genetic alterations and identify newly-generated mutations in the main target and in other signalling pathways proved to be safer and more effective than traditional chemotherapies when used in an appropriate patient population<sup>11</sup>

In summary, efficient and successful medicine depends on proper diagnosis of the disease and selection of the appropriate therapeutic regimen. Thus, drug-induced phenotypic alterations observed under appropriate conditions that reflect the desired outcome can be chosen to select and adjust the proper therapy to reach better outcome. Hence, better understanding of the mechanism of the disease and a better clinical diagnosis of the disease may help in achieving the best therapeutic regimen and most efficient outcome for patient treatment. It is anticipated that by exploring the many members of the kinome and understanding the function of those unknown, the interest in kinases as a drug target and source of great revenue for the pharmaceutical industry will continue. Hopefully, the newly-developed drugs will also be more efficient in slowing the progression of tumours and may even result in patient cure with less toxicity and less cost to patients who are desperately in need for help with their malady. **DDW**

*cell signalling and the role phosphorylation plays in normal and abnormal cell growth. Said has written for more than 80 peer reviewed publications and co-edited a book on kinase screening and profiling. He holds more than 15 issued and several pending patents.*

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