Published in final edited form as:

Curr Top Med Chem. 2009 ; 9(13): 1159–1171.

Cancer and Virus Leads by HTS, Chemical Design and SEA Data Mining

Pahk Thepchatri^{a,b,e}, Jaeki Min^{a,b,e}, Thota Ganesh^{a,b,e}, Yuhong Du^{a,c}, Iestyn Lewis^{a,c}, Serdar Kurtkaya^{a,b}, Andrew Prussia^b, Lian Li^a, Richard K. Plemper^d, Haian Fu^{a,c}, Dennis C. Liotta^{a,b}, James P. Snyder^{a,b}, Raymond Dingledine^{a,c}, and Aiming Sun^{a,c,e,*}

^aChemical Biology Discovery Center, 1510 Clifton Road, Emory University, Atlanta, GA 30322

^bDepartment of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, GA 30322

^cDepartment of Pharmacology, Emory University, 1510 Clifton Road, Atlanta GA 30322

^dDepartment of Pediatrics, Division of Pediatric Infectious Diseases, Emory Children's Center, 2015 Uppergate Drive, Emory University School of Medicine, Atlanta, GA 30322

Abstract

A variety of medicinal chemistry approaches can be used for the identification of hits, generation of leads and to accelerate the development of drug candidates. The Emory Chemical and Biology Discovery Center (ECBDC) has been an active participant in the NIH's high-throughput screening (HTS) endeavor to identify potent small molecule probes for poorly studied proteins. Several of Emory's projects relate to cancer or virus infection. We have chosen three successful examples including discovery of potent measles virus RNA-dependent RNA polymerase inhibitors, development of Heat Shock Protein 90 (Hsp90) blockers and identification of angiogenesis inhibitors using transgenic Zebrafish as a HTS model. In parallel with HTS, a unique component of the Emory virtual screening (VS) effort, namely, substructure enrichment analysis (SEA) program has been utilized in several cases.

INTRODUCTION

High throughput screening, when coupled to synthetic chemistry and computational analysis, has become an effective tool in the hit-to-lead drug discovery process on the path to the clinic. Until recently, the practice was confined to the pharmaceutical industry. However, with the emergence of highly efficient screening hardware, easily accessible compound libraries and user-friendly informatics and molecular modeling tools, the methodology has moved beyond the industrial sector [1, 2]. As a result, the NIH has made an extraordinary effort to engage academic institutions and government (NCGC) in the practice as part of the NIH roadmap to understanding and exploiting the human genome. The Emory Chemical and Biology Discovery Center (ECBDC) has been an active

participant in the NIH's high-throughput screening (HTS) endeavor to identify potent small molecule probes for poorly studied proteins during the past 3 years. Several of Emory's projects relate to cancer or virus infection. Diagnostic assays have not only included target-specific screens (heat shock protein 90 (Hsp90), estrogen α co-activator binding inhibitor, 14-3-3 protein), but also phenotypic screens (measles virus inhibitors, angiogenesis blockers in transgenic zebrafish). In addition to high-throughput screening, the Emory center integrated strong synthesis and molecular modeling teams into the program. In parallel with HTS, virtual screening (VS) has been pursued in each project. *De novo* design approaches have also been used in several appropriate cases. We have chosen three successful projects involving both phenotypic and non-phenotypic screening to illustrate the work and the philosophy behind it.

Discovery of Potent Measles Virus RNA-Dependent RNA Polymerase Inhibitors

Measles virus (MV) is one of the most infectious pathogens known. In spite of the existence of a vaccine, approximately 350,000 deaths/year result from MV or associated complications. To date, Ribavirin is the only drug available for the treatment of some paramyxovirus infections [3, 4]. It has been used experimentally for the treatment of measles but with limited efficacy [5]. Recently, benzimidazo-thiazole derivatives have been reported to be more potent and less cytotoxic compared with Ribavirin against the Leningrad 16 strain, when assessed in Vero (African green monkey kidney carcinoma) cells. The most active compound in this series demonstrated a selectivity ratio (CC_{50}/EC_{50}) of 245.5 compared with 14.4 for Ribavirin. [6].

In previous work we reported the structure-based development of a MV entry inhibitor AS-48 with an EC_{50} of 0.6–3.0 μ M as a shelf-stable alternative (Fig. 1) further attempts to increase the activity within this series of compounds proved to be problematic [7, 8]. As a consequence, we broadened our search by turning to cell-based high throughput screening (HTS) to capture small molecules capable of netting both entry inhibitors as well as compounds operating against other proteins critical for viral infection and reproduction. The exercise identified 1-methyl-3-(trifluoromethyl)-*N*-[4-(pyrrolidinylsulfonyl)phenyl]-1H-pyrazole-5-carboxamide **1** (SID:16677) (Fig. 1) (EC_{50} = 250 nM) as a well-behaved, target-specific inhibitor of MV replication [9]. Bypassing the fusion protein, **1** represents a first-in-class non-nucleoside inhibitor of the MV RNA-dependent RNA polymerase (RdRp) complex. Singly or in combination with the fusion inhibitors, this novel compound class has high developmental potential as a potent therapeutic against MV and can possibly be employed towards further mechanistic characterization of the viral polymerase complex.

Hit Re-synthesis and SAR Study

Following-up secondary assays and independent chemical synthesis of compound **1** (16677) have demonstrated that the compound represents novel, highly potent non-nucleoside inhibitors of the MV polymerase complex with desirable drug-like properties. Hit compound **1** was prepared by coupling of 1-methyl-3-trifluoro-pyrazole-5-acetyl chloride **2** [10] with 4-amino-prolidinyl sulfonamide **3** in very good yield using pyridine as base (Scheme 1).

A structure-activity profile has begun to emerge by examination of the four molecular fragments of compound **1** circumscribed in Fig. 1; namely the pyrazole ring on the right, the central phenyl ring, the amide linker and the pyrrolidine ring to the left. Around 100 analogs have been synthesized by modification of the four different parts of the hit. Modification of the pyrrolidine ring proved to be effective for generating active analogs. Several compounds show low nanomolar activity with very low toxicities. SAR studies of compound **1** have been described in great detail a summary of which is given below [11, 12]. Several representative compounds chosen from the previous publications are listed in Table 1 along with their antiviral activities and cytotoxicities. The best compounds in the series exhibit IC₅₀ around 10 nM with low cell toxicity.

Molecular Field Topology Analysis (MFTA) QSAR

A quantitative structure-activity relationship (QSAR) was developed from analogs of **1** using molecular field topology analysis (MFTA) [13, 14] this 2-D QSAR method was used to perform a topological analysis for all analogs in the training set (26 compounds), generating a molecular supergraph (Fig. 2A) with descriptor values for each compound mapped at each atom vertex. MFTA was then used to perform PLS-based correlation statistics to generate predictive QSAR models. Leave-25%-out cross validation allowed assessment of predictivity for a variety of descriptor sets. The best model ($Q^2=0.66$, $R^2=0.77$, RMSE=0.49) was obtained with descriptors for Gasteiger-Marsili atomic charge (Q), the effective environment van der Waal radius (R_e), and group lipophilicity based on the sum of the Ghose-Crippen atomic contributions for an atom and attached hydrogens (L_g) (Fig. 2B, blue points). An explicit test set (7 compounds) with a range of activities and diverse structures was mapped onto the previously generated molecular supergraph and their descriptor values calculated. These values were applied to the QSAR equation and the test set activities were predicted reasonably well ($R^2=0.45$, RMSE=0.86) (Fig. 2B, red points).

In conclusion, high-throughput screening followed by chemical synthesis has provided the first highly potent small molecule blockers of the measles virus accompanied by low cell toxicity. Synthetic modification of hit compound **1** yielded several derivatives bearing sub-nanomolar activity with low toxicity. The best compound in this series, namely the eight-membered ring analog **1c**, shows an EC₅₀ around 5 nM with no detectable toxicity. This behavior is captured by a three-descriptor QSAR model developed using molecular field topology analysis (MFTA). This novel compound class can possibly be employed towards further mechanistic characterization of the viral polymerase complex and has high developmental potential as a potent therapeutic against MV.

Identification of Heat Shock Protein 90 Inhibitors by FP assay

The heat shock protein 90 (Hsp90) is a ubiquitously expressed molecular chaperone. In cells, Hsp90 functions as a multiprotein chaperone complex with co chaperones such as Hsp40, Hsp70, HOP, TRP1 and TPR2A [15, 16]. Intrinsically Hsp90 possess the ATPase activity. Upon ATP binding and hydrolysis, the co-chaperone complex turns into a mature chaperone complex with additional protein members such as p23, which then catalyses the conformational maturation of client protein substrates [17]. These client proteins are

involved in various cellular pathways such as cell-cycle regulation, signal-transduction and apoptosis. For example, several signature cancer proteins such as Akt, Raf-1, HER-2, ErbB2 and p53 heavily depend on the Hsp90-chaperone complex for their stability and maturation [18]. As a result, Hsp90 has emerged as an important biological target for the treatment of cancer, while its implications in neurodegeneration, viral and fungal infections are being explored [19].

Numerous efforts are underway to find inhibitors of Hsp90 and its chaperone function. So far, the natural product geldanamycin analog, 17AAG is showing promising anti-cancer activity in clinical trials [20] while several other compounds derived from purely synthetic sources have also generated lead compounds for clinical evaluations [21, 22, 23, 24]. These compounds were found to bind at the same ATP-binding pocket located at the N-terminal domain of the Hsp90. They exert their anti-proliferative activity through Hsp90 inhibition, followed by subsequent degradation of Hsp90 client proteins.

Various bioassays have been developed to identify novel inhibitors of Hsp90 [25]. Most of these assays are amenable to HTS, since they report the interaction of a small molecule with recombinant Hsp90 α or β . These proteins are derived from yeast or human normal cells, respectively, in which Hsp90 is found to be in a latent, low-affinity form, compared to the high-affinity state present in tumor cells [26]. One way to determine the therapeutically relevant state of Hsp90 is to develop an assay that makes use of lysates derived from human cancer cells instead of recombinant protein. The former permits direct measurement of the interaction between small molecule and tumor-specific Hsp90. Such an assay is capable of leading to identification of molecules that are specific for tumor cell Hsp90. Under the MLSCN program, we developed such an FP assay for HTS and screened 65,000 compounds (early NIH-DPI collection). The screen generated 223 primary hits at a single concentration. Only 6 of the compounds shown in Fig. 3 passed the dose response test with an IC_{50} in the sub-micromolar to low micromolar range (Table 2).

Compound **1**, known as quinocide, was reported to be a promising anti-malarial agent [27]. It's simple and modular structure is ideally suited to treatment by combinatorial methods. Hit compounds **2** and **3** are analogs of the antitumor drug doxorubicin and have been well studied. Due to the complexity of analog synthesis, this molecular class was not given a priority for synthetic attention in our center. The pyrimido-1,2,4-triazine-5,7-dione class of compounds, **4** and **5**, are known for their redox-based inhibition when using DTT, DCEP and other non-thiol-reducing agents [28]. The series is promiscuous and constantly selected as "phony hits" in high-throughput screening. Compound **6** was likewise judged to be an unattractive candidate for investigation. Nonetheless, we placed hits **2–6** in the NIH market place for consideration by the MLSCN community as potential entities for further exploration.

For the optimization of hit **1**, we performed 2D-Tanimoto similarity searching [29] in several commercial databases followed by selective compound purchase. Designed derivatives were also synthesized as depicted in Scheme 1 based on the 6-methoxy-8-aminoquinoline scaffold of hit **1**. A number of 6-methoxyquinolines and 6-hydroxyquinolines with 8-alkylamino chains were identified as potent Hsp90 inhibitors

during this optimization process. Some of these were provided by courtesy of the NCI. From this set, the four compounds shown in Fig. 6 have emerged as active in both FP and WB assays with IC₅₀ values in the low micromolar range (Table 2). However, the Ki values derived from the FP assay for compounds **14** and **15** were in the low nanomolar range, while **12** and **13** showed micromolar Ki value (Fig. 4).

Although the NCI compounds **12–15** showed western blot activities in the low micromolar range (1–4 μ M), only compound **15** has reproduced this activity by degrading the HER-2 client protein after re-synthesis (Scheme 2). The compound also showed approximately equal IC₅₀ and Ki values measured by the FP assay as shown in Table 2 and Figure 5 [30].

In the meantime to confirm its activity, hit compound **1** was re-synthesized in both neutral and dihydrochloride salt forms using a previously reported procedure [31] to confirm its activity. Surprisingly, both synthesized forms of **7** showed only moderate biological activity in either assay by comparison with the original sample (**1**, collected from Discovery Partner International (DPI)). Likewise all the synthetic derivatives depicted in Scheme 1, including 4-chloroquinocidine **9** and a structural homolog primaquine (**7**), were found to be inactive in the FP assay. To resolve this discrepancy, the activities of several batches of quinocidine HCl stock solutions (stored for various periods of time) were assayed to show increased activity with prolonged storage. The purity of the original DMSO stock solution of quinocidine HCl salt was examined and compared to that of the resynthesized hit compound by LC/MS analysis. By careful analysis of the LC/MS data, one major impurity was detected from the DPI stock solution. Further research to identify, characterize, optimize and biologically evaluate this active degradant is underway and will be published separately.

Molecular modeling was also performed to aid synthetic efforts in optimizing the activity of the aminoquinolines by providing a 3D visualization of the protein-ligand interactions. We have hypothesized that the aminoquinolines bind to the N-terminal region based on their activity in the *in vitro* FP assay, which suggests that they bind competitively with the known N-terminal inhibitor, geldanamycin. X-ray crystallography was used by previous investigators to solve the binding modes of five structurally diverse N-terminal Hsp90 inhibitor classes. All are currently available from the Protein Databank [32]. (PDB Codes: 1YET, 1BGQ, 2BRE, 2BZ5, & 2FWZ) [33, 34]. The observed protein-ligand interactions in these crystal structures were used as guides to model the aminoquinolines into the N-terminal ATP-binding site. Despite the wide structural diversity of these five inhibitors, it was observed that they share interaction in at least two regions of the pocket: a hydrophobic cluster consisting of residues M98, L103, L107, F138, Y139, V150, and W162, and a hydrogen-bonding cluster consisting of Asp93 and crystallographic waters, Wat02 and Wat18 (Numbering based on Protein Databank (PDB) code: 2FWZ).

Schrodinger's Induced Fit protocol [35] was used to dock hit **15** into the ATP-binding pocket of Hsp90. The receptor's atomic coordinates were downloaded from the Protein Data Bank (pdb code: 2FWZ). Restraints were implemented to maintain hydrogen-bonding interaction with crystallographic water 02. All graphic renderings were generated using PyMol (DeLano Scientific).

Results of the induced fit docking for hit compound **15** are shown in Figure 7 overlapped with the experimentally determined pose of the known Hsp90 inhibitor, PU-H71. The latter (green) is a potent Hsp90 N-terminal inhibitor that shows nanomolar activity in the FP and western blot assays and is currently used as a control in both. The X-ray crystal model shows that PU-H71 maintains hydrogen-bonding interactions with Asp93, Thr184 (not labeled), Wat02, and Wat18. PU-H71 also fills the hydrophobic pocket with an aryl group. Docking results suggest compound **15** also maintains hydrogen-bonding interactions with Asp93, Wat02, and Wat18. The substance also fills the hydrophobic cavity with its long alkyl side chain as well as maintaining an additional hydrogen bond with Tyr139. The docking model shows that the planar PU-H71 adenine and compound **15**'s quinoline moieties display planar overlap in the binding site. The model suggests that potential replacement of the alkyl side chain with an aryl group has the potential to maintain interaction with the hydrophobic site as well as to reduce the conformational variability inherent in the long flexible side chain.

To conclude, we have identified hit **1** as an Hsp90 inhibitor by using an HTS based fluorescent polarization assay. Follow-up derivative design, synthesis and SAR study delivered **15** as the most promising inhibitor in the series with IC₅₀ in the low micromolar range in both FP and cell-based western blot assays. The use of aminoquinoline **15** as a lead for further SAR exploration may well yield optimized and therapeutically useful molecules.

Discovery of Novel Angiogenesis Inhibitors Using Transgenic Zebrafish as a High-throughput Screening Model

Angiogenesis plays a significant role in tumor progression and metastasis of the great majority of human solid tumors. Therefore, the angiogenesis process could be an important therapeutic target for the treatment of a variety of diseases, including cancer, macular degeneration, psoriasis, and diabetic retinopathy [36]. For example, solid tumors require an adequate supply of blood vessels in order to survive, grow and metastasize [37, 38]. Thus, as a therapeutic strategy, depriving a tumor of its vascular supply by means of anti-angiogenic agents has attracted attention since its proposal in the 1970s. In addition, a link between angiogenesis and Alzheimer's disease has also been postulated recently possibly opening another clinical use for anti-angiogenesis drugs [39].

Several *in vitro* and *in vivo* angiogenesis assays are currently being used for drug discovery. Each of these assays has distinct advantages and disadvantages [40, 41]. However, most *in vitro* assays are amenable to high throughput screening format but lack the biological complexity of *in vivo* systems. Animal models, including the chick chorioallantoic membrane assay, corneal neovascularization assay and Matrigel plug assay preserve biological complexity but are costly and low throughput. In addition, genetic manipulation, such as gene knockout/knockdown and over expression, is not feasible for most *in vivo* assays. Therefore, there is demand for a novel angiogenesis assay that combines the relevance of an *in vivo* environment with the potential for moderate-to-high throughput drug screening and rapid gene knockdown.

In collaboration with Zygon, LLC, we developed the first quantitative, automated assay for antiangiogenic compound identification using zebrafish embryos. The zebrafish is a well-accepted model for the study of vertebrate development [42]. Transgenic zebrafish expressing green reef coral fluorescent protein (GRCFP), under the control of the vascular endothelial growth factor receptor 2 gene (*VEGFR2*, also referred to as Flk-1 or KDR) for blood vessels restricted expression, were used for this assay [43]. A schematic of the assay is shown in Fig. 8. As a result of screening the LOPAC library (1280 compounds), three hits were identified in this screen, representing a 0.23% hit rate. Two known angiogenesis inhibitors (SU4312, a VEGFR and platelet derived growth factor receptor antagonist, and AG1478, an epidermal growth factor receptor antagonist) were identified as hits. A third compound with no previously known antiangiogenic activity, indirubin-3'-monoxime (IRO, CID 5326739), was also identified.

IRO inhibits angiogenesis in zebrafish embryos with an IC_{50} of 0.31 μ M and inhibits two major components of the angiogenic process, endothelial tube formation and cell proliferation, but does not significantly affect endothelial cell migration in human endothelial cells assay. This part of the work has been included in a previous publication (Table 3) [44].

The hit compound **1** (IRO), and some of its analogs are reported to be inhibitors of glycogen synthase kinase 3 beta (GSK-3 β), cyclin-dependent kinases Cdk1 and Cdk5 [45, 46]. However, their antiangiogenic activity have not been reported previously. Encouraged by this previous result, we commenced structural modification to explore the structural features around IRO that are important for anti-angiogenic activity to discover novel and more potent angiogenesis inhibitors structurally related with IRO. Modifications of IRO were made to both phenyl rings, namely sector A, C, and the 3'-oxime part (sector C) as shown in Scheme 4. A total of 34 analogs were designed and synthesized as a small focused library for testing in the zebrafish angiogenesis assay. According to a published paper, a new class of 3'-substituted 7-halogenoindirubins induced cell death in a diversity of human tumor cell lines, and it was suggested that some indirubins may interact with new, yet unidentified, target proteins, distinct from their well characterized targets such as CDKs and GSK-3 [47]. We applied this strategy in the context of our new design and synthesized a small focused library of indirubin derivatives of 5- or 7-substituted indirubins with various substitutions on position 3' based on the reported synthetic procedure [48]. The chemical structure of IRO, representative modification strategy and synthesis of IRO derivatives are shown in Scheme 4.

During the course of structural modification for IRO **1**, 5- or 7-substituted indirubins (**2**, **3**, and **4**) were identified as potential antiangiogenic agents against the zebrafish angiogenesis model (Table 4). Compounds **3** and **4** were structurally novel and potent. Substitution of fluoride at the 7-position or methyl at the 5-position maintained the antiangiogenic activity with IC_{50} values less than 1 μ M. Adding a bromide group (compound **10**) at the 5'-position diminished activity compared with IRO. Introducing other functional groups such as chloride, nitro, sulphonic acid at the same 5- or 7-position reduced inhibition activity. Interestingly, compound **14** is a well known potent inhibitor of Cdk1 (IC_{50} = 5 nM), Cdk5 (IC_{50} = 7 nM) and glycogen synthase kinase-3 β (GSK-3 β ; IC_{50} = 80 nM) similar to IRO.

However, it was inactive in the zebrafish angiogenesis assay. This implies that the kinase activity might not be related to antiangiogenic activity in this model.

As a conclusion, we identified the antiangiogenic activity of indirubin-3'-monoxime (IRO) using an *in vivo* zebrafish angiogenesis assay by screening the LOPAC library. Utilizing a small focused library which was designed and synthesized based on IRO, we also discovered several novel synthetic analogs which show levels of inhibition similar to IRO against zebrafish angiogenesis. Further biological evaluation of newly discovered synthetic derivatives and ligand-based novel scaffold generation based on IRO is underway to develop more potent antiangiogenic agents.

Substructure Enrichment Analysis (SEA)

When examining the results of a primary HTS screen, such as the Hsp90 primary FP screen shown in Fig. 9, the investigator often establishes an arbitrary cutoff that designates the actives from the inactives. The figure illustrates the existence of around 100 hits between 50–100% inhibition in the present case. In general, we choose those hits above 50% for secondary assay evaluation. While one expects the majority of false positives to fall between the three standard deviation and 50% inhibition lines, many are present above the 50% line as well. Generally, the latter false positives are sufficiently structurally unrelated to be of no informative SAR value for future medicinal chemistry work. Alternatively, it would be ideal to be able to identify groups of compounds that share a common scaffold and send them into secondary assays instead. This would offer medicinal chemistry groups the advantage of correlating varying activity levels with functional group differences observed around a common structural core.

The SEA program is a unique component of the Emory virtual screening effort. It is an approach to discovering molecular fragments or scaffolds that confer an advantage to a group of molecule experimentally screened in a large scale assay. Its current application is to examine primary assay data in such a way as to distinguish structural scaffolds that represent true positives from false positives, which are well known to be highly prevalent in primary HTS screens.

The SEA protocol identifies and aggregates all compounds having a specific shared substructure. The aggregates can be determined to be true positives or false positives based on their biological responses. Aggregates with a range of activity are likely to be true positives since the slight variance in activity may be due to functional changes, an example of which is shown in Fig. 9B. However, aggregates with essentially base levels of activity are most likely inactive scaffold classes (Fig. 9C).

Analysis of the NIH compound library showed that analogs of a known Hsp90 inhibitor resided in the library. This provided an opportunity to test the SEA algorithm. Fragment S11 was used as a query for SEA, and the results of the SEA analysis are shown in Fig. 9D. Compounds containing scaffold S11 vary in their percent inhibition values, suggesting that this scaffold is an excellent lead compound for synthetic workup. In fact, this structural element has been exploited effectively by the Workman group and has spawned a clinical candidate [48].

The first step in implementing SEA is to computationally fragment the structures of the screening library into its scaffold components. The fragment algorithm currently used is available from Pipeline Pilot's fragmentation module [29]. Different fragmentation parameters should be considered to avoid overly simplistic scaffolds (methane, benzene, etc) as well as to keep the scaffolds from being highly complex. Library compounds are then grouped according to a shared scaffold. Finally, the assay data is averaged for each scaffold group to determine whether any scaffold set shows an advantage over other scaffolds in the screen.

The output of the program is a list of mean scores, one for each scaffold, along with a p-value describing the significance of the difference of means. These data may be used as-is to determine which scaffolds show an advantage in a single screen, or the data can be combined with results from SEA analyses on other assays to form a "fingerprint" of the substructure against a range of assays.

ACKNOWLEDGEMENTS

This work was financially supported by the US National Institutes of Health 1 U54 HG003918-02. We also gratefully acknowledge National Cancer Institute (NCI) for the generously supplying the samples listed in Figure 6.

REFERENCES

1. Fox S, Farr-Jones S, Sopchak L, Boggs A, Nicely HW, Khoury R, Biros M. High-throughput screening: update on practices and success. *J. Biomol. Screen.* 2006; 11:864–869. [PubMed: 16973922]
2. Liu B, Li S, Hu J. Technological advances in high-throughput screening. *Am. J. Pharmacogenomics.* 2004; 4:263–276. [PubMed: 15287820]
3. Shigeta S, Mori S, Baba M, Ito M, Honzumi K, Nakamura K, Oshitani H, Numazaki Y, Matsuda A, Obara T, Shuto S, Clercq E. Antiviral activities of ribavirin, 5-ethynyl-1-beta-D-ribofuranosylimida-zole-4-carboxamide, and 6'-(R)-6'-C-methyneplanocin A against several ortho- and paramyxoviruses. *Antivir. Chem. Chemother.* 1992; 36:435–439.
4. Gabrielsen B, Phelan MJ, Barthel-Rosa L, See C, Huggins JW, Kefauver DF, Monath TP, Ussery MA, Chmurny GN, Schubert EM, Upadhy K, Kwong C, Carter DA, Secrist JA III, Kirsir JJ, Shannon WM, Sidwell RW, Kini GD, Robins RK. Synthesis and antiviral evaluation of N-carboxamidine-substituted analogs of 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride. *J. Med. Chem.* 1992; 35:3231–3238. [PubMed: 1507208]
5. Barnard DL. Inhibitors of measles virus. *Antivir. Chem. Chemother.* 2004; 15:111–119. [PubMed: 15266893]
6. Pokrovskii AG, Il'icheva TN, Kotovskaya SK, Romanova SA, Charushin VN, Chupakhin ON. Fluorinated Derivatives of Benz[4,5]-imidazo[1,2-*b*][1,3] thiazole-Inhibitors of Reproduction of Measles Virus. *Doklady Biochem. Biophys.* 2004; 398:285–287.
7. (a) Plemper RK, Erlandson KJ, Lakdawala AS, Sun A, Prussia AJ, Boonsombat J, Aki-Sener E, Yalcin I, Yildiz I, Temiz-Arpaci O, Tekiner B, Liotta DC, Snyder JP, Compans RW. A target site for template-based design of measles virus entry inhibitors. *Proc. Natl. Acad. Sci. USA.* 2004; 101:5628–5633. [PubMed: 15056763] (b) Sun A, Prussia A, Zhan W, Murray EE, Doyle J, Cheng LT, Yoon JJ, Radchenko EV, Palyulin VA, Compans RW, Liotta DC, Plemper RK, Snyder JP. Nonpeptide inhibitors of measles virus entry. *J. Med. Chem.* 2006; 49:5080–5092. [PubMed: 16913698]
8. Plemper RK, Lakdawala AS, Gernert KM, Snyder JP, Compans RW. Structural features of paramyxovirus F protein required for fusion initiation. *Biochemistry.* 2003; 42:6645–6655. [PubMed: 12779319]

9. White LK, Yoon JJ, Lee JK, Sun A, Du Y, Fu H, Snyder JP, Plemper RK. Nonnucleoside Inhibitor of Measles Virus RNA-Dependent RNA Polymerase Complex Activity. *Antimic. Agents Chemother.* 2007; 51:2293–2303.
10. Schlosser M, Volle J, Leroux F, Schenk K. Switchable Reactivity: The site-Selective Functionalization of Trifluoromethyl Substituted Pyrazoles. *Eur. J. Org. Chem.* 2002:2913–2920.
11. Sun A, Chandrakumar N, Yoon JJ, Plemper RK, Snyder JP. Nonnucleoside inhibitors of the measles virus RNA-Dependent RNA polymerase activity: Synthesis and *in vitro* evaluation. *Bioorg. Med. Chem. Lett.* 2007; 17:5199–5203. [PubMed: 17643302]
12. Sun A, Yoon J-J, Yin Y, Prussia A, Yang Y, Min J, Plemper RK, Snyder JP. Potent Non-Nucleoside Inhibitors of the Measles Virus RNA-Dependent RNA Polymerase Complex. *J. Med. Chem.* 2008; 51:3731–3741. [PubMed: 18529043]
13. Palyulin VA, Radchenko EV, Zefirov NS. Molecular Field Topology Analysis Method in QSAR Studies of Organic Compounds. *J. Chem. Inf. Model.* 2000; 40:659–667.
14. Melnikov AA, Palyulin VA, Zefirov NS. Generation of molecular graphs for QSAR studies. *Dokl. Chem.* 2005; 402:81–85.
15. Yi F, Regan L. A Novel Class of Small Molecule Inhibitors of Hsp90. *ACS Chem. Biol.* 2008
16. Chiosis G, Neckers L. Tumor selectivity of Hsp90 inhibitors: the explanation remains elusive. *ACS Chem. Biol.* 2006; 1:279–284. [PubMed: 17163756]
17. Neckers L. Heat shock protein 90: the cancer chaperone. *J. Biosci.* 2007; 32:517–530. [PubMed: 17536171]
18. Shames DS, Minna JD. IP6K2 is a client for HSP90 and a target for cancer therapeutics development. *Proc. Natl. Acad. Sci. USA.* 2008; 105:1389–1390. [PubMed: 18230718]
19. Solit DB, Chiosis G. Development and application of Hsp90 inhibitors. *Drug Discov. Today.* 2008; 13:38–43. and reference cited therein. [PubMed: 18190862]
20. Ramanathan RK, Trump DL, Eiseman JL, Belani CP, Agarwala SS, Zuhowski EG, Lan J, Potter DM, Ivy SP, Ramalingam S, Brufsky AM, Wong MK, Tutchko S, Egorin MJ. Phase I pharmacokinetic-pharmacodynamic study of 17-(allylamino)-17-demethoxygeldanamycin (17AAG, NSC 330507), a novel inhibitor of heat shock protein 90, in patients with refractory advanced cancers. *Clinical Cancer Res.* 2005; 11:3385–3391. [PubMed: 15867239]
21. Chiosis G, Timaul MN, Lucas B, Munster PN, Zheng FF, Sepp-Lorenzino L, Rosen NA. A small molecule designed to bind to the adenine nucleotide pocket of Hsp90 causes Her2 degradation and the growth arrest and differentiation of breast cancer cells. *Chem. Biol.* 2001; 8:289–299. [PubMed: 11306353]
22. (a) He H, Zatorska D, Kim J, Aguirre J, Llauger L, She Y, Wu N, Immormino RM, Gewirth DT, Chiosis G. Identification of potent water soluble purine-scaffold inhibitors of the heat shock protein 90. *J. Med. Chem.* 2006; 49:381–390. [PubMed: 16392823] (b) Immormino RM, Kang Y, Chiosis G, Gewirth DT. Structural and quantum chemical studies of 8-aryl-sulfanyl adenine class Hsp90 inhibitors. *J. Med. Chem.* 2006; 49:4953–4960. [PubMed: 16884307]
23. Kasibhatla SR, Hong K, Biamonte MA, Busch DJ, Karjian PL, Sensintaffar JL, Kamal A, Lough RE, Brekken J, Lundgren K, Grecko R, Timony GA, Ran Y, Mansfield R, Fritz LC, Ulm E, Burrows FJ, Boehm MF. Rationally Designed High-Affinity 2-Amino-6-halopurine Heat Shock Protein 90 Inhibitors That Exhibit Potent Antitumor Activity. *J. Med. Chem.* 2007; 50:2767–2778. [PubMed: 17488003]
24. Sharp SY, Prodromou C, Boxall K, Powers MV, Holmes JL, Box G, Matthews TP, Cheung KM, Kalusa A, James K, Hayes A, Hardcastle A, Dymock B, Brough PA, Barril X, Cansfield JE, Wright L, Surgenor A, Foloppe N, Hubbard RE, Aherne W, Pearl L, Jones K, McDonald E, Raynaud F, Eccles S, Drysdale M, Workman P. Inhibition of the heat shock protein 90 molecular chaperone *in vitro* and *in vivo* by novel, synthetic, potent resorcinyl pyrazole/isoxazole amide analogues. *Mol. Cancer Therap.* 2007; 6:1198–1211. [PubMed: 17431102]
25. (a) Moulick K, Clement CC, Aguirre J, Kim J, Kang Y, Felts S, Chiosis G. Synthesis of a red-shifted fluorescence polarization probe for Hsp90. *Bioorg. Med. Chem. Lett.* 2006; 16:4515–4518. [PubMed: 16797988] (b) Llauger-Bufi L, Felts SJ, Huezo H, Rosen N, Chiosis G. Synthesis of novel fluorescent probes for the molecular chaperone Hsp90. *Bioorg. Med. Chem. Lett.* 2003; 13:3975–3978. [PubMed: 14592488] (c) Rowlands MG, Newbatt YM, Prodromou C, Pearl LH,

- Workman P, Aherne W. High-throughput screening assay for inhibitors of heat-shock protein 90 ATPase activity. *Anal. Biochem.* 2004; 327:176–183. [PubMed: 15051534] (d) Avila C, Kornilayev BA, Blagg BS. Development and optimization of a useful assay for determining Hsp90's inherent ATPase activity. *Bioorg. Med. Chem.* 2006; 14:1134–1142. [PubMed: 16213144] (e) R Barril HX, Dymock BW, Grant K, Northfield CJ, Robertson AGS, Surgenor A, Wayne J, Wright L, James K, Matthews T, Cheung K-M, McDonald E, Workman P, Drysdale MJ. A fluorescence polarization assay for inhibitors of Hsp90. *Anal. Biochem.* 2006; 350:202–213. [PubMed: 16460658] (f) Zhou V, Han S, Brinker A, Klock H, Caldwell J, Gu X-j. A time-resolved fluorescence resonance energy transfer-based HTS assay and a surface plasmon resonance-based binding assay for heat shock protein 90 inhibitors. *Anal. Biochem.* 2004; 331:349–359. [PubMed: 15265741]
26. Smith GK, Barrett DG, Blackburn K, Cory M, Dallas WS, Davis R, Hassler D, McConnell R, Moyer M, Weaver K. Expression, preparation, and high-throughput screening of caspase-8: discovery of redox-based and steroid diacid inhibition. *Arch. Biochem. Biophys.* 2002; 399:195–205. [PubMed: 11888206]
 27. <http://www.drugs.com/dict/quinocide-hydrochloride.html>
 28. Smith GK, Barrett DG, Blackburn K, Cory M, Dallas WS, Davis R, Hassler D, McConnell R, Moyer M, Weaver K. Expression, preparation, and high-throughput screening of caspase-8: discovery of redox-based and steroid diacid inhibition. *Arch. Biochem. Biophys.* 2002; 399:195–205. [PubMed: 11888206]
 29. (a) Pipeline Pilot. San Diego, CA: SciTegic; 2005. (b) MDL Public Keys. San Ramon CA: Elsevier MDL Inc.; (c) Nettles JH, Jenkins JL, Bender A, Deng Z, Davies JW, Glick M. *J. Med. Chem.* 2006; 49:6802–6810. [PubMed: 17154510]
 30. Ganesh T, Min J, Thepchatri P, Du Y, Li L, Lewis I, Wilson L, Fu H, Chiosis G, Dingledine R, Liotta D, Snyder JP, Sun A. Discovery of aminoquinolines as a new class of potent inhibitors of heat shock protein 90 (Hsp90): Synthesis, biology, and molecular modeling. *Bioorg. Med. Chem.* 2008; 16:6903–6910. [PubMed: 18571929]
 31. Bhat B, Seth M, Bhaduri AP. A new convenient synthesis of Quinocide. *Chem. Ind.* 1983:899.
 32. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The Protein Data Bank. *Nucleic Acids Res.* 2000; 28:235–242. [PubMed: 10592235]
 33. Cheung KM, Matthew TP, James K, Rowlands MG, Boxall KJ, Sharp SY, Maloney A, Roe SM, Prodromou C, Pearl LH, Aherne GW, McDonald E, Workman P. The identification, synthesis, protein crystal structure and *in vitro* biochemical evaluation of a new 3,4-diarylpyrazole class of Hsp90 inhibitors. *Bioorg. Med. Chem. Lett.* 2005; 15:3338–3343. [PubMed: 15955698]
 34. (a) Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell.* 1997; 89:239–250. [PubMed: 9108479] (b) Roe SM, Prodromou C, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J. Med. Chem.* 1999; 42:260–266. [PubMed: 9925731] (c) Barril X, Brough P, Drysdale M, Hubbard RE, Massey A, Surgenor A, Wright L. Structure-based discovery of a new class of Hsp90 inhibitors. *Bioorg. Med. Chem. Lett.* 2005; 15:5187–5191. [PubMed: 16202589]
 35. SchrödingerSuite2008InducedFit Dockingprotocol; Glideversion 5.0. New York, NY: Schrödinger, LLC; 2005. Prime version 1.7. New York, NY: Schrödinger, LLC; 2005.
 36. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature.* 2000; 407:249–257. [PubMed: 11001068]
 37. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell.* 1996; 86:353–364. [PubMed: 8756718]
 38. Li CY, Shan S, Cao Y, Dewhirst MW. Role of incipient angiogenesis in cancer metastasis. *Cancer Met. Rev.* 2000; 19:7–11.
 39. Vagnucci AH, Li WW. Alzheimer's disease and angiogenesis. *Lancet.* 2003; 361:605–608. [PubMed: 12598159]
 40. Jain RK, Schlenger K, Höckel M, Yuan F. Quantitative angiogenesis assays: Progress and problems. *Nat. Med.* 1997; 3:1203–1208. [PubMed: 9359693]

41. Auerbach R, Akhtar N, Lewis RL, Shinnars BL. Angiogenesis assays: Problems and pitfalls. *Cancer Met. Rev.* 2000; 19:167–172.
42. Doan TN, Eilertson CD, Rubinstein AL. High-throughput target validation in model organisms. *Drug Discov. Today Targets.* 2004; 3:191–197.
43. Cross LM, Cook MA, Lin S, Chen JN, Rubinstein AL. Fluorescent zebrafish assay for anti-angiogenesis drug screening. *Arterioscler. Thromb. Vasc. Biol.* 2003; 23:911–912. [PubMed: 12740225]
44. Tran TC, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, Baranowski TC, Rubinstein AL, Doan TN, Dingleline R, Sandberg EM. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. *Cancer Res.* 2007; 67:11386–11392. [PubMed: 18056466]
45. Hoessel R, Leclerc S, Endicott JA, Nobel MEM, Lawrie A, Tunnah P, Leost M, Damiens E, Marie D, Marko D, Niederberger E, Tang WC, Eisenbrand G, Meijer L. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat. Cell Biol.* 1999; 1:60–67. [PubMed: 10559866]
46. Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, Greengard P, Biernat J, Wu YZ, Mandelkow EM, Eisenbrand G, Meijer L. Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/P25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease - A property common to most cyclin-dependent kinase inhibitors. *J. Biol. Chem.* 2001; 276:251–260. [PubMed: 11013232]
47. Ferandin Y, Bettayeb K, Kritsanida M, Lozach O, Polychronopoulos P, Magiatis P, Skaltsounis AL, Meijer L. 3'-substituted 7-halogenindirubins, a new class of cell death inducing agents. *J. Med. Chem.* 2006; 49:4638–4649. [PubMed: 16854069]
48. DeLano, WL. The PyMol Graphics Systems (2002) on the World Wide Web <http://www.pymol.org..>

Fig. 1.
Structures of measles virus entry inhibitor and hit **1**.

Fig. 2.

(A) MFTA molecular supergraph (top) formed by the 26 compounds of the training set. Compound 1 (bottom) is outlined to exemplify the alignment. (B) MFTA correlation for the 26 compounds of the training set (blue) and the 7 compounds of the test set (red) based on charge (Q), effective vdW radius (R_e) and lipophilicity (L_g) descriptors.

Fig. 3.
High-throughput screening hits from the Hsp90 screen.

Fig. 4.

The bioactivities of aminoquinolines **12–15** in the Hsp90 FP assay.

Data shown are $n = 1$ (mean $\pm$ SEM of triplicate readings) representative of 3–5 experiments

Fig. 5. Her2 degradation Western Blot assay. A) dose-dependent effect on degradation of the Her2 protein by compound **15** obtained from the NCI collection; B) dose-dependent effect on degradation of the Her2 protein by re-synthesized **15**. PU is the known Hsp90 inhibitor used as a positive control. The IC_{50} determined for **15** from these experiments (A) was $0.8 \mu\text{M}$, B) $2\text{--}3 \mu\text{M}$.

Fig. 6.
Structures of aminoquinolines obtained from the NCI showed activity in both FP and WB assays

Fig. 7.
The carbon atoms of the N-terminal Hsp90 ATP receptor are shown in blue. The carbon atoms of ligands PU-H71 and compound **15** are colored in green and orange, respectively. Hydrogen-bonding interactions are represented as yellow dashes.

Fig. 8.

Assay for angiogenesis in zebrafish embryos expressing GFP in the endothelial cells that line blood vessels. A. Partial view of a 384-well plate with embryos arrayed one per well. B. After overnight treatment with a VEGF receptor antagonist, there are no observable vessels outside the spinal area. C. Embryo treated with water overnight shows robust angiogenesis. D. The spinal area is isolated with a box cursor and the number of intersomitic vessels is counted as a measure of angiogenesis.

Fig. 9.

A) The primary HTS screening results of Hsp90, AID 429. Yellow dots denote a single compound and their relative percent inhibition in the Hsp90 FP assay. The arbitrary cutoff in this procedure was 3 standard deviations above the mean, which is denoted by the dashed red line. B) Hsp90 results highlighting compounds containing scaffold 132, which is highlighted in red. The percent inhibitions of this aggregate are varied, suggesting structural differences around the common scaffold are affecting protein-ligand interactions. C). Compounds containing scaffold 1 are highlighted in blue. SEA analysis of the results show a lone compound with inhibitory activity of ~50%. This suggests scaffold 1 is probably not responsible for the observed Hsp90 activity and is potentially an artifact given that all other compounds containing scaffold 1 show baseline activity. D). Test case for the SEA analysis. Scaffold S11 is a fragment of a known Hsp90 inhibitor identified previously by the Workman Group.³³ Compounds containing the S11 scaffold are highlighted in blue.

Scheme 1.
Synthesis of hit compound **1** and analogs.

Scheme 2

Synthesis of quinocidine derivatives.

^aReagents and conditions: a) H₂, Pd-C, 10%, MeOH, 12Psi. 1h. b) Aryl-SO₂Cl, Py, rt. 6–12 h. c) 4-Bromobutyronitrile, Et₃N, EtOH, microwave, 180 °C, 3 h. d) sat. HCl/MeOH, 12 h, then sat. NH₃/MeOH, 24 h. e) 4-nitrobenzylbromide, Et₃N, EtOH, reflux, 24h, then H₂, Pd-C, 10%, ethylacetate/MeOH, 12 Psi. 1h. f) cis- or trans-N-(4-chlorobutenyl) phthalimide, Et₃N, n -BuOH/EtOH, microwave, 190 °C, 1 h. g) hydrazine hydrate, EtOH, reflux, 3h. h) N-(4-Chloro-2-butyryl)phthalimide, Et₃N, n -BuOH/EtOH, microwave, 190 °C, 1h. i) 5-chloropentan-2-one, Et₃N, MeOH, reflux, 24h. j) NH₂OH. HCl, EtOH reflux. k) Raney-Ni, H₂, 45 psi. l) HBr 48%-AcOH, reflux, 36 h. m) Boc₂O, K₂CO₃, THF, 12 h. n) ArSO₂Cl, Py, 6–12 h. o) TFA, DCM, 12 h. p) Alkyl-SO₂Cl, Py, RT, 12h. q) 4-Me-Ph-CH₂SO₂Cl, Py, 6 h.

^a**Scheme 3**

^aReagents and conditions: a) Br(CH₂)₄CN, Et₃N, EtOH; b) LAH, THF, c) CH₃CHO or Acetone, NaBH(OAc)₃; d) 48% aq.HBr

Scheme 4^a

^aReagents and conditions: a) 3-acetoxyindole, $\text{Na}_2\text{CO}_3/\text{MeOH}$, 25 °C; b) $\text{H}_2\text{NOH.HCl}$, Py, 120 °C; c) $\text{H}_2\text{NNH}_2.\text{H}_2\text{O}$, 70~80 °C; d) $\text{BrCH}_2\text{CH}_2\text{Br}$, Et_3N , anhydrous DMF, 25 °C; e) R_3 , anhydrous DMF, 25 °C

Table 1

Antiviral MV EC₅₀'s, Cytotoxicities and Selectivity Indices for 1-Methyl-*N*-(4-(Piperidin-1-ylsulfonyl)phenyl)-3-(trifluoromethyl)-1H-pyrazole-5-Carboxamide (1a) and other Sulfonamide Analogs

Comp.	R	EC ₅₀ [μM] ^a	CC ₅₀ [μM] ^b	SI ^c
1		0.25	500	>625
1a		0.014 ± 0.02	199 ± 27	14,214
1b		0.029 ± 0.031	54 ± 3	1,862
1c		0.005 ± 0.0003	425 ± 65	85,000
1d		0.045 ± 0.034	16 ± 0.8	ND
1e		43 ± 24	ND	ND
1f		0.85 ± 0.05	159 ± 40	ND

^a 50% effective concentration, at which a 50%-reduction of virus yields were observed in the virus titer reduction assay. Determined only when CPE inhibition-based EC₅₀ concentration < 2.3 μM. Values represent averages of two to four experiments ± SEM; highest concentration assessed 1 μM.

^b 50% cytotoxic concentration, determined for Vero cells using a Cyto Tox 96 Non-radioactive Cytotoxic Assay (Promega). Average and SD values are based on four experiments. (ND: not determined).

^c Selective index (CC₅₀/EC₅₀). (ND: not determined).

Table 2

Bioactivity of the Initial and Re-Synthesized hits Identified by by an FP Assay Employed in the HTS-Screen

Comp.	Pubchem SID	IC ₅₀ (μM)	
		FP-assay	WB-assay
1	850375	5.8	5
1(re-syn.)	JM1-135B	>30	>25
2	855543	0.17	5
3	855944	0.27	3
4	845167	0.14	5
5	850758	0.1	5
6	847157	10.63	8
9	TG2-26-2	>22	>Not active
12	24724348	4.9	1.6
13	24724372	29	6.0
14	24724310	2.4	6.0
15	24724290	1.3	1.0

Table 3

Biological Effects of Indirubin-3'-Monoxime Related to Angiogenesis

IC ₅₀ (μM)	Effect
0.31	Angiogenesis in zebrafish
>30	Vasculogenesis in zebrafish
>30	regression of existing angiogenic vessels in zebrafish
0.36	HUVEC: inhibition of [3H]-thymidine incorporation
8.7	HUVEC: reduction of ATP levels (Cell-titre Glow assay)
6.5	HUVEC: inhibition of tubule formation
>30	HUVEC: inhibition of migration

Table 4

Antangiogenic Effects of Indirubin Derivatives Obtained by Use of an In Vivo Zebrafish Model

Compound	x	R ₁	R ₂	R ₃	IC ₅₀ (μM)
1	NOH	H	H	H	0.3
2	NOH	H	H	F	0.4
3	NOH	H	CH ₃	H	0.7
4	O	H	CH ₃ #	H	1.2
5	O	H	H	F	2.2
6	NOH	Br	H	CH ₃	2.5
7	NOH	Br	CH ₃	H	4.0
8	O	H	Cl	Cl	4.2
9		H	CH ₃	H	6.3
10	NOH	Br	Cl	Cl	>30
11	O	Br	H	H	>30
12	NOH	H	H	Cl	>30
13	NOH	H	H	SO ₃ H	>30