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Reciprocal chemical genetics: correlation between chemical profile of *in vivo* phenotypes and *in vitro* data of potential target genes leads to swift target identification.

Introduction

Biologists try to elucidate biochemical processes within a cell, tissue or organism. Any biological phenotype will be the result of multiple components participating in complex networks. Genetic screens can be used to unravel such networks. The recent development of RNA silencing technologies has made great impact on the development of such genetic screens. In the last decade chemical genetics has come to the fore as a complementary technology. Chemical genetics employ small molecules for studying biological processes even though the molecular targets for these compounds may be poorly defined. Merging the field of genetics and cell biology with chemistry can identify compounds and their corresponding targets for a particular phenotype. Such combinations should allow swift lead development in areas not obvious from defined target-based screens. In this highlight we describe the reciprocal chemical genetics pipeline allowing rapid phenotype/genotype/target identifications as the result of integrated chemistry, cell biology, biochemistry and RNA silencing strategy. The merits of reciprocal chemical genetics are demonstrated by the definition of novel antibiotic targets and corresponding leads.

Screening[1–4]

Genetics-based screens are used to elucidate biological processes and can be divided into *forward genetics*[5] and *reverse genetics*. *Forward genetics* follows a three-step process to identify proteins involved in a defined biological process. Firstly, an assay is developed to report the selected biological process. Subsequently, random mutagenesis is performed to identify phenotypes that have affected this biological process. Finally, the mutated (target) gene is identified. With *reverse genetics* the function of a gene of interest is elucidated after genetic alteration. The gene of interest can be suppressed in expression (*e.g.* knockdown mutation) or altered in its activity by selective mutations (Fig. 1).

The approach where small molecular compounds are used to modify biological processes by interfering with protein function is called *chemical genetics*. Chemical genetics comes in two flavors, namely, *forward chemical genetics*[6,7] and *reverse chemical genetics*.[8] *Forward chemical genetics* follows a similar three-step procedure as forward genetics: assay development, random compound screening and target identification. *Reverse chemical genetics* starts with a protein (gene product) of interest for which small molecules are sought. Such compounds are then further modified to generate high affinity and specific inhibitors.

Reverse genetic screening

Before drug development for any disease can start it is important to know which gene or class of genes should be targeted. Once a target gene is known, reverse chemical genetics can be applied to identify a drug. Targets can be identified by classical cell biology, exemplified by the identification of PKB/Akt[9] or by genetic screening. Target identification with forward genetic screening is more laborious than with reverse genetics, but in principle straightforward. Forward genetic screening has already been covered in excellent reviews[10–13] and will not be further discussed. With the arrival of the gene silencing techniques employing shRNA (short hairpin RNA) or siRNA (small interfering RNA), expression of

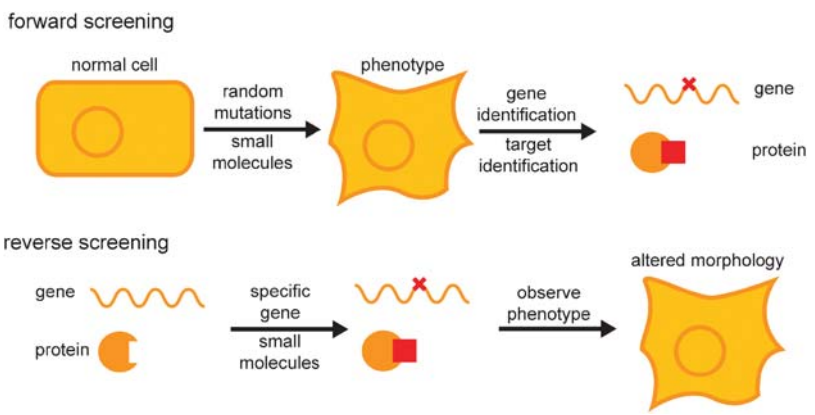

Fig. 1 Overview of screening methodologies. Forward screening starts with creating a phenotype by inhibiting or inactivating a target, followed by target identification. Reverse screening selectively inhibits/inactivates a target (usually in *in vitro* assays) before the effect is assayed in biological systems.

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specific gene products can be downregulated. Reverse genetic screening with the aid of gene (actually transcript) silencing with siRNA has become popular now efficient silencing techniques have become available. With the availability of genome-wide shRNA or siRNA libraries involvement of proteins in a particular cell process can thus be systematically assessed. With this reverse genetics approach target identification can be swift, which does not imply that such targets are druggable however. Reverse genetic screens to identify proteins involved in cell division have been performed by various laboratories. Kittler *et al.*[14] describe how high content (microscopy based) screening cannot only identify genes in cell division, but also places these genes into specific functional clusters. This functional assignment can be achieved by measuring multiple parameters followed by hierarchical clustering. Another study investigating cytokinesis[15] performed a parallel siRNA screen and chemical genetic screen to identify both target and lead. The biological effect of the compound (Binucleine 2) identified mimicked siRNA mediated knockdown of Aurora B which is involved in cytokinesis. This suggested that Binucleine 2 acts in the Aurora B pathway though not necessarily directly on Aurora B. These two examples of reverse genetic screens illustrate the feasibility of target identification in defined biological processes of interest. The route to specific drugs then still has to begin.

Forward chemical genetics

The cell cycle has been the subject of many chemical genetic screens because of the potential use of identified compounds in cancer treatment. Many of the chemical genetic screens performed are live/dead screens. Large libraries of chemical compounds are added to bacteria/yeasts or mammalian cells and effects on cell viability are measured. This approach has yielded many drugs used in the clinic today such as taxol and doxorubicin. Young *et al.*[16] did not use a binary (live/dead) but a multiparameter readout to identify such compounds. Parameters such as nuclear size, DNA replication and chromosome condensation were extracted from microscopy images and used to cluster chemical compounds with a similar mode of action. After screening 6547 compounds, the authors found that structurally unrelated compounds can occasionally lead to similar phenotypes. Similar compounds however usually yielded similar phenotypes as assessed by the multiparameter readout. High content (microscopy based) chemical genetic screening can give insight in the detailed mode of action of classes of compounds, but without information on the actual target.

The bottleneck in many chemical genetic screens is target identification. A biochemical approach is often used where small molecules are modified for cross-linking or affinity purification of targets.[17] This method has two drawbacks. Firstly, modifications of small molecules may affect affinity and specificity of that compound for the target protein. Secondly, identification of target proteins can be difficult for low abundant proteins. Other methods can be applied but may lack specificity as well without further validation. These include phage display, yeast three-hybrid and protein microarray-based assays. A genetic approach can also identify the drug target. The compound then does not require modifications but should yield a biological effect allowing high throughput analyses for genetic screening. Most genetic drug screenings have been performed in yeast and often screen for genetic enhancers or suppressors of the small molecular compound. Although yeast is a well-established model system for many biological processes, proteins and pathways obviously differ from higher eukaryotic organisms which could affect drug specificity. In addition, yeast has four times fewer genes than humans and many (human) targets may thus be missed. Sometimes yeast libraries expressing human genes are used[18] but the biological readout may then be complicated when these genes do not interfere in yeast biological pathways.

Genetics and chemistry: reciprocal chemical genetics

Genetic screens lack the promiscuous effects of small molecules that hardly ever are entirely specific for one target only (which may be even impossible to prove). Still chemical genetics has advantages. Small molecules can be easily applied to different cell types or organisms whereas genetics usually requires labor-intensive genetic modifications in each cell type or organism tested. In addition, mutations are unconditional. The function of a gene cannot be turned on or off at will (inducible systems do exist, but their mode of action is slow compared to that of small molecules). Furthermore, cells may compensate for genetic inactivation of a gene by expressing alternative proteins for their survival. Small molecules are conditional, which means they may be applied or removed with great temporal precision, for instance in a time span in which cells may fail to compensate for the inactivation of a target protein. Finally, temporal application of a small molecule that inhibits a gene product does not have to be lethal, whereas genetic deletions can be lethal to the cell or organism. Genetic and chemical screenings thus have complementary (dis)advantages, and ideally should be used in complementary assays to identify and validate essential cell biological pathways.

By combining forward *chemical* genetic screens with reverse *genetic* screens, many of the problems outlined above in identifying targets and chemical leads can be solved. The two techniques thus combine overlapping benefits with non-overlapping disadvantages. This approach that we term *reciprocal chemical genetics* allows rapid and simultaneous identification of both drug and target. Of note, inhibition of a target by chemical compounds should yield similar effects as silencing the target. Once a lead compound is identified in a forward chemical genetic screen, potential targets can be identified with genome-wide shRNA or siRNA libraries or libraries containing the genetic family corresponding to the compound clan (*e.g.* the 650 members of the human kinome, when the compound is a kinase inhibitor-like molecule). Usually multiple gene targets are identified and *in vitro* assays are required to determine the genuine target. For further validation, derivatives can be synthesized of the lead molecule. Derivatives are expected to have similar but not identical effects. The resulting structure–activity relationship (SAR) of the lead and derivatives in *in vitro* assays against possible targets is then cross-correlated with the effects of the lead and derivatives in

in vivo assays. A correlation is then expected between the *in vitro* results of the lead + derivatives for the genuine target and the effects on cell systems. The SAR of the lead structure is instrumental in target identification and validation within the reciprocal chemical genetics approach (Fig. 2).

Proof-of-principle: kinase inhibitors as novel antibiotics

With the emergence of multidrug resistant (MDR) bacteria, new intervention strategies have to be developed.[19] Current antibiotics target pathogen- rather than host-specific biochemical pathways and only few potential bacterial pathways are as yet unexplored for antibiotic development.[20] If host proteins are critical for bacterial infectivity or intracellular survival, in principle many more proteins are available for anti-bacterial drug development. Pathogenic bacteria that reside or multiply in the host cell are known for their ability to manipulate host cells to survive.[21] If host processes are activated by intracellular bacteria for survival, inhibition of such pathways may be an option to eliminate these bacteria. The interplay between host and pathogenic proteins is the topic of many studies but has,[22,23] until now, not yielded usable drug candidates.

S. typhi that causes typhus and *M. tuberculosis* causing tuberculosis are bacteria that survive inside the host cell (mostly macrophages). Many intracellular pathogenic bacteria survive in a membrane-enclosed endosomal compartment called the phagosome.[24,25] Phagosomes containing dead bacteria are usually transported to—and fuse with—lysosomes where bacteria are degraded along with material derived from the extracellular space. Obviously, bacteria aim to prevent entering such lysosomes for survival.[25] Indeed, bacteria actively avoid these compartments by employing different mechanisms of host cell manipulation. Targets for bacteria include motor proteins,[26,27] small GTPases[28] and kinases as they are likely involved in controlling transport of phagosomes to lysosomes. Kinases are a well-studied group of proteins and a large number of kinase inhibitors have been developed in academia and pharmaceutical industry. Kinases are druggable and various kinase inhibitors are in Phase I and II trials as oncology drugs. In fact, inhibitors of the kinase Abl are applied for treatment of leukemia (CML) patients.[29] Kinase inhibitors may however have broader applications beyond cancer treatment, for example as antibiotics when kinases are defined as involved in intracellular survival of pathogens.

This was studied using an interdisciplinary approach combining cell biology, bacteriology, biophysics, high throughput siRNA screening with automated microscopy and organic chemistry. First the effects of a series of well-known kinase inhibitors on intracellular growth of *Salmonella* were tested. Only one kinase inhibitor (H-89) eliminated intracellular *Salmonella* without any effect on the viability of the infected cell. H-89 (Table 1, small molecule **1**) is an often-used Protein Kinase A (PKA) inhibitor, but is known to target other kinases as well. In fact, another PKA inhibitor (rp-cAMP) did not affect intracellular *Salmonella* growth suggesting that kinases other than PKA are involved in this process.

Subsequently H-89 and ten H-89 derivatives were tested for inhibition of intracellular growth of *Salmonella* in human macrophages. The chemical profile or SAR indicated that only particular H-89 variants affected the kinase of interest, the genuine target. To further assess whether the putative kinase was only active on *Salmonella* or whether a more general cellular mechanism was targeted, the same set of H-89 variants was tested for inhibition of two unrelated intracellular bacteria: *M. smegmatis* and *M. tuberculosis*. Identical chemical profiles were found suggesting that particular kinase(s) were critical for intracellular survival of a set of bacterial pathogens and that a general cell biological mechanism was targeted by H-89 and some variants.

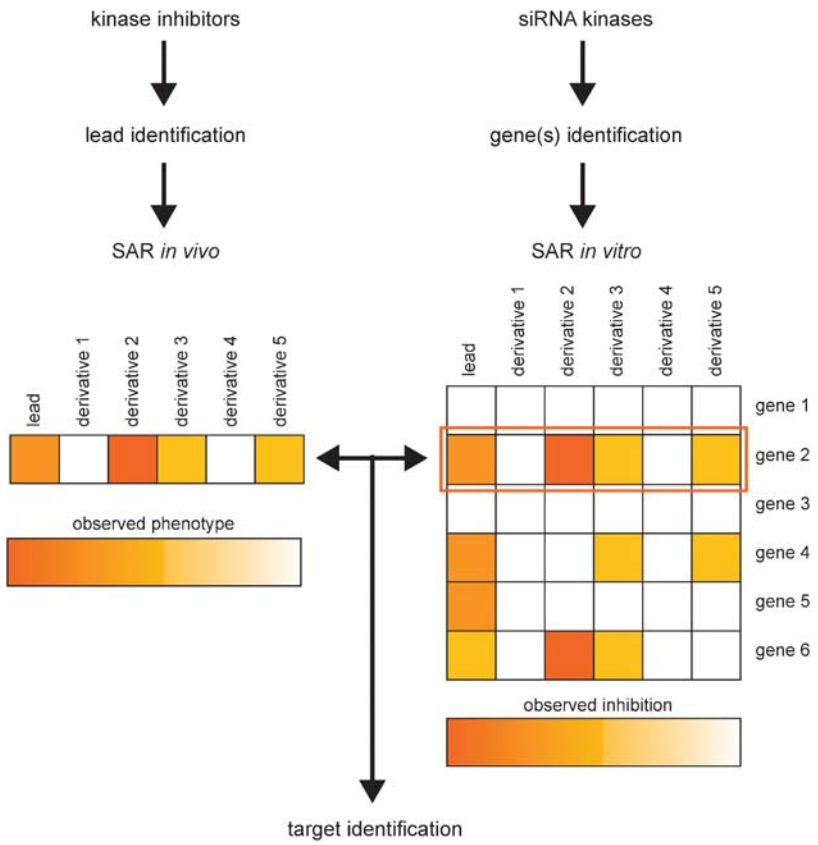


Fig. 2 Flowchart for target identification with reciprocal chemical genetics exemplified for kinases. The SAR from *in vivo* assays of the lead identified in a forward chemical genetic screen is compared to the SAR from *in vitro* assays with purified enzymes identified in an siRNA based screen. The genuine target is identified when the observed SAR from *in vivo* experiments with the small molecules correlates with the observed *in vitro* SAR of one of the identified genes in the siRNA screen (gene2 in this example).

Table 1 ATP competitive PKB/Akt inhibitors

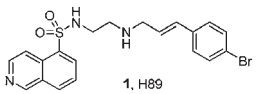
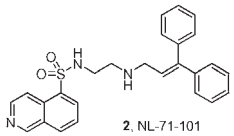
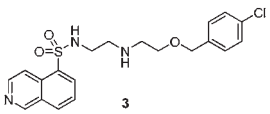
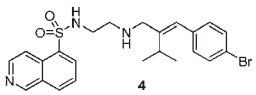
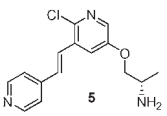
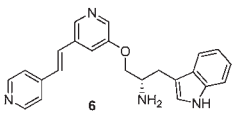
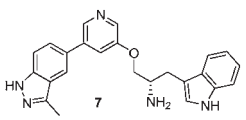
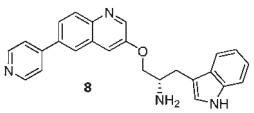
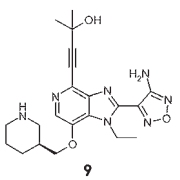
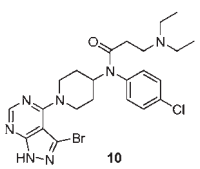
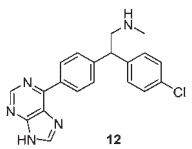
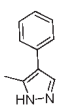
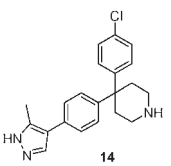
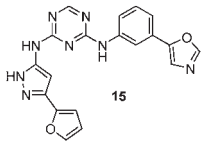
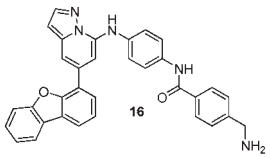
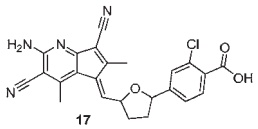
Lead structure	IC_{50}/nM[a]	Hit structure	IC_{50}/nM
 1, H89	PKA: 35 AKT: 2500	 2, NL-71-101	PKA: 9000 AKT: 3700
		 3	PKA: 170 AKT: 260
		 4	PKA: >10 000 AKT: 1500
 5	AKT1: 5290	 6	PKA: 38 AKT1: 14
		 7	PKA: 6.3 AKT1: 0.16
		 8	PKA: N/A AKT1: 300
		 9	PKA: N/A AKT1: 2 AKT2: 8 AKT3: 9
		 10	AKT: <50
 11		 12	PKA: 15[a] AKT2: 10
 13	AKT2: 80 000	 14	PKA: N/A AKT2: 18

Table 1 (*continued*)

Lead structure	IC_{50}/nM[a]	Hit structure	IC_{50}/nM
		 **15**	AKT3: 1000[36]
		 **16**	
		 **17**	AKT1: 2600

Structures **1–4** are described in the text. **5** bis-pyridinylethylene,[37] modification yielded **6** with 15-fold preference for Akt1 compared to other PKB/Akt isoforms. Replacement of the terminal pyridine with a methyl substituted indazole moiety in **6** resulted in **7**[38] with improved activity (not selectivity). Modifications of the central pyridine ring in **5** did not improve (compound **9**[39]) or even reduced PKB/Akt inhibition (**8**[40,41]). A related series of compounds with the central pyridine replaced by piperidine (**10**[42]) are active against PKB/Akt but not selective. Structure-based design resulted in 6-phenylpurine **11**[43] and phenylpyrazole **13**[44] based PKB/Akt inhibitors. **12** and **14**, were designed starting from either purine or pyrazole as hinge region binders, extended with a building block resembling H-89 (**1**). Pyrazolopyrimidine **16**[45] was found to inhibit Akt1 with low micromolar activity. Virtual screening of 50 000 compounds and experimentally testing the 200 best hits resulted in the identification of **17** as a PKB/Akt inhibitor.[46] [a] Tested as racemate.

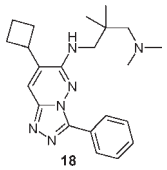
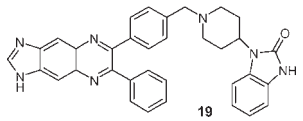
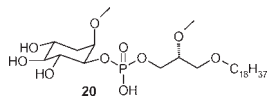
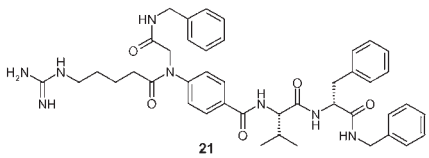
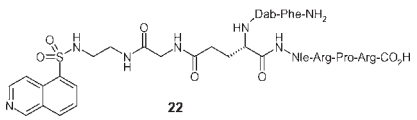
Genetic screens were subsequently employed to identify this kinase. The chemical inhibitors already revealed that inhibition of the target kinase allowed infection without notable effects on cell survival. This was critical information for the genetic screen because it showed that long lasting downregulation of 'our' target by siRNA silencing techniques could be combined with infection. The chemistry was thus used to define the conditions of the genetic screen. Since the chemistry also pointed towards the class of enzymes (kinases) involved in controlling intracellular infection of bacterial pathogens, only the 'kinome' was silenced. Therefore 650 pooled siRNAs selectively silencing each kinase in the human genome were tested for effects on intracellular *Salmonella* growth. Cells were first transfected with the different siRNAs and infected with GFP-expressing *Salmonella* two days post-transfection (these two days are required for target proteins to 'disappear' and usually reflects two to four times their natural half-life). The effects on intracellular bacterial growth were monitored by automated microscopy, luminometry and FACS. This reverse genetic siRNA screen yielded ten kinases that reduced intracellular salmonella growth. The ten kinases were subsequently tested for interactions using programs containing updated literature information and allowing network formation (like Ingenuity or STRING). The ten kinases clustered in one network only, a network around the kinase PKB/Akt1. This suggests that the PKB/Akt1 pathway is critical for intracellular bacterial survival, at least for the bacterial species tested.

Then the actual target for H-89 was defined. Of the ten kinases identified in the genetic screen, five were selected (based on sequence homology to PKA) for *in vitro* SAR. The inhibition profile from *in vitro* kinase assays was subsequently cross-correlated with the biological effects of the compounds and only one kinase significantly cross-correlated: PKB/Akt. Due to the efforts of the pharmaceutical industry and academia to develop PKB/Akt inhibitors, the biological effects on control of bacterial infection could be confirmed with a competitive (Table 1, **2**) and non ATP competitive PKB/Akt inhibitor (Table 2, **19**), strengthening the feasibility of our reciprocal chemical genetics approach. Thus, moving from chemistry-to-chemical profiling-to-genetic screenings-to-biochemistry yielded a chemical lead, structural variants and a target critically involved in control of intracellular growth of a series of bacterial infections. One H-89 variant was further tested for biological activity in mice infected with *Salmonella typhimurium*. A delay in infection was observed without notable effects on tissues or mice, but the H-89 variants obviously have to be improved for further *in vivo* experiments.

The protein kinase PKB/Akt and the development of inhibitors

PKB/Akt provides survival signals in many tumor cells. Inhibiting these survival signals may prevent proliferation of cancer cells. The pharmaceutical industry has therefore great interest in developing small molecule inhibitors for PKB/Akt. Besides the treatment of cancer, these inhibitors might be applied in fighting infectious disease. The development of small molecule PKB/Akt inhibitors is mainly focused on ATP competitive

Table 2 Non ATP competitive PKB/Akt inhibitors

Structure	IC_{50}/nM	Target
 18	AKT1: 4600 AKT2: >250 000 AKT3: >250 000	PH domain
 19	AKT1: 58 AKT2: 210 AKT3: 2119	PH domain
 20	AKT: 4.100	PH domain
 21	AKT1: 1400	Substrate binding groove
 22	PKA: 210 AKT1: 70	ATP pocket and substrate binding groove

Allosteric inhibitors **18**[30] and **19**[47] target the PH domain of PKB/Akt with good potency and excellent selectivity. Compounds mimicking the natural ligands for the PH domain are composed of a polyhydroxylated carbacycle linked to a lipid tail *via* a phosphate or carbonate group (**20**[48,49]). Small molecule **21** mimics the PKB/Akt substrate binding groove.[50] A combination of strategies is exemplified by compound **22**, which contains an ATP pocket binder and a substrate mimetic.[51]

inhibitors targeting the ATP-binding site. Other domains of PKB/Akt are being explored as potential targets for inhibitors; these include the Pleckstrin Homology domain, located at the N-terminus, and the substrate-binding site located at the C-terminus of PKB/Akt. Several factors make the ATP binding site of PKB/Akt a challenging target for inhibitors. The ATP pocket of PKB/Akt shows high sequence homology with other kinases of the AGC kinase family.[30,31] The strong homology between the three PKB/Akt isoforms and in particular the ATP-binding domain, makes this even more challenging when aiming to generate isoform specific inhibitors.[32] This is especially important in fighting infectious disease as simultaneous inhibition of Akt1 and Akt2 will be lethal. Despite these challenges, several lead structures have been defined as PKB/Akt inhibitors. One lead structure is H-89 (Table 1, **1**) which has selectivity for Akt1 and 3 over Akt2. Notably, H-89 is also a potent PKA inhibitor, since the ATP-binding site is very similar to that of PKB/Akt. H-89 has been modified in different regions of the core structure as exemplified by **2**,[33] **3**[34] and **4**.[35] To generate higher affinity inhibitors and inhibitors more selective for PKB/Akt over PKA various H-89 homologues have been tested for chemical profiling. Modification of the isoquinoline moiety or the secondary amine abrogated PKB/Akt inhibition, whereas modifications in the bromophenyl part effected affinity but not selectivity over PKA. The introduction of bulky substituents on the double bond increased specificity for Akt1 over PKA. Further modifications are required to arrive at high affinity and more specific Akt1 inhibitors.

Several pharmaceutical companies have recently disclosed information on PKB/Akt inhibitors. Screening large compound libraries has yielded several promising lead structures, which are now further modified to create more potent and selective PKB/Akt inhibitors (Table 1). The compounds exemplify a wide range of PKB/Akt inhibitors with a similar SAR. They are characterized by a hinge binder, a crucial basic amine functionality, a connecting group, and in most cases a substituted aryl moiety to improve activity. A few compounds that dock in the ATP binding site of PKB/Akt, do not fit in this profile as exemplified by **16** and **17**. These novel structures may be used as further building blocks for modification to improve the PKB/Akt inhibitors.

Although some very potent ATP competitive PKB/Akt inhibitors have been published, none of these have pharmaceutically desirable selectivity with respect to other members of the AGC kinase family, let alone between the three PKB/Akt isoforms. Given the high degree of homology of the ATP binding pocket between these kinases, this is not surprising. One strategy to improve selectivity is to target other, more distinctive, regions of the kinase like the PH

domain or the substrate binding groove. Akti-1/2 (**19**), for example, inhibits PKB/Akt activity by binding to the PH domain.[47] This PKB/Akt inhibitor blocks intracellular growth of *Salmonella* in analogy to H-89 (**1**) and NL-71-101 (**2**).

Conclusion

High throughput gene silencing with siRNA libraries now allows identification of protein targets involved in defined biological processes. Chemical libraries can be screened for overlapping phenotypes. Combining these technologies then defines potential targets for chemical leads. If the lead is further modified for chemical profiling, then the profile can be used for *in vitro* enzyme assays to definitively identify the target of the compounds. This flowchart allows rapid identification of lead and target and, since chemistry and genetics are combinatorially applied, is termed reciprocal chemical genetics. Currently the number of lead compounds and genes identified in chemical genetic and siRNA screens may be overwhelming. It is therefore often helpful to reduce the number of lead compounds and genes for follow-up in subsequent assays. In the near future high content (multiple parameters) screening with compounds and siRNA for the desired phenotype should be able to achieve this reduction. The multiple parameters (*e.g.* nuclear size, nuclear shape, vesicle distribution *etc.*) obtained in a chemical genetic screen exemplified by Young *et al.*[16] can be cross correlated with the same parameters obtained in a genetic screen (*e.g.* siRNA or shRNA). Lead compounds and genes resulting in similar parameter values are then selected for further reciprocal chemical genetic screening.

In a proof-of-principle study, we thus identified Akt1 as a key target in the inhibition of intracellular growth of pathogenic bacteria like *Salmonella* and *M. tuberculosis* by H-89 derivatives. Since PKB/Akt inhibitors are already in various phases of development as anti-cancer drugs, Akt1 inhibitors may successfully enter clinical practice. Such drugs may then not only be applied as anti-cancer drugs but also as antibiotics. Since PKB/Akt defines a new class of antibiotic targets (a host target rather then one of the pathogen) multidrug-resistant bacteria will still be susceptible to these compounds, as indeed shown by us for MDR-*M.tuberculosis*.[35] Given the growth in numbers of patients with multidrug-resistant bacteria, definition of new targets and corresponding inhibitors is urgent.

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