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Selectively Nonselective Kinase Inhibition: Striking the Right Balance

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1. Introduction

Protein kinases have become the second most exploited group of drug targets after G-protein-coupled receptors (GPCRs), accounting for 30% of drug discovery projects at many pharmaceutical companies with dozens of compounds in clinical development.[1] Most early kinase inhibitors exhibited poor selectivity between kinases, and the trend in recent years has been toward ever more selective inhibitors in an attempt to minimize the risk of side effects. The risk with highly selective inhibitors is that their efficacy for treating complex diseases like cancer might be compromised by the redundancies in signaling pathways. The increasing interest in multitarget drug discovery (MTDD[a]) stems from a belief that modulating more than one target can provide superior efficacy and safety profiles compared to single target drugs. Currently, there are two contrasting MTDD philosophies. The first involves combining agents that are selective for a single target to achieve an additive or synergistic effect. The second involves discovering agents that are simultaneously capable of addressing two or more targets. Although this perspective focuses primarily on the latter, the advantages and disadvantages of both approaches will be highlighted.

Very few drugs are truly selective for a single target, and in reality most biologically active small molecules have a degree of promiscuity by their very nature. Many clinically useful drugs are now known to have multiple activities, but most of these multitarget drugs (MTDs) were discovered serendipitously and their mechanisms of action were only established retrospectively. The deliberate and prospective design of ligands that act in a "selectively nonselective" manner on multiple targets of therapeutic interest is an emerging trend in drug discovery.[2,3] Increasing numbers of these so-called designed multiple ligands (DMLs) are being reported in the medicinal chemistry literature.[4] In particular, identifying multikinase inhibitors (MKIs) with specific multiple activity profiles is currently an area of great interest in the pharmaceutical industry, especially for the treatment of cancer. Five years ago there were few examples of DMLs in the medicinal chemistry literature for kinase targets, but the period since has witnessed an explosive growth in interest in this area.

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[a]Abbreviations: MTDD, multitarget drug discovery; MTD, multitarget drug; DML, designed multiple ligand; MKI, multikinase inhibitor; STD, single target drug; FBDD, fragment-based drug discovery; SPR, surface plasmon resonance ; AS−MS, affinity selection−mass spectrometry; PDB, Protein Data Bank; ITC, isothermal titration calorimetry; FDC, fixed dose combination.

Marketed MKI drugs vary with respect to the number of kinases they are known to inhibit, with some inhibiting only a small number of kinases, whereas others appear to be highly promiscuous. These apparent differences in selectivity are to an extent influenced by the amount of selectivity screening that has been performed, with some inhibitors appearing to be more promiscuous simply on the basis of having been profiled more rigorously. As the title of this Perspective delineates, the aim for the medicinal chemist working in the MKI field should be to strike the right balance between the nonselectivity (promiscuity) that may be required for efficacy and the selectivity that is required for safety. At present it is difficult to intentionally design a MKI with activity only at the kinases of interest, but increasingly rational and elegant medicinal chemistry approaches are being applied to solving this difficult problem. This Perspective aims to capture the current state of the art and to explore the future challenges and strategies in this area. The terminology used herein, illustrated using known inhibitors, is summarized in Figure 1.

2. The First Marketed Kinase Inhibitor Drugs

2.1. BCR-ABL Inhibitors for Treating Chronic Myelogenous Leukemia (CML). Since the launch of imatinib (Gleevec) **1** (Figure 2) in 2001 for treating chronic myelogenous leukemia (CML), a number of other kinase inhibitors have been approved for the treatment of cancer. Imatinib was originally developed to selectively inhibit the platelet-derived growth factor receptors (PDGFR) α and β but was later found to inhibit several structurally related tyrosine kinases like c-Kit and BCR-ABL.[5] The multikinase activity of imatinib has led to its exploitation in different cancer types showing significant clinical activity against malignancies dependent on all three of the receptors: BCR-ABL in CML, c-Kit in gastrointestinal stromal tumors (GIST), and PDGFR in dermatofibroma sarcoma protuberans (DFSP).

While imatinib is extremely effective in treating chronic CML, patients with late-stage disease often have a less durable response due to acquired resistance, attributable to mutations in the ABL kinase domain that prevent the binding of imatinib.[6] The newer BCR-ABL1 inhibitors, nilotinib (Tasigna) **2** and dasatinib (Sprycel) **3**, are not only significantly more potent against the wild-type form of the kinase but also inhibit virtually all the known mutants, with the exception of the problematic ABL1 "T315I" mutant which increases the size of the gatekeeper residue from threonine to isoleucine (Figure 2).[7,8] One MKI that inhibits the T315I mutant is the aurora kinase inhibitor

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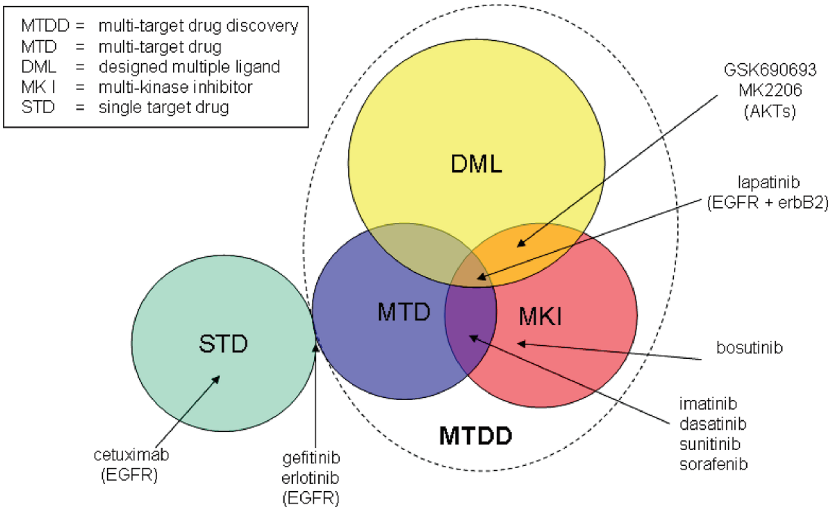


Figure 1. Overview of the terminology used in this article with examples.

VX-680/MK-0457 whose binding mode evades these gatekeeper mutations, leading to its successful use in imatinib-resistant CML patients.[9]

Drug resistant mutant kinases will likely present an ever greater challenge in the future because of natural selection pressures as ever larger patient populations are treated. As well as being active against multiple forms of BCR-ABL, dasatinib **3** and bosutinib **4** also inhibit a second family of kinases, SRC. Dual SRC/ABL inhibitors may have two separate roles in overcoming imatinib resistance, first combating BCR-ABL mutations by hitting multiple mutant forms of the same target and second hitting a second target, SRC, that has also been implicated in BCR-ABL signaling. Bosutinib is now in phase III clinical trials, and phase II studies have shown good activity in patients resistant to imatinib or other tyrosine kinase inhibitors.[10]

2.2. Treatment of Solid Tumors. Where a specific target exists within a solid carcinoma, a highly target-selective drug might be useful.[11] The monoclonal antibody cetuximab (Erbitux) is a single target drug (STD, Figure 1) that acts selectively via the epidermal growth factor receptor tyrosine kinase (EGFR). It is used to treat metastatic colorectal cancer and head and neck cancer. Similarly, the small molecule kinase inhibitors gefitinib (Iressa) **7** and erlotinib (Tarceva) **8**, which are highly selective for EGFR, are used as a monotherapy for patients with non-small-cell lung cancer (NSCLC) who have activating mutations of EGFR. While the efficacy of gefitinib and erlotinib is generally regarded as being driven by inhibition of a single kinase, there is evidence that these inhibitors interact with other kinases at physiological concentrations and it is unknown if these contribute to the therapeutic effect.[12] Truly target-specific small molecule kinase inhibitors probably do not yet exist, and even if they are achievable, their value in the cancer area is questionable.

Clinical experience suggests that selective targeting of a single kinase will produce fewer successful results in solid carcinomas than in leukemias and lymphomas and for shorter durations when they occur. A comparison of imatinib in CML and GIST demonstrates its superior response in the former condition.[5] The 10% major response rate for non-small-cell lung cancer is disappointing compared with the 90% response rate to imatinib for CML. Most solid tumors are not "addicted" to a single pathway for survival in the way that CML is "addicted" to the BCR-ABL pathway. There is increasing evidence that inhibiting multiple targets produces greater benefit over single-target inhibition where no specific pathway drives tumor proliferation and survival. Structural and architectural factors that are absent from leukemia have a profound influence on drug penetration into three-dimensional tumors. Once tumors grow beyond ~2 mm^3, a new network of blood vessels is required to sustain them (angiogenesis) and there appears to be an advantage to multitargeted agents that target the vasculature in addition to the tumor itself. In theory, the stable genome of endothelial cells compared to tumor cells should make resistance to an antiangiogenic drug less likely than for a drug that targets the tumor itself.[13]

The signaling pathways generated by receptor tyrosine kinases that are activated by vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) collectively control angiogenesis as well as tumor growth and survival. Combined inhibition of VEGF and PDGF receptors might result in broader antitumor efficacy, since multitargeted inhibitors should help to overcome the redundancies in signaling pathways.[14,15] The recent approval of the multitargeted agents, sunitinib (Sutent) **5**[16] and sorafenib (Nexavar) **6**,[17] demonstrates that clinical benefit in the treatment of solid tumors with manageable side effects is possible with broad-acting kinase inhibitors (Figure 2). While both sunitinib and sorafenib have a much wider spectrum of activities than imatinib, these two agents differ both in their potency against their targets and in their spectrum of activities. Which of these drugs proves to be ultimately superior will require testing in a wider range of cancer types, especially common forms such as breast and lung cancer. The future challenge will be to ascertain which specific spectrum of targets produces a significant clinical benefit with respect to specific tumor types. One of the main concerns with MKIs is their safety window, so it is encouraging that these broad spectrum inhibitors seem to be generally well tolerated with the most common side effects being generally manageable, e.g., gastrointestinal toxicities, skin reaction, and hypertension for sunitinib and rash and diarrhea for sorafenib.[18]

The first generation of multikinase drugs to show activity in the clinic were not intentionally designed to have their particular multitarget profiles. Indeed many MKIs were originally envisaged as single kinase inhibitors until they

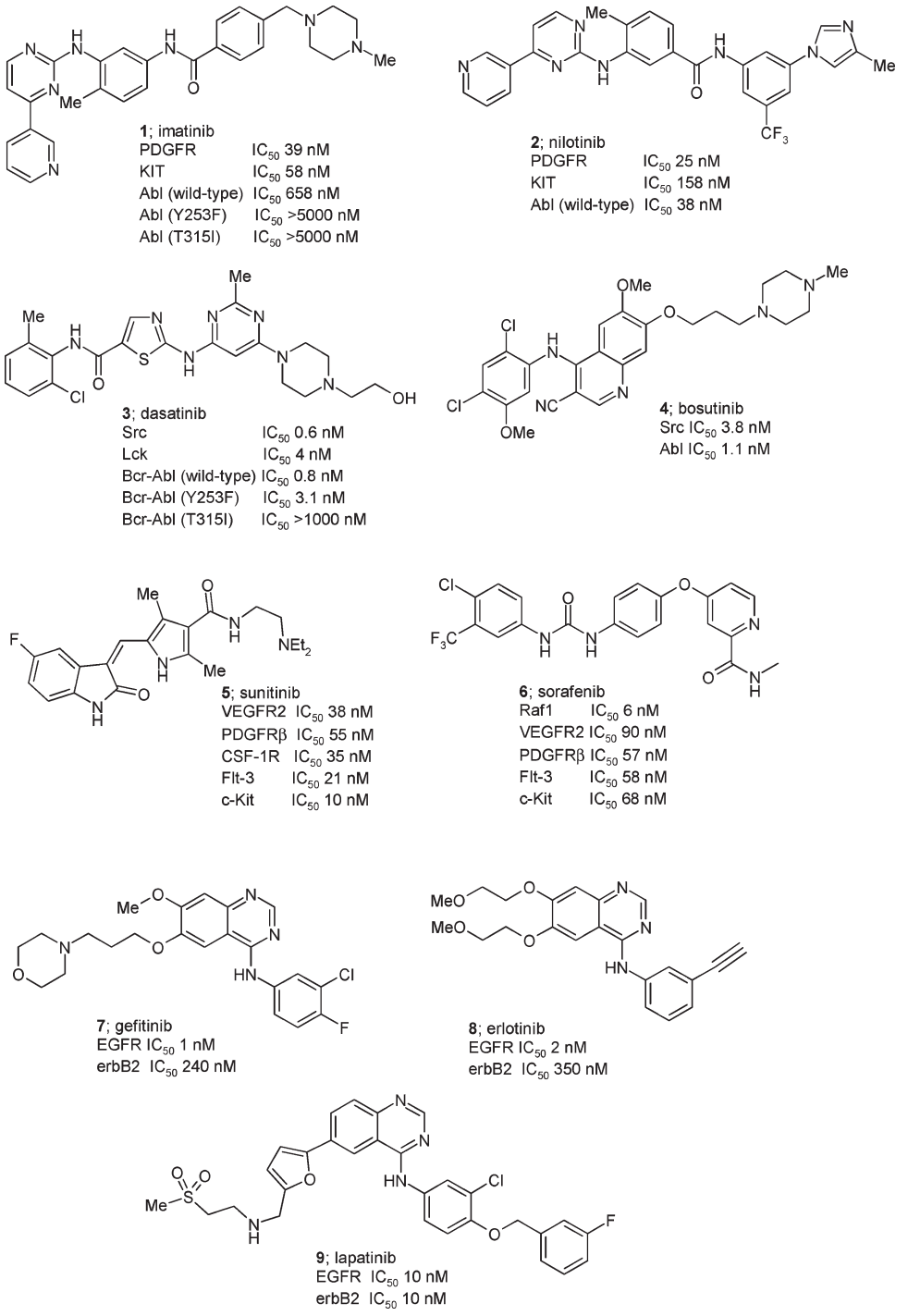


Figure 2. Kinase inhibitors used for treating cancer.

were found to inhibit other kinases as well. For example, sorafenib **6** was originally developed as a mutant B-RAF inhibitor[19] but was later found to inhibit tyrosine kinases as well, whereas bosutinib **4** was originally identified as a SRC inhibitor and was later found to have ABL-inhibitory activity[20] and another ABL-inhibitor dasatinib **3** was derived from a LCK inhibitor program.[21] Sunitinib was initially envisaged as a dual VEGFR and PDGFR inhibitor[22] but has been found to have one of the most promiscuous MKI profiles.[23] The risk with such unintentional MKIs is that they are more likely to hit other, perhaps unknown, kinases associated with undue host toxicity. To minimize this risk, the next generation of MKIs with predefined profiles are increasingly being sought. For a MKI to be defined as a "designed" multiple ligand (Figure 1), it must address only those desired kinases that are associated with the disease while avoiding undesired kinases that are associated with side effects.

3. New Generation of Designed MKIs

3.1. Discovery of Lapatanib. The first marketed MKI drug that comes close to fulfilling the definition of a designed multiple ligand is lapatinib (Tykerb) **9**. Here, the advantage of a dual inhibitor was rationalized prospectively and the optimization was conducted in such a way as to balance the

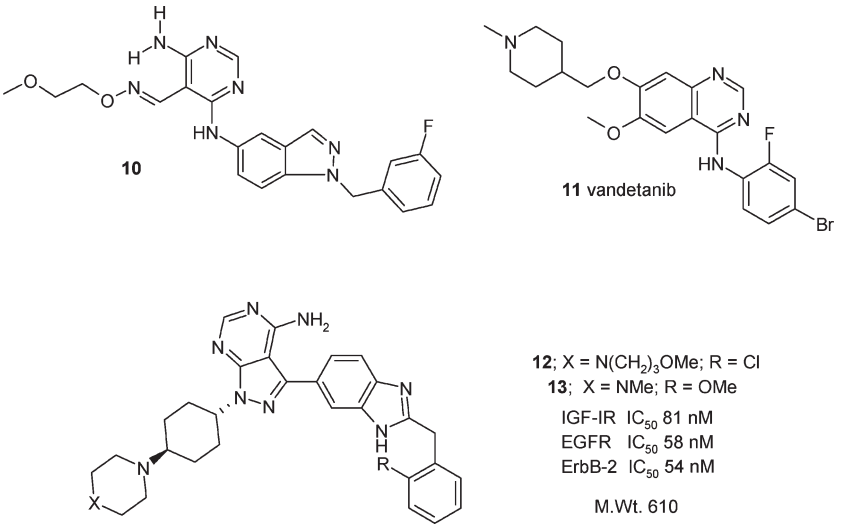


Figure 3. EGFR family inhibitors.

desired activities and exclude undesired side activities.[24,25] EGFR and ErbB2 are both overexpressed in many cancer cell types. Because of their differing receptor expression patterns in human tumors, the inhibition of both kinases was anticipated to provide a broad profile of anticancer activity.[26]

The starting point was a series of 4-anilinoquinazolines with a structure similar to those of the selective EGFR inhibitors gefitinib **7** and erlotinib **8** (Figure 2). It was found that increasing the size of the group on the aniline, by replacing the 4′-fluorine with a benzyloxy group, introduced potent erbB2 activity **9**. A wide variety of linear substituents were tolerated on the furan, and a range of substituent patterns gave good isolated enzyme activity, but the 2,5-disubstituted furan ring spacer was found to give especially good activity in cells. A sulfonylamine group was introduced to improve aqueous solubility.

The crystal structure of lapatinib bound to EGFR revealed a closed ("unactivated") conformation with a large back pocket containing the benzyloxyaniline headgroup associated with high ErbB2 potency.[27] The quinazoline functions as the hinge-binder, and the heteroaryl linker and hydrophilic side chain extend into a solvent exposed region. This unusual conformation (αC-Glu-out conformation) was found to be associated with a slow off-rate that in turn produced a prolonged inhibition of signal transduction in tumor cells with a half-life of 5 h (compared to < 10 min for erlotinib), which is assumed to contribute to lapatinib's impressive efficacy. Another unusual feature of lapatinib is the cleanness of its dual activity profile. It did not show any activity in a panel of 119 kinases at Ambit.[24]

Lapatinib was efficacious in the HN5 and BT474 cancer cell lines overexpressing EGFR and erbB2 respectively. In vivo efficacy was also demonstrated against the same cell lines grown as subcutaneous xenograft models. Lapatinib was approved in 2007 for the treatment of breast cancer, and studies in many other conditions are now ongoing.

Analogues of lapatinib with alternative hinge-binding cores have recently appeared in the literature. For example, an arylaminopyrimidine-5-carbaldehyde oxime scaffold effectively mimics the well-known quinazoline core of gefinitib **7** and lapatinib **9** (Figure 3).[28] The amino group forms an intramolecular hydrogen bond with the oxime nitrogen atom, mimicking the quinazoline phenyl ring. Like lapatinib, compound **10** was found to be highly selective for the EGFR subfamily.

3.2. Other EGFR Family Inhibitors. The development of lapatinib has stimulated much activity in the EGFR family area, and there has been increasing interest in combining activity at EGFR family receptors with activity at other kinases. Vandetanib **11** (Zactima) inhibits both the EGFR and VEGFR pathways with triple inhibition of EGFR, ErbB2, and VEGFR-2 (Figure 3).[5] The recent literature has suggested that a combination of selective EGFR and insulin-like growth factor receptor 1 (IGF1R) inhibitors (gefitinib and NVP-ADW742, respectively) affords a synergistic decrease in cellular proliferation across a diverse set of cancer cell lines compared to the single agents.[29] A group at Abbott aiming to develop a dual inhibitor started from compound **12** (Figure 3) that showed potent IGF-1R inhibitory properties but displayed poor cellular activity versus EGFR and ErbB-2. Identifying 2-OMe as the preferred substituent on the benzimidazole and a methyl-substituted piperazine provided balanced inhibition of IGF-1R, EGFR, and ErbB-2 in both isolated enzyme and cellular assays. In a murine PD model, triple inhibitor compound **13** completely inhibited receptor phosphorylation of both IGF-1R and EGFR. The compound showed modest oral bioavailability of 12% in the mouse, which is consistent with its high molecular weight of 610.

4. Lead Generation Approaches

Increasingly rational approaches are being followed in the development of MKIs. Lapatinib itself was discovered by starting from an EGFR-selective compound and then increasing activity at erbB2 to achieve a balanced inhibitory profile. Indeed the most common approach to discovering MKIs is the cross-screening of focused sets of compounds originating from "selective" kinase programs.

4.1. Focused Screening. In focused screening, compound classes that are already known to be active against one of the targets of interest are screened against another target. This simplifies the logistics of screening against multiple targets and improves screening hit rates. The large number of kinase projects in many companies lends itself to a large-scale

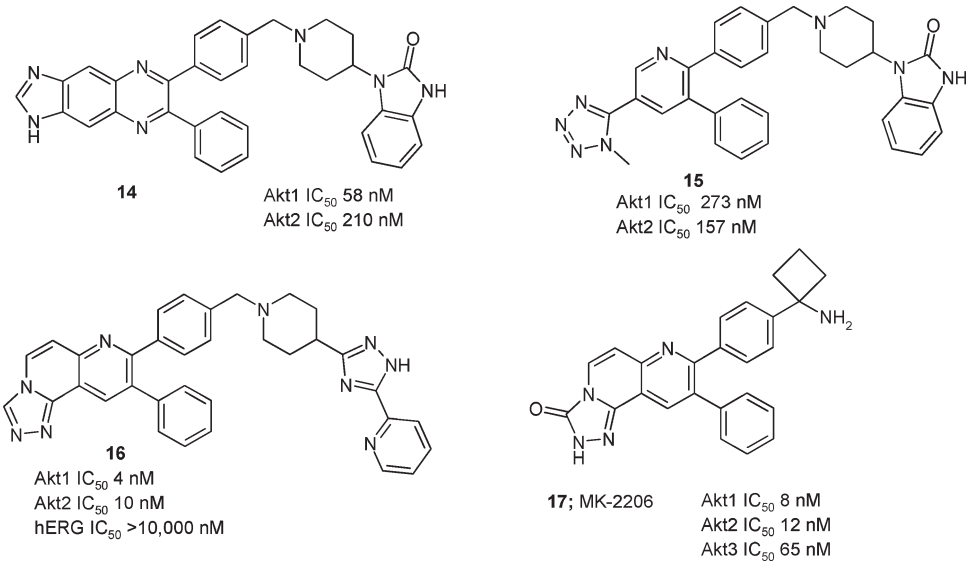


Figure 4

chemogenomics effort whereby chemotypes for one target are recycled as screening hits for others. The value of serendipitous cross-screening for MKIs has been vindicated by the history of the development of the dual ABL/SRC inhibitors starting from compounds designed as selective SRC inhibitors.[30] The task of developing these MKIs was aided by the fact that the SARs for the two enzymes have been observed to be similar, with the most potent SRC inhibitors also being the most potent ABL inhibitors.

The big advantage of cross-screening is the high hit rate due to the high similarity of the ATP binding site between kinases, and in a recent GSK study, at least one hit was found against every kinase when a kinase-like library was screened.[31] For most targets, multiple active compounds covering different chemotypes were found. This hit rate is very high compared to that normally obtained from diverse or focused screening. Certain motifs within MKIs have been correlated with promiscuous inhibition across kinase families, and a five point pharmacophore combination has been proposed.[32] This suggests that starting from scaffolds that fit this pharmacophore, two strategies might be pursued, incorporating additional components to render it selectively nonselective or excising moieties responsible for the undesired interactions, hoping the desired activities are retained.

A major disadvantage of cross-screening is that the intellectual property (IP) space is becoming increasingly congested for compounds that bind at the ATP site. One way around this is to use "scaffold-swapping" in which structural information is used to guide the replacement of the hinge-binding scaffold with a novel heterocycle.[33] A second way is to use diversity-based screening as described below.

4.2. Diversity-Based Screening. Diversity-based screening can generate novel chemotypes, possibly binding to sites other than the ATP-site. To date, there have been few reported examples of MKIs being discovered via diversity-based HTS approaches, which could be due to the complexity of screening large numbers of compounds at multiple targets. It could be due, however, to the fact that HTS is a relatively new lead discovery paradigm and there is an inevitable time lag to publication. Perhaps the best known example of a multikinase drug being derived from HTS is sorafenib, although, as mentioned earlier, this compound was originally developed as a B-RAF inhibitor and its multitarget profile was not conceived prospectively. In a more recent HTS example, dual AKT inhibitors were discovered by a team at Merck.[34] Increased AKT1 and AKT2 kinase activity has been observed in various cancer cell types such as breast, ovarian, pancreatic, and prostate cancers. An AKT1-selective inhibitor was obtained from the HTS screen, and analogue synthesis also produced an AKT2 selective inhibitor. A mixture of the two selective inhibitors was shown to induce a superior apoptotic response compared to either inhibitor alone, and this provided the team with the motivation to develop a dual inhibitor **14** (Figure 4). The allosteric mechanism of action within the series gave good selectivity over other kinases. Importantly, these AKT inhibitors displayed selectivity versus the closely related AGC family (PKA, PKC, SGK) of kinases as well as selectivity with respect to the individual AKT isozymes.

Further work from the same group describes efforts to improve physical properties, thereby enhancing cellular potency.[35] Increasing the basicity of the heterocyclic core, by moving from a quinoxaline to a pyridine template **15**, improved aqueous solubility and cell permeability and reduced molecular weight (MW). Unfortunately compounds from the series showed hERG activity.[36] Introducing a tricyclic triazole scaffold in **16** significantly reduced hERG binding affinity and provided more potent and balanced activity against AKT1 and AKT2, which was important for in vivo efficacy. These inhibitors do not seem to be ATP or substrate competitive and appear to occupy a novel allosteric binding site, the so-called pleckstrin homology domain. Another member of this series, the pan-AKT inhibitor **17** (MK-2206), is reported to be in phase 1 clinical trials for the potential oral treatment of solid tumors.[37]

There are a range of biophysical affinity-based methods that also have applicability in the search for MKIs, such as NMR, surface plasmon resonance (SPR), and mass spectrometry. For example, affinity selection−mass spectrometry (AS−MS) techniques enable the screening of large compound libraries as potential ligands for any binding site on the protein surface and not just the "active site," enabling the

discovery of ligands that act through allosteric binding and other mechanisms.[38] It has also been suggested that fragment methods could be useful for the discovery of DMLs.[39]

4.3. Framework Combination. A knowledge-based approach, known as framework combination, is another lead generation strategy for DMLs frequently reported in the literature.[4] This approach is based on a hybridizing of the frameworks and the underlying pharmacophores of two molecules, each selective for a different target of interest, into a single molecule with both activities. There are a large number of literature examples of the framework combination approach being applied to other proteomic families like GPCRs, transporters, nuclear receptors, proteases, and oxidases. The rarity of the framework combination approach for kinases probably reflects the fact that obtaining selective ligands for kinases is still a major challenge, and this step precedes the rational "designing in" of multiple activities, driven by knowledge of the selective ligand SARs. Whether this approach will be applied in the kinase area is uncertain, and it is likely that the predominance of screening approaches will endure.

5. Challenges of Lead Optimization

Medicinal chemists working on a DML project must optimize the desired ratio of activities, remove any undesired activities associated with side effects, and attain the pharmacokinetic (PK) profile required for oral administration. The last two goals are also common to most single target projects, but it is their combination with the multiactivity goal that creates the added challenge in MTDD.

5.1. Importance of Appropriately Balanced Potency. In any multitarget project, establishing an optimal ratio of the desired activities is critical in order to maximize efficacy and safety. For most multikinase cancer drugs, it is not clear to what extent the inhibition of each kinase contributes to the overall therapeutic effect. Moreover, it is possible that the efficacy of some of these drugs is being primarily driven by targets other than the designated targets. This lack of knowledge concerning how these drugs are actually working greatly complicates clinical development and makes the identification of groups of patients who are more likely to respond very difficult. Even for relatively selective dual inhibitors such as lapatinib, can we be sure that both targets are being inhibited to the appropriate extent in patients?[40] There is a distinct lack of available biomarkers to indicate whether an agent is hitting each intended target to an appropriate degree or indeed whether unintended targets are also being hit. An unbalanced potency against the various targets might lead to inadequate inhibition of one or more targets with a consequential reduction in efficacy. Even worse, the result of suboptimal inhibition might be the more rapid or more complete emergence of therapeutic resistance. The optimal balance of inhibitory activity is not necessary equivalent for each target, although in most published work in this field the aim has been to achieve equal or similar activity for all targets in the first instance. This is normally to provide a clear if somewhat arbitrary goal for the medicinal chemist. Without extensive testing in predictive animal models, and ultimately clinical feedback, determining the optimal balance of activities will be guesswork. There will be an optimal level of inhibition for each kinase in a multitarget profile that is associated with maximum efficacy and safety. These occupancy relationships are rarely discussed in the literature and in most cases are probably unknown.

Knowledge generated during clinical studies can help researchers to identify an optimal profile. The wider availability of target engagement biomarkers, such as PET ligands, that provide occupancy data associated with efficacy and adverse effects mediated through on-target and off-target activities will aid future developments in the field of MTDD. Matching individual MKI drugs to individual patients is important to maximize efficacy, since kinase expression profiles differ between cancer patients.[41]

As the number of targets to be balanced increases, the complexity of the task will increase supraproportionally. Furthermore, measuring the potency balance in both biochemical and cell-based assays is important to avoid misleading effects. Another complication facing the medicinal chemist is the possibility of active metabolites that have a significantly different profile from the parent compound. There are several examples of MTDs that give rise to metabolites that are thought to contribute to their efficacy.[42] For the currently marketed MKI drugs, it is unclear to what extent this is an issue. The des-benzyl metabolite of lapatinib is known to lack activity at the erbB2 receptor.[43] The des-ethyl metabolite of sunitinib is described as having a profile similar to that of sunitinib itself toward VEGFR, PDGFR, and KIT, but given the highly complex profile of the parent compound, it is difficult to be sure that there is no contribution to the efficacy or safety profile from such metabolites.[44]

5.2. Physicochemical Properties of MKIs. The detrimental influence of high MW and lipophilicity (cLogP) on the PK behavior of orally administered drugs has been the subject of intense interest since the publication of the "rule-of-5" (RO5) in 1997.[45] A comparison of the physical properties of DMLs in general, relative to marketed drugs or preclinical compounds, indicated that DMLs have poorer properties.[46,47] Many kinase ligands are extended linear structures because they need to reach away from the ATP hinge region to neighboring regions that provide selectivity. A few MKIs such as the highly promiscuous sunitinib **5** are relatively small, but many others such as imatinib **1** and lapatinib **9** which are more selective are significant larger. Another example is the VEGFR ligand **19** (Figure 5) that was produced from a HTS-derived hinge-binding template **18** and elaborated with H-bonding and hydrophobic functionality to access additional binding sites.[48]

Since only a limited number of kinase drugs have been approved thus far and these are heavily biased toward oncology, it has been argued that the optimal physical property profile for kinase inhibitors is still evolving. The average MW of oral drugs in general tends to decrease with progression through clinical development,[49] whereas the average MW of kinase drug candidates shows the opposite trend.[50] However, the optimal properties that determine absorption across the gut wall should be independent of target family, and consequently, kinase inhibitor design should be influenced by the property profiles of oral drugs in general and not just kinase drugs. The unusual MW trend for kinase inhibitors may be related more to the fact that larger compounds are more potent and selective inhibitors and therefore more likely to progress to the later phases.

The high cLogP of many MKIs is a consequence of the primarily hydrophobic binding pockets adjacent to the small polar hinge recognition site (Figure 6). There are many examples in the kinase inhibitor literature of trying to reduce cLogP, since this property is a primary determinant of drug

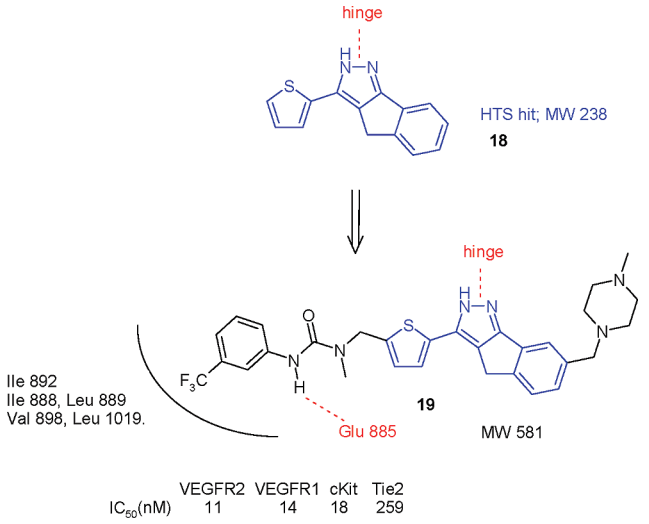


Figure 5

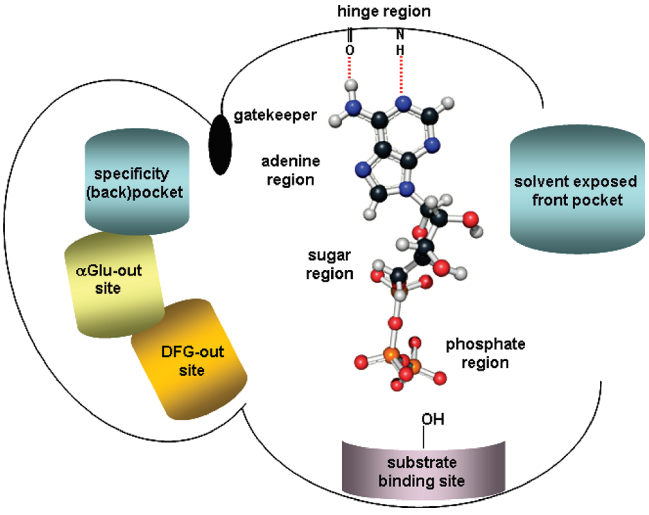


Figure 6. ATP and inhibitor binding sites of kinases.

metabolism, distribution, and off-target activity. For example, the cLogP of lapatinib analogues needed to be reduced to reduce plasma protein binding.[51]

Lipophilicity and the presence of a positive charge in a molecule have been shown to be positively correlated with undesired promiscuity.[52,53] Reducing cLogP for multitarget ligands has been shown to have a beneficial effect on off-target activities such as hERG blockade.[54]

Most MKIs have low brain exposure which may be an advantage in some cases, but it is an issue for treating brain metastases which can arise from tumors in the periphery. Lapatinib **9** is a substrate for the efflux transporters P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP) at the blood−brain barrier, which is consistent with its relatively high MW (581) and cLogP (5.1).[55] Nonetheless, lapatinib has been shown in clinical studies to reduce CNS tumor growth possibly via disruption of the blood−brain barrier by tumors or via inhibition of efflux by lapatinib upon repeat dosing.

The inherently challenging physicochemical property profiles of MKIs are less problematic if the goal of a project is a parenterally administered drug or a biochemical probe rather than an oral drug. Intravenous agents can be an option for treating certain forms of cancer, as illustrated by compound **21** (Figure 7). The development of high quality pharmacological tools to explore and validate the potential therapeutic value of novel target combinations is an important area of future research in this field. Establishing the ground rules for designing such chemical probes is the subject of much current interest within the chemical biology community.[56] The ideal set of properties for probe compounds remains to be defined, but certainly potency in cellular assays and the wider selectivity profile of these tools will be more important than oral drug developability criteria.

5.3. Selectivity Challenge. Gaining selectivity for a single kinase, or in the case of MKIs a limited subset, is widely recognized as the principal challenge facing medicinal chemists working in the kinase field. The risk of a MKI project is that such compounds are by their very nature probably more likely to hit a wider range of kinases. Several recent papers describe the profiling of large numbers of compounds against large numbers of kinases. A recent report looked at the selectivity of approved kinase drugs and candidates across 317 different kinases.[21] The most selective of the currently approved multikinase drugs was lapatinib **9**, and the least selective was sunitinib **5**; the latter bound >15% of kinases tested with K_d < 100 nM. Fedorov et al. profiled a diverse set of 156 commercially available, widely used kinase inhibitors against 60 serine/threonine kinases.[57] Bamborough et al. screened 577 diverse compounds versus 203 protein kinases and found that two-thirds of the compounds bound to more than 10 kinases, thereby clearly illustrating the extent of the selectivity challenge in the kinase area.[58] These screening exercises have provided many useful insights and lessons for medicinal chemists engaged in kinase drug discovery.

In cases where a large number of closely related kinase isozymes exist, some of which may be critical to normal cellular function, the task of achieving wider selectivity will be particularly intricate. At the present time, it is difficult, if not impossible, to rationally design a compound with absolute selectivity for two or three kinases and with no affinity for any off-target. So this current reality has led to a more pragmatic approach whereby MKIs are developed that are deemed to be "selective enough" to be progressed into toxicity testing in animal studies. Even if absolute selectivity cannot be achieved, it would be worthwhile to determine if particular off-target activities are detrimental before terminating the development of an otherwise promising lead compound. The consequences of inhibiting most kinases are poorly understood, raising the question of how to proceed if an off-target activity is detected.

Studying clinical drugs can reveal which kinases can safely be inhibited and which are critical to normal cellular function and should be avoided. Off-target kinases that are significantly inhibited by clinical compounds without overt safety concerns could be classified as posing lower selectivity risks. Unanticipated activities, even for well studied inhibitors, are still being found via panel screening.[59] For example, the receptor tyrosine kinase DDR1 was recently reported as a novel target of imatinib **1**.[60] Although sunitinib **5** was originally viewed as a dual VEGFR2 and PDGFRβ inhibitor, it was later found to inhibit no less than eight kinases with IC_{50} values of less than 100 nM and yet still has an acceptable side effect profile in man. It is also instructive to look at selective kinase inhibitors that failed for safety

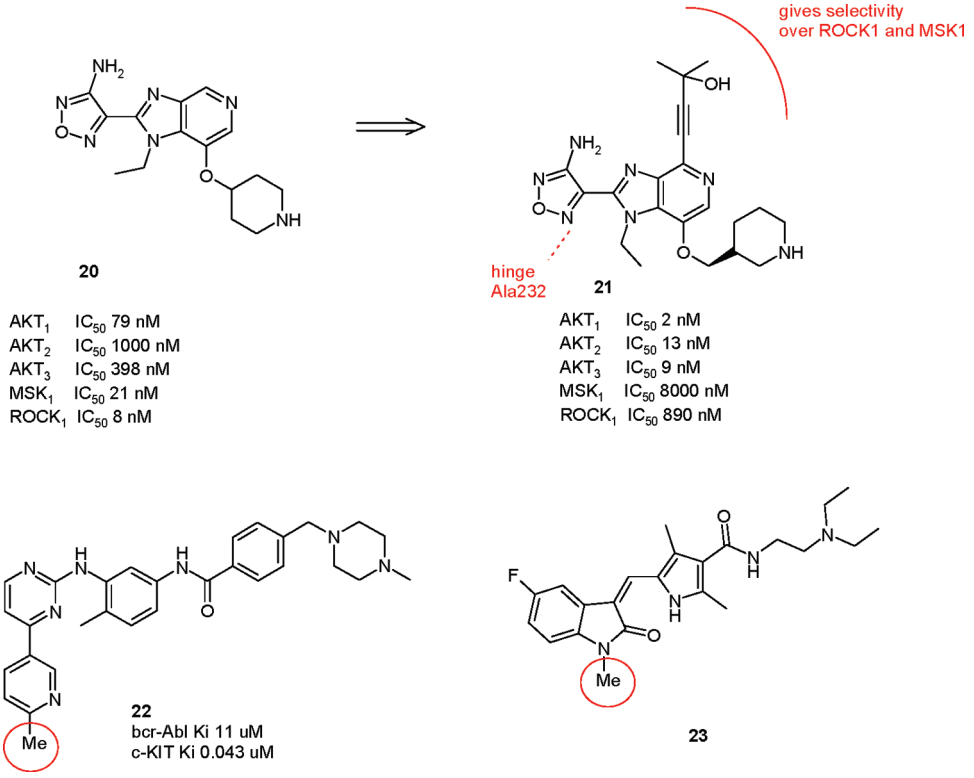


Figure 7

reasons in the clinic, which could give an indication of kinases to avoid.

Kinase selectivity should thus be treated in the same manner as any other off-target activity that might translate into undesirable biology. If an inhibitor hits a target in vitro, it does not mean that it will be an issue in vivo. It will depend upon the administered dose required to achieve efficacy compared to that producing unacceptable side effects.

It remains to be seen whether such a pragmatic approach to kinase selectivity profiles can be extended beyond oncology to non-life-threatening disease areas such as inflammation, where side effect liabilities will be particularly critical (see section 10). Cardiotoxicity associated with multikinase inhibition is one area of concern.[6,61] Imatinib has been reported to have mechanism-based cardiotoxic effects traceable to its impact on the C-ABL kinase, and nilotinib carries a black box warning for possible heart complications.[62] Often it is not clear which kinases are responsible for the cardiotoxicity because of the complex profiles of MKIs, but hypothetical mechanisms have been proposed.[59] MKI-associated cardiac complications represent a potentially serious toxicity and underscore the need for careful monitoring of cardiac function in cancer patients.

5.3.1. Measuring Selectivity and Off-Target Risk. The measurement of selectivity in the kinase inhibitor field is a controversial issue, in part resulting from the empirical nature of the assays used and their lack of standardization. To avoid arriving at erroneous conclusions, selectivity should be evaluated not just at the protein level but also at the whole cell level and preferably also at the level of the whole organism.

Initially, kinase inhibitors are typically evaluated at the protein level for their potential to inhibit kinase-catalyzed phosphotransfer from ATP to a substrate protein or peptide. Kinase selectivity profiles can be determined by a number of service providers using kinase enzymatic or binding assays for most of the known kinases. These studies provide a useful glimpse of potential off-target activity with the caveat that the kinase assays are often run under varying nonphysiological conditions using multiple ATP/K_m ratios, primarily truncated protein constructs (usually only the kinase domain) and artificial substrates. It is thus necessary to confirm on-target versus off-target kinase activity in a more physiological context through further analysis in cell-based and ultimately in vivo settings.

There are dangers in comparing IC_{50} values that have been determined using different concentrations of ATP, substrate, or enzyme. Some service providers tailor the ATP concentration to the K_m of each individual kinase, whereas others use a fixed ATP concentration closer to the much higher physiological intracellular level of around 1 mM. It is important to be aware of these differences when comparing selectivity data, since drastic shifts in IC_{50}s for an ATP competitive inhibitor can occur. In one example of a dual PIM1/PIM2 inhibitor, the compound appeared either PIM2-selective, if an ATP concentration close to the K_m was used, or PIM1-selective if a fixed concentration of 100 μM ATP was used.[63]

In many published studies describing MKI specificity, only a small number of closely related kinases were selected, and this may lead to erroneous conclusions about an inhibitor's perceived selectivity. Screening as large and diverse a panel as possible will help to minimize safety risks, but even then there will be kinases that are missed, so extended postmarketing surveillance of MKI drugs will be needed to pick up any side effects.[21] Since MKIs are still relatively new to the market, some rare but severe side effects might still be discovered with more prolonged use.

With over 500 human kinases known, it is an expensive, if not impractical, task at present to determine the full selectivity

profile of every active compound in a lead optimization series. Since similar compounds on the whole have a tendency to show similar profiles, this is probably not even necessary. On the other hand, small structural differences can make significant differences to selectivity, so as well as looking at a wide kinase panel at the start of a project, medicinal chemists should check selectivity periodically during the optimization process. Although costs are coming down with increasing automation, the relatively high costs associated with these commercial services means that compounds are typically screened at a single concentration of 1 or 10 μM and then further evaluated in dose−response studies against selected kinases of interest. It has been suggested that large panel screens provide redundant information and that relatively small panels if judiciously selected can give a good impression of the selectivity and promiscuity of compounds across the human kinome.[64]

Another method for examining the wider selectivity of novel kinase inhibitors is chemical proteomics, whereby a nonselective kinase inhibitor is immobilized on a bead and captures on the surface any targets to which it is capable of binding. In this way, new information about the binding profiles of several well-known clinically used kinase inhibitors, such as imatinib, has also been revealed.[60,65] Chemical proteomics has the advantage that it can be performed using extracts from diseased cells and tumor tissue, including from human subjects. The attraction of such selectivity screening relates not just to safety but also to the potential to discover cross-reactivity at targets associated with efficacy. Chemical proteomics, when integrated with the biochemical screening of MKIs in large assay panels, can help elucidate the true mechanism of action of MKIs in clinically relevant samples.

During the course of compound optimization, the importance of monitoring structure−activity relationships in both biochemical and cellular kinase assays cannot be overemphasized, since disconnects between the two types of assays are extremely common for a variety of reasons.[66] The concentration of ATP, substrate, or enzyme is probably not in its physiological context. Cell permeability and intracellular accumulation of the inhibitor can sometimes account for differences between biochemical and cellular assays. In other cases it appears that the physiologically relevant form of the kinase is not accurately reflected by the biochemical kinase assay, especially when only the truncated kinase domain is used. For example, the IGF1R inhibitor AEW541 displayed almost identical potency for inhibition of IGF1R (IC_{50} = 150 nM) and insulin receptor (IC_{50} = 140 nM) in biochemical kinase assays. However, in cellular assays the compound was 25-fold more selective for IGF1R versus the insulin receptor (EC_{50}, IGFR = 86 nM).[67]

The cellular selectivity of kinase inhibitors can be evaluated using cell lines that have been engineered to report on the inhibition of a particular kinase such as the murine Ba/F3 cell line.[68] Model cell lines, though very useful for guiding chemical optimization, can sometimes give misleading information if the kinase expression profile differs from diseased human tumor cells. The kinase target signature of bosutinib, determined via chemical proteomics and a large-scale kinase inhibition panel, varied between cells obtained from patients with CML and a model cell line for CML, the K562 cell line.[69] This signifies the added value of generating such profiles in disease-specific primary cell populations.

Evaluation of kinase inhibitor selectivity in vivo remains a significant hurdle. It is clear that we need new methods to globally monitor the changes in phosphorylation that result from kinase inhibition at the level of the whole organism and the relationship to efficacy and toxicity. The concentrations of protein kinases and inhibitors in specific tissues may exceed concentrations tested in vitro leading to unexpected inhibition of multiple kinases in vivo and the irrelevance of carefully crafted in vitro selectivity profiles.

5.3.2. Chemical Strategies To Design Out Undesired Activities. Focused screening frequently produces nonselective inhibitors with undesired kinase activities. There are as yet few literature examples of a prospective approach to rationally design out side activities, although such an approach is undoubtedly occurring in many laboratories.

Undesired off-target activities fall into two general categories, kinase selectivity issues and non-kinase selectivity issues. In the latter category, binding to promiscuous proteins such as the cytochrome P450s and the hERG channel frequently correlates with lipophilicity, so reducing the global cLogP of a molecule is usually a favored approach. Given the high cLogP of many kinase inhibitors, hERG binding can become a major issue. In the AKT1/AKT2 example above, activity at the hERG ion channel was particularly challenging to remove.[36] Improving kinase selectivity is less likely to be solved by such an approach and instead benefits from a more precise understanding of the differences in the pharmacophores between the desired and undesired targets. Biostructural information can be extremely useful for rationally removing side activities as illustrated in the following example.

In a report from Heerding et al. at GSK, the discovery of a pan-AKT inhibitor is described.[70] The starting compound **20** (Figure 7) was a modest inhibitor of the AKTs, with only 1μM potency for AKT2 and poor selectivity over the related AGC family kinases, MSK1 and ROCK1. A significant feature of this work is that a model of the AKT2 active site was used to guide potency improvements at the AKTs while at the same time reducing activity at ROCK and RSK1. A 2-methyl-3-butyn-2-ol group was used to extend the compound through a narrow opening into the back pocket of AKT2, a change that was not well tolerated by ROCK and MSK1 because of differences in the residues lining the pocket. Side activity at ROCK and MSK1 kinases was removed by incorporation of an additional group into **20** to give compound **21**. Compound **21** was cocrystallized with AKT2 which confirmed the binding mode predicted by the docking study with a key H-bond between the N5 of the oxadiazole and Ala232 in the AKT2 hinge region. While this compound shows good selectivity over ROCK and MSK1, it still shows activity at other AGC kinases, including PKA and PKC isozymes, and AMPK and DAPK3 from the CAMK family. This example shows how MKI design is evolving with the use of biostructural information from the desired and undesired binding sites to improve selectivity. At the same time it demonstrates the difficulty medicinal chemists face when trying to improve selectivity over multiple off-targets. Although compound **21** had poor oral exposure, it was progressed into clinical trials as an intravenous agent (GSK690693) to treat patients with solid tumors or hematological malignancies.

Fernández et al. use nonconserved patterns of shielding of hydrogen bonds between amino acids ("dehydrons") as a basis to design out undesired activities. Addition of an extra

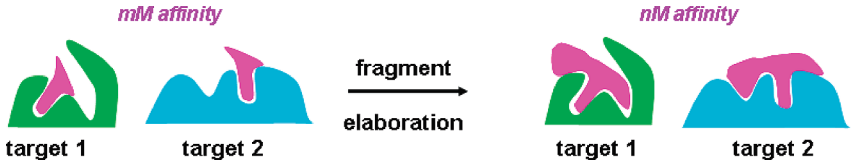


Figure 8. Fragment-based approach to MTDD.

methyl group to imatinib removed activity at BCR-ABL while retaining activity at c-KIT **22** (Figure 7).[71] The aim was to reduce the risk of BCR-ABL-mediated cardiotoxicity while maintaining c-KIT-mediated efficacy against gastrointestinal stromal tumors (GIST). At the same time, activity at JNK1 was introduced to reinforce the prevention of cardiotoxicity. It has been proposed by the same group that a simple modification to sunitinib (N-methylation, **23**) will improve its selectivity over AMPK2 and RSK, targets associated with cardiotoxicity.[72] This evolution of a strategy of identifying kinase off-targets associated with toxicity and then rationally designing that activity out is still at an early stage but mirrors that ongoing in the psychiatry disease area. Here, "dirty" monoaminergic-based schizophrenia drugs such as clozapine are being cleaned of activities associated with side effects such as activities at the adrenergic $\alpha 1$ receptor associated with CV side effects and the histamine H_1 receptor associated with weight gain.[73]

As the number of targets in a profile increases, the complexity of the task of balancing the desired activities while removing undesired activities increases exponentially. This is made somewhat easier if the desired targets are pharmacophorically similar to each other but pharmacophorically dissimilar to the undesired targets. By gaining knowledge of the individual kinase SARs and maximizing the use of biostructural and pharmacophore information for each target, a more efficient path to a ligand with a selectively nonselective profile can be followed. The inherent challenge of achieving finely tuned kinase activity profiles will without doubt be facilitated by the kinase SAR knowledge derived from the screening of many kinase inhibitors against large-scale panels of kinases and a chemogenomic analysis of the resulting data.[31,57]

5.4. Fragment Approach. Fragment based drug discovery (FBDD) has become very popular over recent years as a means of providing compounds with high "ligand efficiency" in terms of their binding energy per heavy atom.[74] Given the physicochemical issues with some MKIs, a FBDD approach to the discovery of DMLs with improved physicochemical properties has clear attractions.[39] Supporting evidence for the relevance of such an approach is provided by recent reports that smaller ligands are more likely to bind to multiple targets than larger ones.[39,75,76]

In a fragment-based approach to MTDD, any of a number of biophysical methods that have been applied to single-target FBDD could be considered, such as NMR, high throughput crystallography, mass spectrometry, or SPR. Alternatively, high concentration biochemical screens could be performed. A basic core scaffold that is capable of binding to both targets would be sought (Figure 8). Subsequent "growing" of the fragment, guided by biostructural data, could provide high affinity and ligand efficiency at both targets. A recent example is the use of a FBDD approach to pan-PPAR inhibitors.[77]

In the MKI field, one viable approach could be to screen for a hinge-binding fragment and then grow the compound into the neighboring back pockets to increase potency and selectivity ("front-to-back" approach). Care needs to be taken to avoid growing fragments into excessively decorated, high MW compounds which may provide better potency and selectivity but at the expense of poor PK. Encouragingly, Hajduk et al. found that most hinge-binding fragments exhibit at least some level of selectivity even without decoration.[33] An alternative to screening for hinge-binding fragments is the so-called "back-to-front" approach whereby a fragment is selected that is shown by X-ray crystallography to bind in the neighboring hydrophobic pockets (Figure 6). This fragment is then grown into a potent inhibitor by adding only the necessary groups to provide the essential interactions in the kinase active site and thereby provide more druglike final structures.[78] This approach was used by Pargellis et al. to discover a deep pocket-binding fragment that was later extended into the ATP-site to give the p38 inhibitor BIRB-0796.[79]

If improving the affinity of a fragment for two or more targets simultaneously proves to be impossible, there exists a tantalizing possibility that the high-affinity binding that is characteristically required for target-selective agents may not be essential for a multiple ligand by virtue of the synergy that can exist between the targets.[39]

5.5. Usefulness of Biostructural, Calorimetric, and Kinetic Data in MKI Discovery. One advantage for medicinal chemists working in the multitarget kinase area, compared to those working on membrane targets like GPCRs, is the availability of three-dimensional protein structures for many of the targets. These valuable assets should enable a more rational approach to both the lead generation and optimization of MKIs. In lead generation, large-scale multitarget virtual screening is a promising protein structure-based method that complements diversity-based screening.[80] In lead optimization, access to X-ray information can help guide the process of improving activity and selectivity as illustrated by the pan-AKT inhibitor **21** above (Figure 7). The availability of multiple crystal structures of kinases in various conformational states can further enhance the value of crystallography and pave the way for designing MKIs with more finely tuned multitarget profiles.

However, there are limits to the value of biostructural information. Although many cocomplex structures of MKIs have been solved, in most cases it is difficult to understand the structural basis of the observed selectivity or promiscuity. Indeed, predicting kinase selectivity from structures in the Protein Data Bank (PDB) is complicated by the wide range of dynamic flexibility (plasticity) of kinase structures for different inhibitor classes. In a recent article, Hajduk et al. conclude that guidance from crystallography and modeling is very useful for driving initial potency gains, but exploiting the subtle differences between the kinases structures to achieve selectivity could only be achieved in some cases by the fine-tuning of substitution patterns through an empirical medicinal chemistry approach.[33] Part of the problem is that there are, as yet, few examples in the

Table 1. Classification Scheme for Kinase Inhibitors

kinase class	ATP-competitive	activated state	reversibility
activated (type 1)	√	√	√
unactivated (type 2)	√	x	√
allosteric (type 3)	x	√/x	√
covalent irreversible	√/x	√/x	x

PDB of the same compound bound to different kinases. However, from the limited number of structures available, we already know that the same compound can bind to two different kinases in different modes, illustrating the complexity and subtlety of kinase−inhibitor interactions. For example, imatinib binds to ABL and SYK kinases in quite different modes.[81] Small fragments are known to be able to adopt different binding modes with different kinases.[33] A fragment-based approach to MTDD may be complicated by the fact that hits may bind to each kinase differently, so a crystal structure of each protein−ligand complex would be needed to guide the optimization process.

Other techniques can complement the use of crystallography in lead generation and optimization such as calorimetric and kinetic analyses of inhibitor binding and catalytic activity. Isothermal titration calorimetry (ITC) is useful for determining the enthalpic and entropic contributions to inhibitor binding. It has been suggested that enzyme inhibitors that are discovered via an enthalpy-driven optimization approach can have advantages over entropy-driven inhibitors by adopting a higher quality fit to the binding site with optimal alignment of hydrogen bonds specific for the desired target.[82] It is tempting to speculate that enthalpy-driven MKIs may be more likely to attain selectivity for the desired over the undesired targets. Kinase inhibitors that exhibit unusually slow off-rates have been attracting increasing attention in recent years because of their potential to have a prolonged duration of action even when the compound clears rapidly from the systemic circulation.[83] Techniques such as SPR can be used to study the kinetics of binding to multiple targets.

6. Influence of Binding Mode on Selectivity and the Feasibility of MKI Design

There are a number of different ways of classifying kinase inhibitors, and the best way of doing so is a topic of ongoing debate within the kinase community. A commonly employed classification scheme stratifies inhibitors depending upon whether they are ATP-competitive, the activity state of the kinase, and their reversibility (Table 1). In this section, the influence of these factors on selectivity and promiscuity is discussed.

6.1. Activated State Inhibitors. Classical kinase inhibitors, sometimes known as type 1 inhibitors, bind in the ATP-binding site and are ATP-competitive, with the kinase in its activated state. At least one hydrogen bond is formed with the hinge region as well as one or more interactions with the surrounding front, ribose, or back pockets, which are largely hydrophobic in nature (Figure 6).[84] The gatekeeper residue controls access to the second hydrophobic back pocket, and most ATP-competitive kinase inhibitors exploit this pocket to gain extra potency. Sunitinib's especially broad activity is likely to be connected with the fact that it binds in the ATP site without extending into the more kinase-specific regions of the binding site. In contrast, the high selectivity of gefitinib **2** for EGFR is most likely due to its ability to exploit the back pocket which is occupied by the 3-chloro substituent. Not all type 1 kinase inhibitors can access the back pocket due to the potential blocking effect of the gatekeeper residue.

A potential advantage of type 1 inhibition is that the activated state will certainly be present in diseased cells because kinases are constitutively active in cancer and the cells may be less likely to become resistant because of conserved nature of the activated state. The high degree of conservation of the activated conformation between kinases should make it easier to obtain the desired multiple activities, but at the same time the likelihood of low selectivity may also be higher. Most compounds in development are of the type 1 variety, and for many kinases activated state binders may be the only option given that there is no evidence so far that they are able to adopt unactivated conformations. A recent review describes the most common hinge-binding scaffolds present in ATP-competitive MKIs.[85]

6.2. Unactivated State Inhibitors. Some MKIs bind to an extended ATP-binding site of an "unactivated" (sometimes called an "inactive") form of the kinase.[86] These so-called type 2 inhibitors open up a new hydrophobic pocket in the back of the protein (Figure 6) and form new hydrogen bond interactions. The clinical importance of type 2 compounds is illustrated by the fact that five out of eight approved kinase inhibitors bind to unactivated states: imatinib, nilotinib, sorafenib, sunitinib, and lapatinib.[87]

Two prominent examples are the "DFG-out" and the "helix-C-out (αC-Glu-out)" unactivated conformations. The "DFG-out" state is so called because the phenylalanine of the DFG motif flips "out" toward the solvent, blocking access of ATP and creating a new pocket for compounds to bind. Examples of the "DFG-out" state are the interaction between c-ABL and imatinib and nilotinib, as well as sorafenib binding to VEGFR and Raf-1.[88,89] The C-helix-out conformation retains the general DFG-in form but leads to inactivation by rotating and shifting the C-helix outward, which opens up an additional pocket.[90] The C-helix-out form is most commonly associated with EGFR and binds lapatinib **9**.

There has been a noticeable trend in the pharmaceutical industry toward identifying type 2 unactivated state binders driven by a desire for higher efficacy, selectivity, and a better IP position than is possible for a type 1 inhibitor. Higher affinity may arise from binding to the new pockets opened up by the conformational rearrangement. The fact that there is less conservation among the unactivated conformations of different kinases may lead to better selectivity, and the high selectivity of imatinib and lapatinib has been rationalized in this way. However, the common belief that type 2 inhibitors are more selective is challenged by compounds such as **24** (Figure 9) that binds to the DFG-out state but is less selective than **25** which binds in the DFG-in mode.[31] The DFG pocket itself is actually highly conserved between kinases, and any selectivity advantage may be more due to the fact that not all kinases can adopt the DFG-out conformation. Aurora inhibitors that preferentially bind either activated or unactivated conformations have been reported to show good selectivity, suggesting that both conformations can contain unique motifs that can be exploited.[91]

The different conformations of a kinase are essentially different drug targets, so the best option for a medicinal chemist is to keep an open mind with respect to which type of inhibitor affords the best opportunity for a particular multitarget profile. Researchers at ARIAD Pharmaceuticals

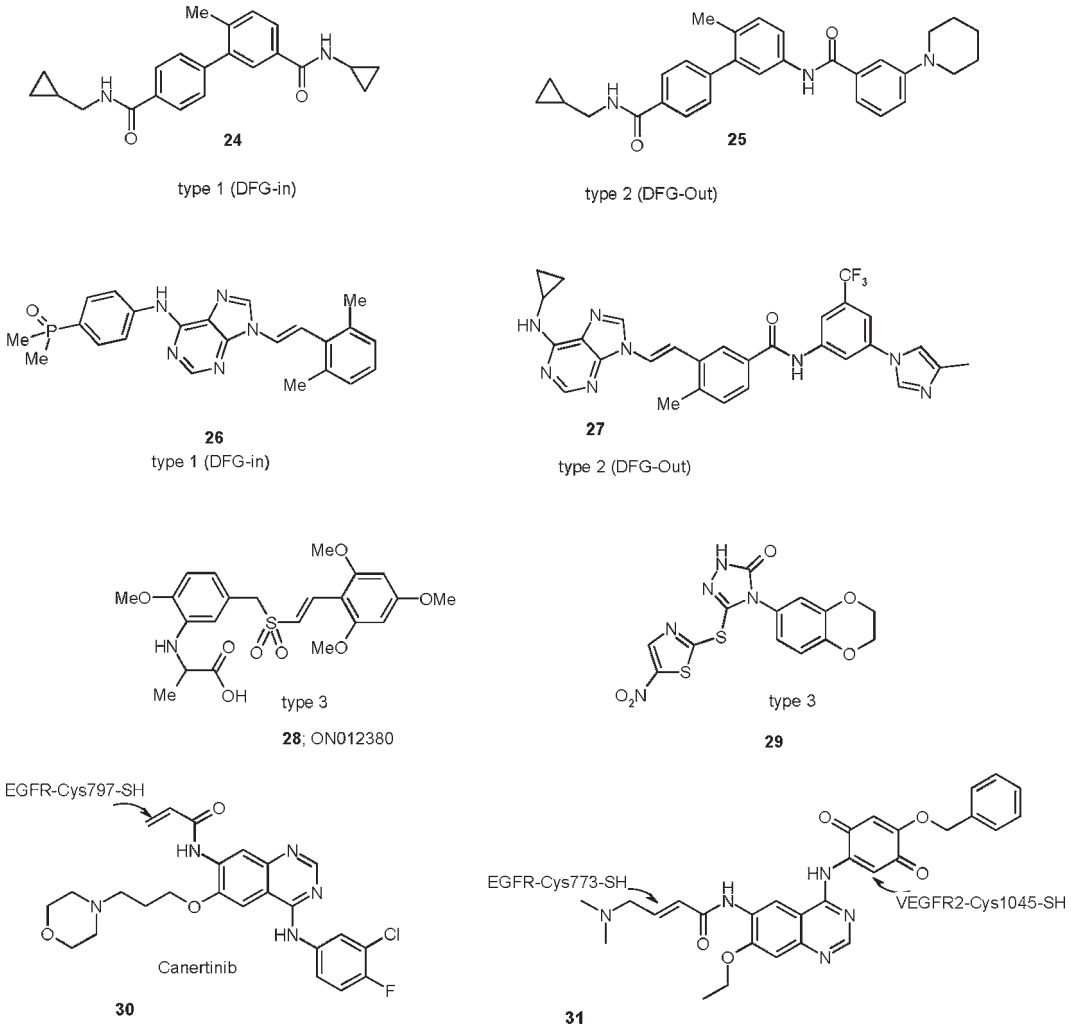


Figure 9. Inhibitors with different binding modes.

described two parallel projects to obtain both type 1[92] and type 2[93] dual SRC/ABL inhibitors from the same chemical series, represented by **26** and **27** (Figure 9), respectively. Interestingly, most type 2 ABL inhibitors like imatinib do not bind to SRC because of differences in the hydrogen bonding requirements in the hinge region in the DFG-out conformation. Nonetheless, compound **27** possesses good activity against SRC ($IC_{50} = 8$ nM) and wild-type ABL ($IC_{50} =$ 25 nM) and has some activity against the normally intractable ABL T315I mutant ($IC_{50} = 478$ nM).

Both imatinib and lapatinib arose from starting compounds that were classical type 1 ATP site inhibitors.[81] In each case, enlargement of the structures caused the switch of the kinase conformation with the *N*-methylpiperazinomethyl and 3-fluorobenzyl moieties being the responsible groups in imatinib and lapatinib, respectively. Similarly, the type 2 dual SRC/ABL inhibitor **27** is noticeably larger than the type 1 inhibitor **26**, with the diarylamide group considered as a privileged DFG-out inducing fragment (Figure 9).[93]

Kinase inhibitors that are deliberately designed to target multiple mutated isoforms can, in a broad definition of the term, also be regarded as DMLs, and one strategy is to design type 2 inhibitors that bind away from the mutation-prone gatekeeper region. Mutation-resistant type 2 SRC inhibitors have been rationally designed by hybridizing hinge-binding (type 1) and allosteric (type 3) fragments using crystal structures to design a linker that evades gatekeeper mutations.[94]

Structural biology will be the key to developing a better understanding of the different structural/conformational states that different kinases can display and assessing which enzymes can be successfully bridged with a MKI while attaining acceptable wider kinase selectivity. It is not clear for the five marketed drugs that bind to an unactivated state that the same conformation is adopted for all the kinases in each profile. There is emerging evidence from X-ray structures that this may not necessarily be the case. Imatinib binds to cKit and ABL in the same DFG-out conformation but to SYK in the DFG-in mode.[81] BMS-599626 inhibits EGFR in an ATP-competitive manner but inhibits erbB2 in a non-ATP-competitive manner, suggesting that the binding modes are significantly different despite the two binding sites being very similar.[95] Another complication is that an inhibitor can bind to the same kinase in different modes. Sunitinib and dasatinib are able to bind to both activated and unactivated states.[81]

The above observations serve to illustrate some general and important points for MKI design. In addition to the activated state, a wide range of unactivated states may be

available and new inhibitors may bind to one state preferentially or to several, and this may in itself differ between the different kinases in the profile. The biostructural situation is potentially highly complex and difficult to predict with small changes in structure being enough to shift the binding mode as illustrated by compounds **24** and **25** (Figure 9). The importance of having regular access to X-ray structures during an optimization program is paramount.

6.3. Allosteric Inhibitors. Allosteric kinase inhibitors, sometimes known as type 3 inhibitors, target sites on the protein outside the ATP site, even though in some cases they may bind in proximity to it. Unlike lapatinib, which is a type 2 ligand that extends into the ATP region, some DFG-out and αC-Glu-out ligands do not and have been described as allosteric.[94] Compounds that bind to unactivated states can be either ATP-competitive or not depending upon their binding mode. Drawing a distinction between type 2 and type 3 inhibitors is sometimes complicated by the fact that inhibitors that bind outside the ATP site can still be ATP-competitive in kinetic experiments.

Compared to ATP site inhibitors, there are as yet relatively few examples of allosteric inhibitors, but there has been a consistent effort to develop such inhibitors,[96] and novel assay systems are being developed to specifically detect binding outside the ATP pocket.[94] Among the best known allosteric inhibitors are MEK inhibitors such as CI-1040 (PD184352), many of which are highly selective because of the uniqueness of their binding site.[97]

It is unknown at the present time whether targeting allosteric sites will offer any advantages in terms of the discovery of selectively nonselective MKIs. These alternative binding sites can be more structurally distinct than the ATP binding site, so type 3 inhibitors may be more likely to be selective in a broad kinase panel than type 1 inhibitors but less likely to hit all the kinases of interest in the first place. However, it is conceivable that two kinases that cannot be bridged with an ATP-competitive inhibitor could share sufficient structural similarity at an allosteric site to allow development of a dual inhibitor. At present, the structure-based design of allosteric MKIs is hampered by a lack of examples in the PDB.

Historically, there has been a problem in obtaining drug-like, small molecule substrate mimetics, since these protein−protein binding sites are usually solvent-exposed and rather featureless surface patches. However, some of these sites have been shown to be druggable with nonpeptidic small molecules, for example, the non-ATP-competitive inhibitor **28** of BCR-ABL (Figure 9), ON012380, that can override imatinib resistance.[98]

Allosteric inhibitors do not need to out-compete millimolar levels of ATP inside the cell but rather (protein) substrates that are present at much lower concentrations. Thus, it has been argued that acceptable in vivo potency can arise from surprisingly low levels of affinity compared to ATP-competitive inhibitors.[99] Currently we have only limited knowledge of the diversity of allosteric kinase pockets. The use of biophysical screening methods such as AS−MS has the clear potential to identify multitarget hits binding to novel sites, with a profoundly different selectivity profile from ATP-competitive hits.[38] Relatively unexplored binding sites certainly exist at least for some kinases, such as conserved binding sites for common regulatory molecules, and perhaps these will in the future be exploited for MKI design. For example, a novel binding site in p38 that binds a wide range of regulatory lipid-like molecules has been reported.[100] Compounds **14**−**17** (Figure 4) bind to an unusual allosteric binding site associated with the pleckstrin homology domain (PH domain) of AKT kinases, and this confers exceptionally good selectivity over other related kinases.[101] The JNK inhibitor **29** targets a specialized protein interaction site, thereby mimicking the binding of JNK to its partner, the JNK-interacting protein 1 (JIP1) (Figure 9).[102]

6.4. Covalent Inhibitors. No consensus exists in the literature concerning the use of covalent, irreversible inhibitors compared to fully reversible inhibitors. Although toxicity risks may be amplified, it has been suggested that selective covalent binding may be a positive contributor to compound potency as a result of prolonged inhibition of the enzyme, the ability to effectively compete with high concentrations of ATP, and increased activity against mutant kinases.[103]

The most extensively studied covalent inhibitor, canertinib (CI-1033) **30** (Figure 9), is a dual inhibitor of EGFR and erbB2 with a structure similar to that of gefitinib **7**. The thiol group of Cys 797 in the EGFR enzyme catalytic site is trapped by a Michael addition reaction to the acrylamide moiety. Preclinical studies suggest that irreversible EGFR/erbB2 inhibitors may have activity in tumors refractory to erlotinib or gefitinib, although this has yet to be borne out in the clinic.[104]

In terms of the scope for designing irreversible MKIs, a recent analysis suggested that there are over 200 kinases (∼40% of the kinome) with cysteines accessible to targeting with an irreversible inhibitor.[66] Irreversible dual inhibitors of EGFR and VEGFR-2, such as **31** (Figure 9), have been reported incorporating two independent reactive centers each targeting different cysteine residues in the kinase domains.[105] A potential issue arises in the design of a covalently bound irreversible MKI if the reactivity of the covalent warhead varies between the different kinases resulting in unbalanced activity.

7. Current Scope and Future Feasibility of Multikinase Inhibition

7.1. State of the Art. Over the past few years, the medicinal chemistry literature describing multikinase inhibition has grown enormously and an ever increasing number of target combinations of postulated disease relevance have been proposed (Figure 10). This network of connections gives a flavor of the historical activity in the MKI field but will certainly not be comprehensive given the extensive and fast moving nature of this research. Because of its pivotal role in angiogenesis, most work has involved combining VEGFR-2 inhibition with other activities and there are a total of 19 connections from VEGFR-2 in this network. Most of these combinations comprise closely related tyrosine kinase receptors, although a small number of them bridge more distantly related kinases and some even cross the barrier between kinases and another target family. It is probable that the currently exemplified combinations represent merely the tip of a large iceberg of conceivable disease-relevant combinations. An important question to ask therefore is "Where do the outer limits of MTDD lie?" MTDD is frequently a difficult and resource-intensive endeavor for medicinal chemists, so it is important to assess the feasibility of any given project at an early stage to avoid frustration and wasted resources. The feasibility of a particular profile should be dependent on the similarity of the binding sites of the desired

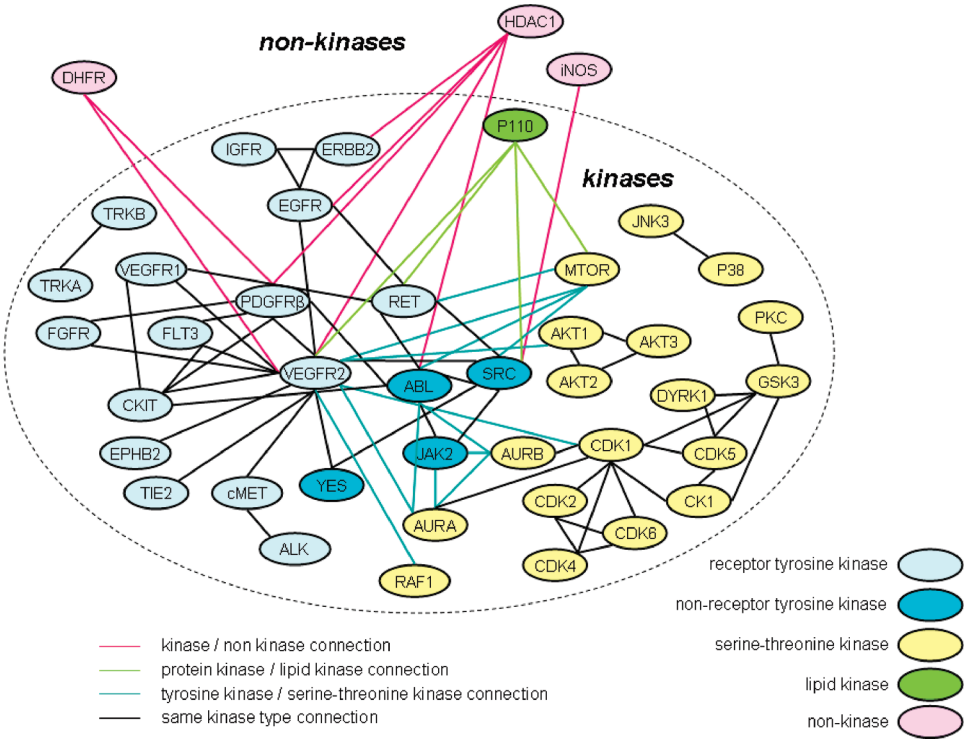


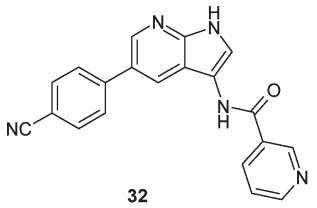
Figure 10. Network of disease-associated MKI connections.

and undesired targets. The similarity between kinases can be assessed from the protein perspective, using either the full or a partial (e.g. ATP-site) sequence identity, or from the ligand perspective, using "SAR" or "chemogenomic" similarity-based methods.

7.2. Feasibility of Combinations within the Kinase Superfamily. Recent studies have compared how the sequence-based similarity of kinases aligns with SAR-based similarity. Vieth et al. found that clustering based on ATP-site sequence identity correlated reasonably well with clustering based on SAR similarity for kinases with >60% sequence identity.[106,107] Similarly, Bamborough et al. found that compounds that inhibit one kinase will often show activity against others from the same branch, provided that these kinases are related by over 40−50% sequence identity in their kinase domains.[31]For example, high sequence identity (83%) and high SAR similarity are observed between EGFR and ErbB2. This suggests that these two kinases are highly likely to bind similar ligands and that the search for a dual inhibitor like lapatinib was a sound decision based on good feasibility. The threshold above which sequence identity and SAR similarity converge seems to be unusually low for tyrosine kinases compared to other kinases, explaining the broad inhibitory profiles of many of the approved tyrosine kinase inhibitor drugs.[31]

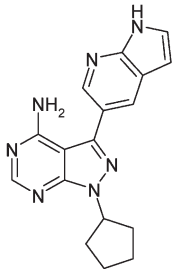
Kinases with dissimilar sequences can show high SAR similarity, but this is rare and not readily predictable.[31]It is also rare for closely related kinases to show low SAR similarity. The implication of these two observations for the feasibility of MKI design seems clear and intuitive. The medicinal chemist's job will be easier if the desired kinases are closely related and the undesired kinases are unrelated by sequence to the desired ones. Bamborough et al. also found that where inhibitors do hit more distant kinases they tend to be broadly nonselective pan-kinase inhibitors.[31] Several caveats apply to this study which used nonphysiological conditions with truncated kinase domains immobilized onto beads in the absence of ATP. Also, the conclusions could be influenced by the historical bias of the kinase library studied. Only the screening of even larger compound sets against the wider kinome may reveal hidden connections between distant kinases. It would clearly be a generic problem for medicinal chemists trying to bridge dissimilar kinases if the only way to do so was using a nonselective compound with a concomitant risk of side effects. There were exceptions though such as the two dissimilar pairs, LKB1/AAK1 and RIPK2/LCK. Compound **32** is a moderately potent LKB1/AAK1 dual inhibitor that is fairly selective over other kinases (Figure 11).

Vieth et al. found other examples where sequence identity and SAR similarity diverge; e.g., ABL clusters next to PKCα in SAR space, despite low sequence identity.[107] The explanation for these unexpected connections between kinases is unclear. It is possible that compounds such as **32** depend strongly on individual residues for their interactions with the ATP sites of LKB1 and AAK1 which are lost when whole sequences are compared. Differences in the size of the gatekeeper residue could be a principal cause of the discrepancies between sequence and SAR similarity, since this residue is a very important determinant of selectivity. Inhibitors of tyrosine kinases show a higher tendency to cross-react with other members of the subfamily compared to other branches of the kinome, such as the MAPK region of the CMGC branch.[58] This could be due to the fact that tyrosine kinases have a more highly conserved gatekeeper residue compared to other subfamilies. In contrast, p38α and p38γ show low SAR similarity despite high sequence identity, which is consistent with their different gatekeeper residues, threonine and methionine, respectively. The fact that so many of the current MKIs are tyrosine kinase inhibitors is consistent with the hypothesis that it should be unusually easy to develop MKIs for this subfamily and unusually hard for



32

LKB1 Kd 380 nM
AAK1 Kd 53 nM



33

p110α	IC_{50} 52 nM
mTOR	IC_{50} 10 nM
Bcr-abl	IC_{50} 18 nM
Src	IC_{50} 14 nM
VEGFR2	IC_{50} 12 nM
PDGFR	IC_{50} 2 nM

Figure 11

the CMGC branch. The counterside is that broader selectivity may be harder for multityrosine kinase inhibitors but the experience of lapatinib, reported to bind to only 3 out of 290 kinases, shows it is possible.[21]

In essence, the identity of the gatekeeper could be a key determinant of the feasibility of a particular MKI profile, especially where the kinases have relatively low sequence similarity. Nonetheless, kinase pairs with unexpectedly high SAR similarities remain highly unpredictable. It is possible that unusual conformational features such as dynamic flexibility, which are not obvious from either their sequence or gatekeeper identities, are responsible. Activities at two kinases that are not closely related by sequence can sometimes be combined if both enzymes can adopt the same type 2 conformation. For example, dual activities have been observed for EphA1 and FRK but not for EphA1 and SYK despite the latter pair having more similar sequence identities. The explanation for this unusual combination could be due to the fact that both EphA1 and FRK can both adopt the DFG-out conformation whereas SYK cannot.

As a result of the high degree of conservation of the ATP-binding site, it is very common to find cross-reactivity among protein kinase inhibitors and tyrosine kinase inhibitors appear to be particularly prone to cross-reactivity.[31] There are many examples of compounds that inhibit both tyrosine kinases and serine/threonine kinases, so there is no significant barrier to MKI design between these two subfamilies (Figure 10). Sorafenib inhibits the serine/threonine kinase Raf-1 and several tyrosine kinases.

Another family of kinases that share a similar function to the protein kinases are the lipid kinases such as phosphatidylinositol-3-OH kinases (PI3Ks). The pyrazolopyrimidine ligand **33** (Figure 11) is active at both tyrosine kinases (BCR-ABL, SRC, VERGFR2, PDGFR) and PI3Ks (p110α) despite the fact that these two families lack significant sequence similarity.[108] PI3K signaling is a common mechanism of tumor resistance to tyrosine kinase inhibitors,[109] and preclinical studies have shown efficacy by combining inhibitors of these two families.[110] The compounds from this class were inactive at serine/threonine kinases despite the fact that tyrosine kinases are structurally more similar to serine/threonine kinases than to lipid kinases.

While most tyrosine kinase inhibitor templates lack activity against lipid kinases, there appears to be something special about this pyrazolopyrimidine chemotype that allows binding to both families. The surprising activity profile of **33** was also rationalized on the basis of the properties of the gatekeeper residues. The small threonine gatekeeper in tyrosine kinases allows the azaindole substituent of **33** to reach its deep hydrophobic pocket. In both serine/threonine and lipid kinases the gatekeeper is a bulkier isoleucine residue, but only in the case of the PI3Ks can the inhibitor circumvent this residue and gain access to the hydrophobic pocket. This sort of detailed structural understanding will be increasingly necessary to rationally design kinase inhibitors with specific selective profiles.

Identifying such privileged, polymodal templates that can provide a diversity of low energy interactions could be the key to bridging more distant targets, and a fragment-based approach to MTDD has a particular appeal in this respect.[39] Vieth et al. also proposed that the SAR similarity relationships between kinases can be predicted more effectively by looking at the similarity between their constituent fragments within their ligands rather than by looking at sequence comparisons.[111]

Another factor that should influence the feasibility of designing a particular multikinase profile is the widely differing hit rate among kinases in focused or diversity-based screening. In their broad kinase panel screen, Bamborough et al. identified tyrosine kinases that bound many compounds from many chemotypes such as PDGFRα/β, KIT, and FLT3, whereas some others such as ZAP70 bound no compounds at all.[31] Similarly some serine/threonine kinases like MST1, LOOK, and DRAK1 are highly sensitive to inhibition, while for others like ERK1/2, it is very difficult to find inhibitors. While the reasons for these widely different hit rates are not clear at the structural level, some kinases are clearly more tolerant while some have more restrictive binding requirements. It seems logical to propose that those kinases that are less stringent in terms of their SAR requirements will be those that are easier to combine with other kinases of interest. Those refractory kinases with high SAR stringency will be more difficult to combine and may only be inhibited by highly nonselective ligands that also hit undesired kinases.

In summary, taking a chemogenomics-type approach to kinase inhibition can help expand our knowledge of the complex inter-relationships within the kinome. This work is still at an embryonic stage, and the associations made so far are necessarily tentative. A larger data set from more panel screening data is needed to firm up these associations and facilitate the in silico predictions of cross-reactivities. Surprises can arise in terms of both desired and undesired kinase activities, and account should be taken of SAR similarity, as well as sequence. This SAR information could be useful at the earliest stages of target profile selection to choose target combinations with a good chance of finding MKIs with acceptable wider selectivity. If it transpires that two targets cannot be addressed because of high SAR dissimilarity and/or stringency, then the medicinal chemist could look elsewhere in the same signaling pathway for an alternative combination.

7.3. Feasibility between the Kinase and Other Proteomic Superfamilies. The debate surrounding the selectivity and

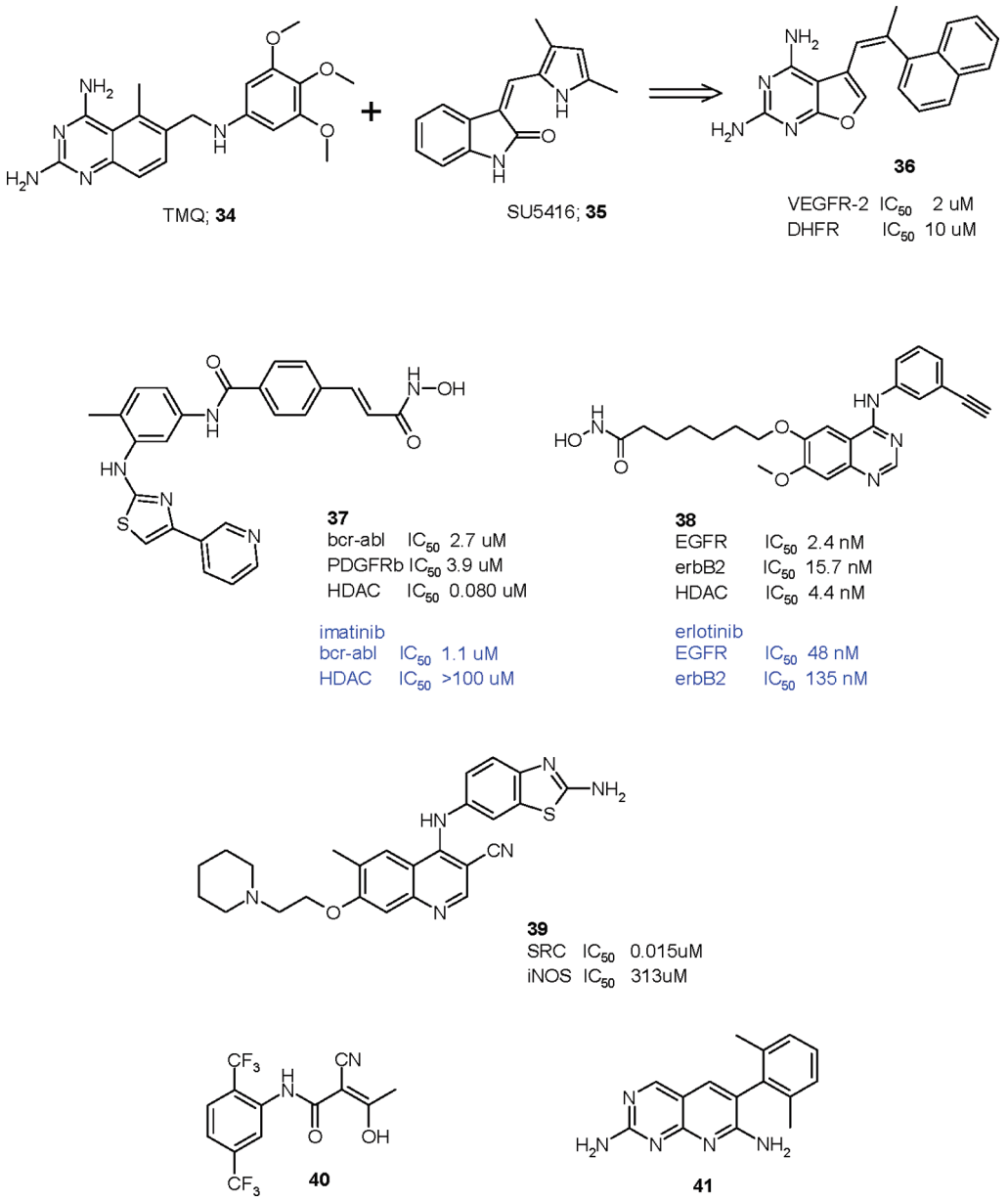


Figure 12

promiscuity of kinase inhibitors is dominated, not surprisingly, by the issue of intrasuperfamily similarity. However, this debate often ignores the fact that kinase inhibitors have some of the greatest intersuperfamily promiscuity.[75] MKIs frequently possess off-target activity at non-kinase targets which needs to be designed out during the optimization phase. Although it has the potential to amplify the risk of off-target effects, on the positive side, the general promiscuity of protein kinase inhibitors also provides an opportunity to design unusual combinations of activities that span different proteomic families. It is also possible that the antitumor efficacy of clinically used kinase inhibitors is in part due to non-kinase activities that may only be discovered serendipitously and retrospectively. Two such examples of non-kinase activity are the recent disclosures that both imatinib and nilotinib strongly inhibit several human carbonic anhydrase (hCA) isoforms[112] and the drug-metabolizing oxidoreductase NQO2.[65] Thus far, the literature contains few examples of ligands that were deliberately designed to selectively target a kinase and a non-kinase. In part, this may be due to medicinal chemists having steered clear of such an endeavor, regarding this as an especially daunting task given the current state of the art in kinase inhibitor research.

Dual inhibitors of VEGFR-2 and dihydrofolate reductase (DHFR) are potential antitumor agents.[113] Pyrimidines such as **36** (Figure 12) were designed by combining structural features of the DHFR inhibitor TMQ **34** and the VEGFR-2 inhibitor SU5416 **35**. The design was facilitated by modeling of the ligands into the ATP-site of VEGFR-2 and the active site of DHFR. Efforts are currently underway to improve the relatively weak activity, but the authors made the point that if there is a strong synergistic effect, a multitarget drug need not be highly potent. Thus, even the low DHFR inhibitory activity of **36** perhaps acts synergistically with the kinase inhibitory activity to provide a viable antitumor effect.

Since the approval of the histone deacetylase (HDAC) inhibitor SAHA, there has been interest in combining HDAC and kinase inhibition. A combination of a HDAC

inhibitor with imatinib showed synergistic effects in terms of inducing apoptosis in CML cell lines and overcame imatinib resistance due to the mutant BCR-ABL T315I protein.[114] A dual inhibitor **37** was designed by incorporating, into the structure of imatinib **1**, a hydroxamic acid warhead that complexes the Zn^{2+} ion in the active site of HDAC. A triple inhibitor **38** of HDAC, EGFR, and erbB2 has been reported with low nanomolar activity against all three enzymes and reasonable selectivity in a 72-kinase panel.[115] Compound **38**, obtained by adding a hydroxamic acid to the EGFR inhibitor erlotinib **8** (Figure 2), is reported to be in clinical development for the treatment of solid tumors.

On the basis that both c-SRC kinase and nitric oxide synthase (iNOS) are key regulatory enzymes in tumorigenesis, dual inhibitors of both enzymes were designed in the hope that they would be more effective than single inhibitors and could be beneficial to overcome drug resistance. Starting from a SRC inhibitor template, the 4-aniline-3-quinolinecarbonitrile skeleton of bosutinib **4** (Figure 2), the existing 4-anilines were replaced with groups that have selective inhibition against iNOS such as 2-aminothiazole. Although compounds such as **39** showed good inhibition of SRC (IC_{50} = 15 nM), the iNOS inhibition was much weaker (IC_{50} = 313 μM). This may imply that the inhibition mechanism of compound **39** is mainly dominated by the inhibition of SRC kinase with no evidence that the dual profile is beneficial in this case.

The challenge of trying to get a balanced profile and good physicochemical properties in an inhibitor spanning dissimilar target families will be profound, and compromises may need to be made where the pharmacophores are fundamentally different. The chance of success might be expected to be higher for a combination of a kinase with another protein with a nucleotide binding site. In addition to the 518 kinases encoded in the human genome, there are over 2000 other nucleotide-dependent enzymes, including polymerases, chaperones, motor proteins, reductases, and methyltransferases.[66] There are also many nucleotide-binding receptors such as the adenosine and purinergic receptors. This cross-reactivity among nucleotide-binding proteins is backed up by screening evidence. Hopkins et al. found that kinase inhibitors frequently inhibit the phosphodiesterases (PDEs).[75] Hajduk et al. reported that kinase-targeted libraries often exhibit increased hit rates against non-kinase targets, with an average enhancement of 3-fold compared to a random selection of compounds.[33] The conserved nature of ATP binding sites among other protein classes implies that exploiting the wide range of ATP competitive kinase inhibitors that are already available could provide starting points for the development of inhibitors that target kinases and other proteins simultaneously. Waldmann et al. screened tyrosine kinase inhibitors against the bacterial enzyme D-alanine−D-alanine ligase on the basis that the ATP binding sites were similar between these different target families and found some hits such as **40** (Figure 12).[116] An inhibitor of SRC, ABL, and LCK **41** was found to bind to the ATP binding site of another bacterial enzyme, biotin carboxylase.[117]

It is noticeable that all of the above examples are enzyme combinations, with kinase inhibition being combined with activity at DHFR, HDAC, or iNOS. Thus far, the literature contains no examples of deliberately combining activity at a kinase and a GPCR for a particular disease, but many accidental examples of such cross-reactivity are known. The prototypical ATP-competitive kinase inhibitor staurosporine has been found to show potent allosteric interactions at muscarinic M1 and M2 receptors.[118] In fact, two of the target families that had the highest cross-target family promiscuity are kinases and the aminergic GPCRs, so it would not be a surprise for a kinase ligand to bind to an aminergic GPCR and vice versa.[75] In fact, only ligands for the notoriously promiscuous cytochrome P450s appear to have higher promiscuity than the ligands for kinases and aminergic GPCRs. The high cross-target family promiscuity of kinase ligands is a double-edged sword. On the one hand it should provide an opportunity to discover unusual combinations of therapeutic relevance, but at the same time, the task of achieving sufficient wider selectivity over undesired targets could be complicated.

7.4. Predicting Unexpected Connections between Targets from Remote Target Families. A big challenge for the future development of MTDD is to correlate the promiscuity of targets and compounds with discrete structural features.[119] The promiscuity of proteins could either be related to their having multiple discrete binding sites that coexist on the protein surface concurrently or be due to plasticity of the protein creating an assortment of competing sites that can bind ligands in low energy conformations. Protein promiscuity may be due to a binding site being able to accommodate multiple ligands in a variety of different binding modes with high affinity, perhaps due to the ability of water to act as a bridge in different ways between the protein and ligand. Promiscuity within a target family could be due to the overwhelming importance of a single interaction to the binding energy, such as the conserved salt bridge between the basic nitrogen of an aminergic GPCR agonist and the Asp residue in the transmembrane TM3 domain.

The promiscuity of compounds is influenced by physicochemical properties, and the role of MW and cLogP has already been documented.[39,52] DMLs also seem to be more flexible than preclinical compounds in general.[47]An optimal level of flexibility may be important to allow the binding of compounds to different targets in different conformations. Imatinib is known to bind to NQO2 in a different conformation from BCR-ABL.[120] ATP is known to bind in different conformations to different types of proteins in the PDB, and even within the kinase family there are differences, suggesting that similarity in protein function does not necessarily imply similarity in the binding mode of the endogenous ligand.[121]

The example above of compound **33** (Figure 11) that binds to two structurally unrelated kinases with different binding modes illustrates that certain scaffolds are capable of specific binding to different proteins by being able to form different interactions. To maximize the feasibility of a particular combination, proteins should be lenient in terms of their binding requirements and compound chemotypes should be amenable to binding to proteins in multiple ways.

Although the DML field has expanded enormously over recent years, many new combinations await discovery. Given the number of possible permutations, even taking account of the medicinal chemistry challenges, there will likely be a larger number of druggable combinations than there are druggable single targets. New computational methods can help to define the true extent of the "opportunity space" for DMLs. In silico methods have been devised to compare crystal structures, looking for commonalities in binding sites that may provide opportunities for designing new

combinations.[122,123] In the MKI area, protein structure-based search methods often suffer from the disadvantage that kinases readily change conformation, so comparing proteins by looking at ligand similarity is at least as appropriate. Similar compounds can bind distant kinases and structurally unrelated compounds can bind similar kinases, but this is relatively uncommon. Thus, it should be possible to assess the feasibility of bridging two kinases by looking at the similarity of their known inhibitors. The work of Bamborough et al. implies that if two inhibitors of different kinases have a Tanimoto coefficient above 0.6 ($T_c > 0.6$), there is a good chance that they will also hit the other kinase.[31] Ligand-based similarity methods have also been used by other groups to study ligands from a wider range of targets from diverse proteomic families to produce a map of polypharmacology space.[75,124,125] These maps can reveal relationships between targets that are unexpected from sequence data alone and help identify novel target combinations for which it may be possible to find a multitarget agent.

Although they have great potential, it is still early days for such predictive approaches. It is difficult to know how generally useful they will be for assessing the feasibility of those unexpected combinations where the targets are not obviously related by structure or function, and yet it is still possible to obtain a druglike DML. The discovery that imatinib and nilotinib inhibit CA I and CA II carbonic anhydrases would not currently be predicted on the basis of the structures of either the proteins or their known ligands.[112] These kinase inhibitors lack the typical warheads, such as sulphonamides, associated with CA inhibition.

Likewise, chemical similarity does not always translate into biological similarity, so the fact that two targets have similar ligands does not guarantee success. Fusing multiple prediction methods that use different measures of compound similarity is likely to improve the overall success rates of in silico lead identification.

8. Network Pharmacology

In addition to assessing the feasibility of a particular combination from a medicinal chemistry perspective, the second critical determinant of success in MTDD is discovering and validating novel combinations from the disease perspective. Network pharmacology is an emerging and exciting discipline that has the potential to transform the way we discover MTDs.[126]

There are emerging signs of a change in mindset in drug discovery from targeting single targets to targeting disease-relevant pathways. Signaling pathways are not strictly linear processes but rather involve a complex network of interconnected circuits. When only a single pathway is targeted, redundancy and crosstalk between these pathways allow for compensatory effects by alternative pathways. Systems-level and individual target-based research should not be regarded as competing fields. They are complementary. By adopting a single-target mindset, we can better understand how an individual target contributes to the operation of a system as a whole. In addition to biological approaches such as gene knock outs and knock downs, chemical biology approaches using highly selective chemical probes can reveal the contribution of each component to the therapeutic efficacy and safety of a multitarget drug.[56] Isobolographic analyses of the effect on efficacy of different doses of selective ligands can help to identify the individual target contributions.[127]

The targeting of aberrant network states may represent a powerful new paradigm in MTDD. Although the majority of the protein inventory in a cancer cell is the same as a normal cell, the differences in the topology of the biological networks could be targeted to provide an improved therapeutic index.[126] We need to ascertain where the best places to intervene in a network are, from the perspective of efficacy, safety, and druggability.

Known MKIs achieve their superior antitumor effects by simultaneous disruption of different targets in the same pathway or of multiple targets in different pathways. The terms "single-spectrum" and "extended-spectrum" inhibitors have been used to describe agents that respectively target kinases in the same or different signaling pathways.[5] Lapatinib is an example of the former, inhibiting two kinases in the EGFR pathway, EGFR and ErbB2. The blockade of several targets in the same pathway can be useful for overcoming the onset of resistance to inhibition of one component in a pathway. However, it may be necessary to block more than one pathway to prevent signaling simply being redirected via an alternative pathway. Vandetanib **11** is an extended-spectrum agent that blocks both the EGFR and VEGFR pathways with triple inhibition of EGFR, ErbB2, and VEGFR-2.[5] As well as affecting tumor cell proliferation, EGFR stimulation increases angiogenesis, so this combination is potentially synergistic. Another example of an inhibitor that acts on two separate pathways is XL880 which as well as inhibiting multiple receptor tyrosine kinases (RTKs) involved in angiogenesis (VEGFR, PDGFR, c-Kit, Flt3, and Tie-2) also inhibits c-MET, which is indicative of tumor aggressiveness and poor prognosis.[5] Sorafenib has a unique profile among the approved inhibitors, with activity at an intracellular serine/threonine kinase (B-RAF), hence blocking the MEK/ERK pathway and the cell surface RTKs (VEGFR, PDGFR, c-Kit, and RET).

The literature now contains numerous examples where selective inhibition of one kinase appears to lead to compensatory changes in the activity of another target or pathway that counteracts the intended antitumor effect. Developing a network-based understanding of which targets are involved in these feedback loops can lead to the rational design of new MKI profiles. Paradoxically, several kinase inhibitors, such as the allosteric mTORC1 inhibitor, rapamycin, and the ATP-competitive AKT inhibitor, A-443654, have been found to activate rather than inhibit the target pathway owing to inhibition of a negative feedback loop.[128] Because activation of these pathways promotes tumor growth, it is crucial to understand which pathways may have active feedback loops and which kinases are responsible for their control. Selective inhibition of mTOR may lead to PI3 kinase (PI3K) activation which can be overcome with combined inhibition of mTOR and p110α.[129] The efficacy of EGFR/erbB2 inhibitors in treating erbB2-positive breast cancer is reduced by compensatory changes in the phosphorylation of erbB3 that lead to the restoration of PI3K/AKT-mediated signaling.[130] Combining EGFR inhibition with PI3K/mTOR inhibition is a promising approach for improving efficacy.[131] Similarly it has been suggested that MET inhibition should be combined with EGFR inhibition to overcome resistance to gefitinib in lung cancer patients due to MET-induced activation of the PI3K/AKT-mediated pathway.[132]

A better understanding of the role of feedback loops within networks is already helping to avoid safety issues with kinase inhibitors for chronic, nononcology diseases. One of the

potential safety issues with p38 inhibitors is the possibility of tumorigenic side effects via the p38a-TAB1 feedback loop.[133] This potential risk may be avoided by targeting targets downstream of p38 such as MAPK-activated protein kinase 2 (MK2).[134]

9. Combinations of Selective Inhibitors as an Alternative Approach to MKIs

The development of exquisitely selective single target kinase inhibitors, rather than selectively nonselective MKIs, is currently the primary focus in many companies. Single target agents can be effective in treating tumors that are "addicted" to a single oncogenic kinase, the best known example being CML which is dependent on the sustained activation of BCR-ABL. Some selective agents, such as gefitinib **7**, show good efficacy but only in a small subset of patients with EGFR mutations. Thus, there is a general recognition that in many, if not most, cases achieving sufficient efficacy will demand their combination with other agents. Where two or more agents, that are highly selective for a single target, need to be dosed separately in the form of two (or more) individual medications, there is a risk of poor patient compliance due to complex dosing regimens. However, this will be less of a concern for life-threatening diseases such as cancer, especially where patients are hospitalised. Indeed, there are currently a large number of cancer trials ongoing combining small molecule kinase inhibitors as well as combinations of small molecule inhibitors and monoclonal antibodies. To make dosing regimes simpler and improve compliance, particularly for less severe diseases, several small molecules can be coformulated in a single tablet.[135] Recently this fixed dose combination (FDC) approach has started to be applied in the kinase area. For example, AstraZeneca and Merck are developing a combination of the allosteric AKT inhibitor **17** (Figure 4) and the MEK1/2 inhibitor AZD6244.[136]

The question is often asked whether FDCs or DMLs are the preferred approach to MTDD. The answer is not clear at present and is unlikely to be resolved in the near future. Arguing at this point in time that one approach is always better than the other risks stifling the important intellectual debate that needs to take place. In reality, there is almost certainly room for both in the armamentarium of modern drug development. DMLs and FDCs have their own distinctive advantages and disadvantages, and which is preferred will depend upon a diverse range of considerations from the feasibility of the DML approach at the drug discovery stage to the individual needs of the particular patient in the clinic.

An advantage of drug combinations is the ability to titrate the dose for optimal inhibition of each target. While it is not possible with a FDC to fine-tune an exact ratio for an individual, a number of different dose combinations are usually made available based upon the most common doses of the individual drugs. One limitation of FDCs is that concomitant administration of two or more agents may result in drug−drug interactions and unacceptable additive toxicities.

For some disease-relevant target combinations, the medicinal chemist will struggle to achieve the requisite multitarget profile. Particularly where the targets in a combination are distantly related, the danger of the DML approach is that obtaining the multiple activities in a single molecule will only be achieved at the expense of either poor physicochemical properties and low oral bioavailability or poor selectivity and high toxicity. In such cases, a more practical and safer approach might be the combined administration of two highly target-selective compounds. Together they could provide the desired balance of activities, with a wider selectivity profile that translates into an optimal therapeutic window in man.

The question then arises as to how many single agents can be combined in a single tablet. Most current FDCs contain two agents only, but advances in formulation technology are expanding the number of drugs that can be combined. The so-called polypill combines five medicines for treating cardiovascular disease in a single tablet with a similar additive effect to each drug separately and no unexpected interactions between the drugs.[137] However, the difficulties of formulating multicomponent capsules over extended periods of time should not be underestimated and can be very costly and time-consuming.[138] For example, some components may well require different conditions for stability from others, such as a different pH, to avoid problems with chemical degradation.

Another issue with FDCs is the ownership of the IP if the individual drugs are derived from different companies. The AstraZeneca and Merck collaboration combining their MEK and AKT inhibitors is a new type of intercompany collaboration. Rather than combining an experimental therapy with an approved drug as is more common to enhance the drug's therapeutic effect, the two companies are coming together at an earlier stage and planning a phase I trial.[136] Whether this type of agreement marks the start of a new trend in clinical research collaboration remains to be seen.

Even many of the broad spectrum MKIs such as sunitinib and sorafenib may need to be combined with other agents, since neither appears to be curative when used alone. There are several clinical trials ongoing combining these agents with other drugs, for example, combining sunitinib with chemotherapy, combining lapatinib with trastuzumab, and combining sorafenib with bevacizumab, to add another level of inhibition by removing VEGF from the blood. So in the future, a third hybrid strategy could well emerge in addition to conventional DMLs and FDCs in which combinations of DMLs are given to further enhance efficacy.

10. Noncancer Application of MKIs

All kinase inhibitors, approved in the U.S. and Europe, are used in the cancer area, and there is only one nononcology kinase inhibitor, fasudil, used in Japan for the treatment of cerebral vasospasms and ischemia. However, many pharmaceutical companies are putting a significant effort into exploiting this target family in other therapeutic areas. The risk to benefit ratio needs to be appropriate for the disease being studied, and for most other diseases this will inevitably mean cleaner selectivity and side effect profiles will be required. For the current generation of multitargeted anticancer agents, it is not entirely clear which targets are driving efficacy. For less serious and chronic diseases, the clinical development of MKIs without such knowledge is much less likely and the discovery of biomarkers relating target engagement to efficacy will be even more important.

The second most important area for kinase inhibitors after cancer is inflammatory disease, and many kinase inhibitors that modulate proinflammatory pathways have entered clinical trials.[139] However, many have been discontinued, and this is probably related to inadequate selectivity. As a result, the emphasis is presently on obtaining highly selective single kinase inhibitors rather than MKIs. Whereas the side effects

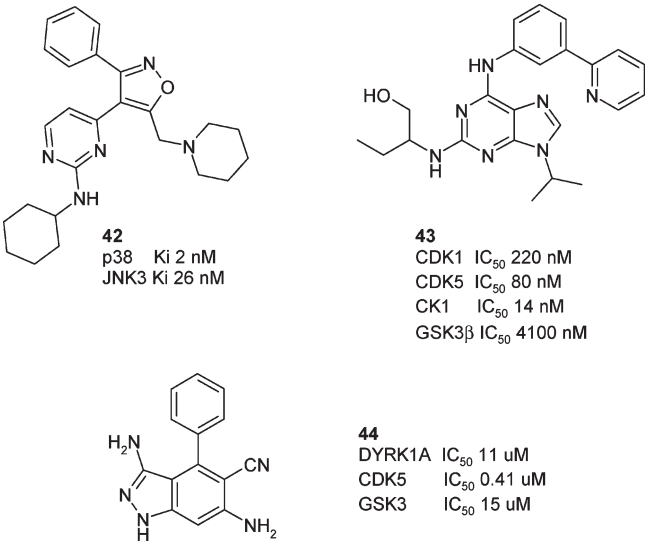


Figure 13

of MKIs may be tolerable in the cancer disease area, combinations of highly selective kinase inhibitors may ultimately prove to be a more valid approach clinically for some non-oncology applications.

Activation of p38 kinases causes downstream up-regulation of cytokine production (TNFα and IL-1) and therefore plays a pivotal role in inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis. Many pharmaceutical companies have sought increasingly selective p38 inhibitors over the past decade or so, but disappointingly, none of them have yet progressed to the market. The p38 family is closely related to the Jun N-terminal kinase (JNK) families, so it is perhaps not surprising that some p38 inhibitors also inhibit the JNK pathway. Dual p38/JNK3 inhibitor **42** developed by Vertex as potential therapies for stroke may address both the inflammatory (p38) and anti-apoptotic (JNK3) components of the disease (Figure 13).[140]

While inhibition of the p38 MAPK pathway remains a major focus of research in the pharmaceutical industry, there is an increasing trend to move away from direct inhibition of p38 kinase, because of concerns over its multifarious roles in cellular signaling, and toward inhibition of downstream targets such as MK2.[133] This may provide a more measured and safer way of inhibiting the production of pro-inflammatory cytokines such as TNF-α. Since MK3 has a parallel role to MK2 in its inflammatory function, it has been proposed that MK2/MK3 dual inhibitors may provide a superior anti-inflammatory effect compared with MK2 inhibition alone.[134]

Like many forms of cancer, Alzheimer's disease is a terminal disease with few treatment options at present, and therefore, the MKI approach may be highly appropriate. Both hyperphosphorylation of tau (leading to neurofibrillary tangles) and amyloid-β production (leading to amyloid plaques) are mediated by a small set of kinases, such as CDK1, CDK5, GSK3, and CK1.[141−144] Multitarget inhibitors acting on this selection of kinases could therefore have great therapeutic value. The GSK3 kinases are more closely associated with the cyclin-dependent kinase (CDK) sub-branch than its location on the phylogenetic tree would suggest, and most CDK inhibitors have been found to be good inhibitors of GSK3β due to the similarity of the ATP binding domains.[107] Oumata et al. prepared trisubstituted purines **43** that were evaluated as inhibitors of CDK1, CDK5, GSK-3, and CK1, and Chioua et al. described pyrazolopyridine **44** as a prototypical inhibitor of CDK5, GSK-3, and DYRK1A (Figure 13).[145,146]

11. Summary

MKIs can deliver superior efficacy compared to inhibitors with high specificity for a single kinase, and the recent introduction of several MKIs to the market opens the door to a new era of safer and effective anticancer therapy. The key to combining high efficacy with acceptable safety is to inhibit multiple targets in a selectively nonselective fashion. Strategies for intentionally designing MKIs are emerging, but the field is still in its infancy and we are as medicinal chemists currently on the steepest part of the learning curve. MTDD can be time-consuming and expensive, and we need to become more proficient first at identifying disease-relevant target combinations and second at discovering MKIs that combine optimal physicochemical and biological properties. Bold and innovative medicinal chemistry strategies are required to tackle "difficult combinations" where the disease rationale is compelling but where it is a struggle to combine all the desired attributes of an oral MKI drug into a single molecule. At present it is unclear to what extent MKIs with highly tuned selectivity profiles can be rationally designed, particularly for targets that are unrelated by sequence. In addition to the well-known selectivity challenge, the physicochemical property profiles of ATP-competitive MKIs can be inherently challenging and limited scope for patentability can also be a serious hindrance. On the plus side, the amount of kinase-specific structural information is growing very rapidly, and ultimately this may reveal distinct features and design rules that enable a medicinal chemist to rationally modify and refine the profile of MKIs. In addition, increasing SAR knowledge is emerging from large scale panel screening with the binding profiles starting to reveal to medicinal chemists how chemical structure affects cross-reactivity across large parts of the kinome.

The merit of MKIs compared with single kinase inhibitors is a subject of controversy in drug discovery that is unlikely to be resolved in the near future. At the start of a new MTDD project, a rigorous debate needs to take place as to whether it makes more sense to seek a combination of highly selective agents or a DML. Many factors need to be taken into account in this decision such as the number, similarity, and promiscuity of the targets in the profile and the disease area.

Conformational plasticity and the occurrence of multiple binding modes complicate the in silico prediction of kinase polypharmacology based solely upon protein structure. The use of ligand-based similarity to assess the feasibility of a given combination can add real value. Currently, serendipity plays a significant role in MKI discovery and many, if not most, MKIs have been discovered by chance during the search for selective inhibitors. Medicinal chemists need to be alert to the possibilities when a surprising combination is found by chance. To exploit such serendipity, you need a good appreciation of when you have a sufficiently high quality starting compound and then you need to be able to make and test sufficient analogues to explore your new disease-based hypothesis.

MKIs are costly to develop and are consequently priced at a premium level, so they will need to show clear improvements in order to get reimbursement. There have already been problems with reimbursement for some MKIs in some markets due to concerns from funding bodies over insufficient efficacy. The true value of MKIs relative to other anticancer drugs still has to be established, and the results from recent

clinical trials have been mixed. Despite the broad activity profile of many MKIs, the patient response can be inconsistent and unpredictable. The identification of predictive biomarkers of response or resistance is a critical step to ascertain which specific combination of targets produces a significant clinical benefit with respect to specific tumor types. More clinical feedback is needed to facilitate the design of the next generation of inhibitors with more precisely defined profiles. Although it might seem immeasurably distant at the present time, the ultimate goal should be to derive the prerequisite knowledge and tools so that MTDD becomes a rational endeavor rather than a black box approach that relies upon serendipity. This will help banish claims that MKIs are merely "dirty", nonspecific drugs with insufficient specificity for treating a wider range of human diseases.

Biography

Richard Morphy completed his Ph.D. at Durham University (U.K.) under the direction of Professor David Parker working on macrocycle synthesis and conjugation to monoclonal antibodies. In 1989 he joined Celltech Ltd. in Slough (U.K.) as a medicinal chemist working on a variety of oncology and inflammation projects. In 1995, he joined Organon Laboratories in Scotland as a team leader in the CNS area, and he is currently a section head in the Medicinal Chemistry Department at Schering Plough's Newhouse site. His involvement with a number of CNS and non-CNS projects over the years has led to a keen interest in the challenging area of discovering multitarget ligands.

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