ACS Combinatorial Science

Research Article

pubs.acs.org/acscombsci

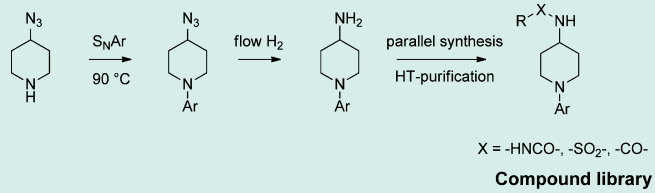
Disubstituted 1-Aryl-4-Aminopiperidine Library Synthesis Using Computational Drug Design and High-Throughput Batch and Flow Technologies

Marian C. Bryan,[†] Christopher D. Hein,[†] Hua Gao,[‡] Xiaoyang Xia,[‡] Heather Eastwood,[‡] Bernd A. Bruenner,[§] Steven W. Louie,[§] and Elizabeth M. Doherty*[,†]

[†]Medicinal Chemistry Research Technologies, [‡]Molecular Structure and Characterization, and [§]Pharmacokinetics and Drug Metabolism, Therapeutic Discovery, Amgen Inc., One Amgen Center Drive, Thousand Oaks, California 91320, United States

Supporting Information

ABSTRACT: A platform that incorporates computational library design, parallel solution-phase synthesis, continuous flow hydrogenation, and automated high throughput purification and reformatting technologies was applied to the production of a 120-member library of 1-aryl-4-aminopiperidine analogues for drug discovery screening. The application described herein demonstrates the advantages of computational library design coupled with a flexible, modular approach to library synthesis. The enabling technologies described can be readily adopted by the traditional medicinal chemist without extensive training and lengthy process development times.



KEYWORDS: *drug discovery, drug-likeness, lead-likeness, computational library design, library synthesis, continuous flow hydrogenation, enabling technologies, aminopiperidines*

INTRODUCTION

The costs associated with drug research continue to rise and many U.S. and E.U.-based pharmaceutical companies are responding by creating greater efficiencies through the use of predictive computational tools and innovative enabling technologies for the design and production of drug candidates. Many of these tools and technologies originated in the combinatorial chemistry world of the 1990s[1] with the more recent addition of the highly popular microwave and, less commonly used, continuous flow reactors.[2] In some larger organizations, dedicated high-throughput (HT) synthesis groups tasked with creating libraries to support drug discovery are often the specialist users of these technologies. Nevertheless, whether an organization has the capacity to maintain a dedicated library synthesis team or not, adoption of these computational tools and enabling technologies by traditional medicinal chemists is highly useful.

As the state of the art advances, ingenious library synthesis platforms have emerged that integrate multiple synthetic steps,[3] integrate synthesis with purification,[4] and even integrate synthesis with biological evaluation.[5] The siren call of the fully integrated synthesis, purification, characterization, and biological evaluation platform continues to drive many research efforts. In contrast to an integrated approach, however, our group places an emphasis on identifying enabling technologies that are modular in design and can be incorporated into a library synthesis platform accessible to every medicinal chemist. Techniques that will readily become a part of the day-to-day toolkit of the medicinal chemist are robust, intuitive, and require minimal specialized training. Because the library synthesis platform is modular, each tool can be utilized independently with no delays or disruptions to the overall workflow. The platform consists of five main features:

(1) a web-based computational library design tool using molecular structure-based calculated properties coupled with a predictive algorithm to select for "drug-like"[6] and "lead-like"[7] molecules,

(2) a custom-weighed reagent service coupled with a proprietary and commercial reagent collection,

(3) a suite of parallel and HT-synthesis and purification tools, each of which is supported by a dedicated "power-user" who provides technical assistance and training on an ad hoc basis,

(4) a HT mass-triggered HPLC purification and reformatting process (HTPP) that is supported by an internal team of HT-purification specialists, and

(5) an electronic laboratory notebook-based (ELN) compound tracking and record keeping system with a batch compound registration process that correlates sample numbers and analytical data to products in bar-coded vials or 96-well plates.

To demonstrate an application of the platform, an aminopiperidine-based 120-member library synthesis and evaluation is described herein. The library production incorporates various commercially available synthesis-enabling tools, including parallel reactor blocks, automated chromatography systems,

Received: June 15, 2013
Revised: August 7, 2013
Published: August 8, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/co400078r | *ACS Comb. Sci.* 2013, 15, 503−511

and a flow hydrogenator. The library was evaluated in vitro for key indicators of drug-likeness (solubility, microsomal stability, cell permeability) and the results compared to those predicted using our predictive algorithm.

RESULTS AND DISCUSSION

Library Design. Because of its representation in many biologically active compounds, the 4-aminopiperidine substructure may qualify as a "privileged" scaffold.[8] As a subset of this class, 1-aryl-4-aminopiperidines appear in a variety of therapeutic agents, for example, the cardiac stimulant Buquineran,[9] and the antidiabetic ABT-279[10] (Figure 1).

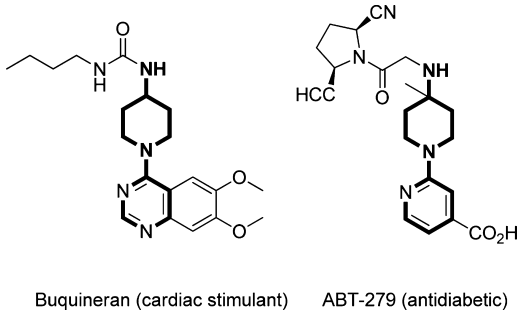
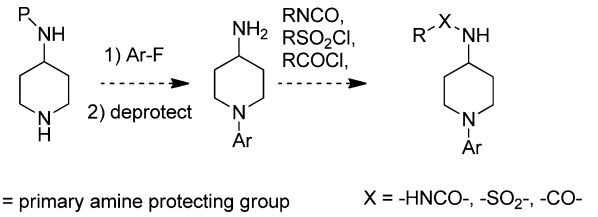


Figure 1. 1-Aryl-4-aminopiperidine scaffold represented in bioactive molecules.

For the purposes of library synthesis, the 4-aminopiperidine scaffold provides a convenient opportunity for diversification at two sites, a primary and a secondary amine, which we proposed to differentiate by means of a suitable protecting group strategy. Thus our synthetic strategy consisted of three steps (Scheme 1): (1) arylation of the piperidinyl amine via thermally induced

Scheme 1. Proposed Synthesis of 1-Aryl-4-aminopiperidine Library



S_NAr, (2) deprotection of the primary amine, and (3) reaction of the primary amine with an isocyanate, sulfonyl chloride, or acid chloride.

For the S_NAr step, a minimal set of eight aryl and heteroaryl fluorides (**1**{1−8}, Figure 2) was selected. The set included aromatic rings with *ortho*- or *para*-activating groups to enhance reactivity and provide an orthogonal functional handle for potential future elaboration (acyl **1**{1}, nitrile **1**{2−3}, ester **1**{4−5}) thus affording "lead-like" structures. Reactive heteroaromatics (2-pyridine **1**{6}, 2-pyrimidine **1**{7}) were included as well as an example incorporating a kinase binding moiety, 2-aminopyrimidine **1**{8}.

A set of putative R-groups for the second point of attachment (urea, sulfonamide, amide) was selected from reagents available in Amgen's proprietary reagent collection, which is inventoried and managed by Frontier Scientific Services, Inc. (FSSI). Located in Newark, Delaware, FSSI also maintains their own commercial reagent collection and custom weighing service

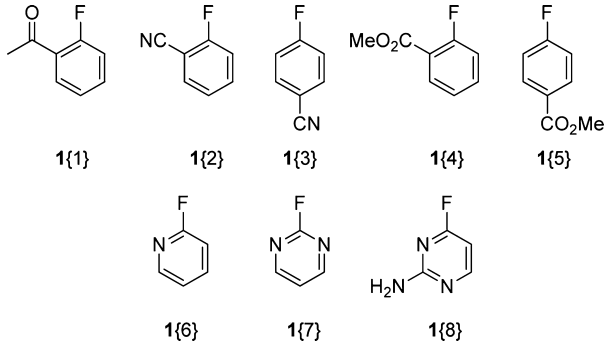


Figure 2. Aryl and heteroaryl fluorides selected for S_NAr step.

available to the public. The reagents can be accessed on demand and delivered to the chemist's bench, within 2−3 days of request, custom-weighed in the requisite reaction vessel. In the selection process, the inventory of potential reagents was searched via a custom web interface using a structural filter based on required functional groups (acid chloride, isocyanate, sulfonyl chloride) and undesired groups (aldehydes, nitro groups, carboxylic acids, primary amines), thus eliminating obvious pharmacokinetic liabilities and competing reactivity. The reduced list (301 acid chlorides, 127 isocyanates, 134 sulfonyl chlorides) in combination with the 4-aminopiperidine core and aryl groups in Figure 2 was used to enumerate a virtual library of 4496 disubstituted 4-aminopiperidines. A script written using Pipeline Pilot software[11] simultaneously generated the structures and associated structure-based calculated properties. The calculated properties were used to refine the list further based on molecular weight (<400 Da), clogP (0−4), polar surface area (45−125 Å), total hydrogen bond donors and acceptors (≤7), and number of aromatic rings (1−3). While consideration of prior SAR, Topliss analysis, and virtual docking programs are routinely used to design libraries at Amgen, for the exercise described herein a simple visual inspection of the truncated list led to the selection of five each from the acid chlorides, isocyanates, and sulfonyl chlorides to apply to library production (**2**{1−15}, Figure 3).

Library Synthesis. The synthesis commenced with S_NAr arylation of a protected 4-aminopiperidine. It was quickly determined that *N*-carbamate protecting groups (Boc or Cbz) were unsuitable because of thermal instability of the carbamate under the S_NAr reaction conditions (DMSO, K_2CO_3, 90 °C), yielding byproducts resulting from decomposition to the isocyanate. We then considered using an azide as an amine precursor which offers the attraction of atom-efficiency, thus reducing the mass required in the first steps of the library synthesis. While many organic azides are known to be thermally unstable, it has been reported that azides may be stable when the number of nitrogen atoms does not exceed the number of carbon atoms.[12] The 4-azidopiperidine (**4**) was prepared from the commercially available Boc-protected precursor (**3**) by acid deprotection (Table 1). Without further purification, the resulting hydrochloride salt was used directly in the arylation step (**1**{1−8}, DMSO, K_2CO_3, 90 °C) under which conditions the azide proved thermally stable. The crude products were readily purified by silica gel chromatography using an automated flash silica gel chromatography system. The overall process afforded the desired 1-aryl-4-azidopiperidines **5**{1−8} in 23−84% isolated yield over two steps.

While the azide may be reduced by chemical means (Ph_3P or $NaBH_4$), catalytic hydrogenation was preferred because of the

dx.doi.org/10.1021/co400078r | ACS Comb. Sci. 2013, 15, 503−511

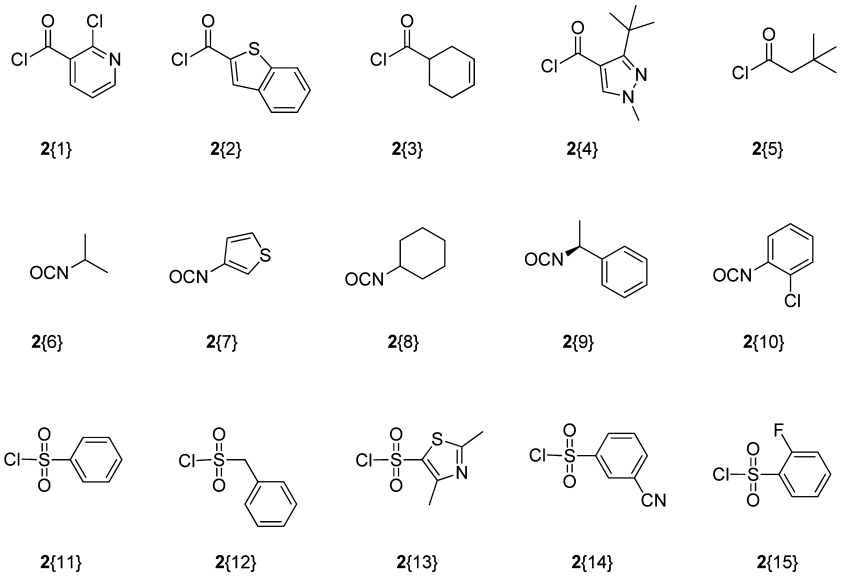
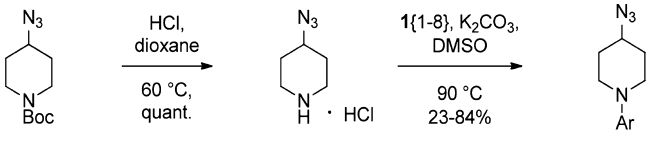


Figure 3. Acid chloride, isocyanate, and sulfonyl chloride reagents.

Table 1. Generation of 1-Aryl-4-azidopiperidines 5{1−8} from 4-Azidopiperidine



3 4 5{1-8}

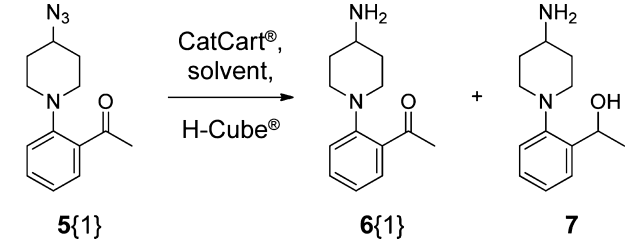
entry	ArF	product	yield (%)[a]	entry	ArF	product	yield (%)
1	1{1}	5{1}	61	5	1{5}	5{5}	47
2	1{2}	5{2}	67	6	1{6}	5{6}	47
3	1{3}	5{3}	84	7	1{7}	5{7}	72
4	1{4}	5{4}	23	8	1{8}	5{8}	70

[a]Isolated yield after purification by silica gel chromatography.

relative convenience of product isolation via filtration. Furthermore, by using an H-Cube continuous flow reactor (ThalesNano, Budapest, Hungary) the time taken from reaction screening to scale-up was minimized. The reactor introduces hydrogen into a flowing stream of substrate in carrier solvent which is then passed through a stainless steel cartridge (CatCart) containing solid catalyst. The product is isolated in high yield from the eluent after evaporation of solvent. The conversion of azide 5{1} to amine 6{1} (Scheme 2) was first examined on a screening scale (0.08 mmol) to optimize conversion and minimize the formation of the over-reduced side product 7. For screening reaction conditions, the H-Cube was equipped with a syringe injector port and a 2 mL sample loop, previous studies having shown that this configuration provides the best correlation between small-scale screening results and larger continuous-flow preparative scale results.[13] Commercial 10% Pd/C, 5% Pd/C, 20% $Pd(OH)_2$, and 10% Pt/C CatCarts were evaluated under standard hydrogenation conditions (1 mL/min flow rate, 30 °C, "full" hydrogen mode) and the effect of MeOH vs tetrahydrofuran (THF) was compared (0.04 M substrate concentration).[14] All three Pd catalysts afforded the undesired alcohol 7 when the reduction was performed in MeOH, though this over-reduction appeared to be mitigated to some extent in THF. In comparison, 10% Pt/C afforded excellent conversion (>90%) and selectivity in both MeOH and THF for all of the azides 5{1−8} on a 2 mL screening scale. Therefore, 10% Pt/C was used for all preparative scale azide reductions.

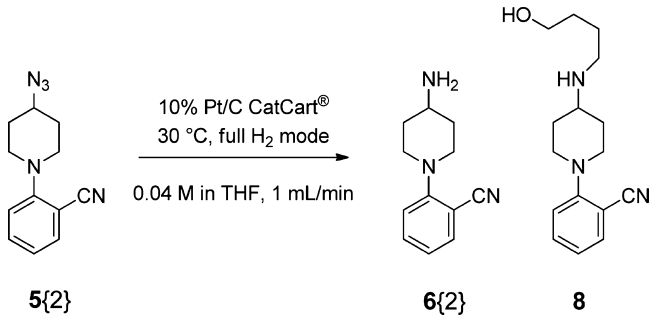
Initially, in the course of screening and scale-up, preservative (BHT)-free THF was used so that the presence of preservative would not interfere with GC analysis. Using this solvent, however, led to varying amounts of a side product with a mass consistent with *n*-butanol addition. In the case of the reduction of azide 5{2} with a 10% Pt/C CatCart, this side product was isolated and identified as alcohol 8 (Scheme 3). The addition of *n*-butanol to amines in the presence of Pd has been reported,[15] though not to our knowledge in the presence of Pt. To prevent the formation of this byproduct, MeOH could be substituted,

Scheme 2. Reduction of Azide 5{1} to Amine 6{1} and Over-Reduced Product 7



5{1} 6{1} 7

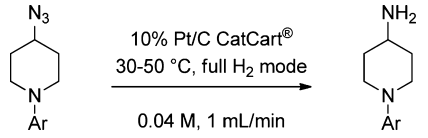
Scheme 3. Alkylation of Amine under H-Cube Reduction Conditions in Preservative-Free THF



5{2} 6{2} 8

dx.doi.org/10.1021/co400078r | ACS Comb. Sci. 2013, 15, 503−511

Table 2. Reduction of Azides 5{1−8} to Amines 6{1−8} on Preparative Scale (1.1−5.5 mmol)

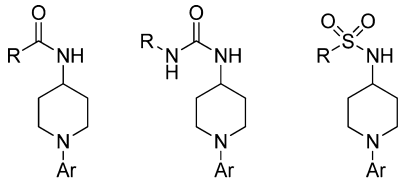


5 **6**

entry	azide	scale (mmol)	solvent	temp (°C)	product	yield (%)[a]	purity (%)[b]
1	**5**{1}	3.3	MeOH	50	**6**{1}	96	89
2	**5**{2}	2.9	MeOH	50	**6**{2}	97	92
3	**5**{3}	2.6	MeOH	50	**6**{3}	96	93
4	**5**{4}	2.5	MeOH	50	**6**{4}	90	>99
5	**5**{5}	2.8	MeOH	30	**6**{5}	98	>99
6	**5**{6}	1.1	MeOH	30	**6**{6}	97	90
7	**5**{7}	4.4	THF	30	**6**{7}	96	95[c]
8	**5**{8}	5.5	0.02 M AcOH/MeOH	50	**6**{8}	84[d]	86

[a]Isolated yield after removal of solvent unless otherwise indicated. [b]Purity by reverse phase HPLC at 254 nm unless otherwise indicated. [c]Purity by GC. [d]Isolated yield after aqueous workup described fully in Experimental Section.

Table 3. Yield and Purity of a Representative Chemset 9{1−2,1−15} from the Final Library



9{1-2,1-5} **9**{1-2,6-10} **9**{1-2,11-15}

entry	amine	*N*-functionalizing reagent	product	purification method[a]	purity (%)[b]	isolated yield (%)
1	**6**{1}	**2**{1}	**9**{1,1}	A	>99	27
2	**6**{1}	**2**{2}	**9**{1,2}	A	98	60
3	**6**{1}	**2**{3}	**9**{1,3}	A	>99	38
4	**6**{1}	**2**{4}	**9**{1,4}	A	>99	45
5	**6**{1}	**2**{5}	**9**{1,5}	A	>99	49
6	**6**{1}	**2**{6}	**9**{1,6}	A	>99	30
7	**6**{1}	**2**{7}	**9**{1,7}	A	94	26
8	**6**{1}	**2**{8}	**9**{1,8}	A	>99	20
9	**6**{1}	**2**{9}	**9**{1,9}	A	93	23
10	**6**{1}	**2**{10}	**9**{1,10}	A	>99	11
11	**6**{1}	**2**{11}	**9**{1,11}	A	>99	34
12	**6**{1}	**2**{12}	**9**{1,12}	A	>99	32
13	**6**{1}	**2**{13}	**9**{1,13}	A/B	>99	10
14	**6**{1}	**2**{14}	**9**{1,14}	A	>99	35
15	**6**{1}	**2**{15}	**9**{1,15}	A	>99	32
16	**6**{2}	**2**{1}	**9**{2,1}	B	>99	84
17	**6**{2}	**2**{2}	**9**{2,2}	B	97	82
18	**6**{2}	**2**{3}	**9**{2,3}	B	96	94
19	**6**{2}	**2**{4}	**9**{2,4}	B	97	100
20	**6**{2}	**2**{5}	**9**{2,5}	B	>99	92
21	**6**{2}	**2**{6}	**9**{2,6}	B	>99	59
22	**6**{2}	**2**{7}	**9**{2,7}	B	98	59
23	**6**{2}	**2**{8}	**9**{2,8}	B	>99	23
24	**6**{2}	**2**{9}	**9**{2,9}	B	>99	64
25	**6**{2}	**2**{10}	**9**{2,10}	B	>99	57
26	**6**{2}	**2**{11}	**9**{2,11}	B	>99	88
27	**6**{2}	**2**{12}	**9**{2,12}	B	98	79
28	**6**{2}	**2**{13}	**9**{2,13}	B	>99	44
29	**6**{2}	**2**{14}	**9**{2,14}	B	>99	89
30	**6**{2}	**2**{15}	**9**{2,15}	B	>99	89

[a]Purification method A = HTPP; B = silica gel chromatography; A/B = HTPP followed by silica gel chromatography. [b]Purity by HPLC at 215 nm.

dx.doi.org/10.1021/co400078r | *ACS Comb. Sci.* 2013, 15, 503−511

Table 4. Properties of Aminopyrimidine Chemset 9{8, 1−15}

chemset	MW g/mol	clogP[a]	PSA[a] Å	HBA[b]	HBD[c]	Ar rings	observed solubility[d] μM	predicted RLM CL μL/min/mg	observed RLM CL[e] μL/min/mg	predicted HLM CL μL/min/mg	observed HLM CL[e] μL/min/mg	predicted P_{app} μcm/s	observed P_{app}[f] μcm/s	predicted ER	observed ER[g]
9{8,1}	332.8	0.00	97.0	4	2	2	500	50	<14	63	<14	14.3	10.1	3.0	5.3
9{8,2}	353.4	2.02	84.1	3	2	3	11	94	18	41	<14	3.5	nd[h]	11.3	nd[h]
9{8,3}	301.4	0.40	84.1	3	2	1	500	29	<14	24	<14	9.7	28.3	7.5	2
9{8,4}	357.5	0.59	102.0	4	2	2	43	15	<14	15	<14	12.6	38	8.5	1.6
9{8,5}	291.4	0.71	84.1	3	2	1	500	26	<14	20	<14	11.7	31.9	6.0	1.2
9{8,6}	278.4	0.22	96.2	3	3	1	500	29	<14	17	<14	1.1	1.1	21.2	18.8
9{8,7}	318.4	0.90	96.2	3	3	2	107	95	16	52	<14	1.7	7.5	31.6	6.5
9{8,8}	318.4	1.42	96.2	3	3	1	497	28	<14	15	<14	7.6	14.3	6.7	2.4
9{8,9}	340.4	1.46	96.2	3	3	2	500	35	<14	21	<14	1.5	10.2	34.5	1.8
9{8,10}	346.8	1.49	96.2	3	3	2	166	44	26	28	<14	9.0	35.5	12.3	1.5
9{8,11}	333.4	0.85	101.2	4	2	2	500	41	61	29	<14	3.0	9.9	29.3	3.8
9{8,12}	347.4	0.71	101.2	4	2	2	500	31	21	17	<14	6.9	12.1	11.0	2.5
9{8,13}	368.5	1.31	114.1	5	2	2	500	118	28	17	<14	2.3	11.5	34.5	4.9
9{8,14}	358.4	0.99	125.0	5	2	2	206	31	22	16	<14	1.2	7.3	36.9	6.2
9{8,15}	351.4	1.30	101.2	4	2	2	500	27	56	23	<14	8.2	27.5	8.6	1.6

[a]Partition coefficients (clogP) and polar surface area (PSA) were calculated using Daylight (V481) toolkit module: Daylight Chemical Information Systems, Inc. [b]Hydrogen bond acceptors. [c]Hydrogen bond donors. [d]Solubility in pH 7.4 phosphate buffer. [e]Rat liver microsomal (RLM) and human liver microsomal (HLM) intrinsic clearance measured in vitro in liver homogenate. [f]Apparent cell permeability (A2B) in LLC-PK1 cells stably overexpressing human P-glycoprotein is measured by LC-MS/MS. [g]Efflux ratio = B2A/A2B. [h]Not determined because of low recovery in cell assay.

dx.doi.org/10.1021/co400078r | ACS Comb. Sci. 2013, 15, 503−511

otherwise the use of preservative (BHT)-containing THF appeared to mitigate the issue.

To reconfigure the H-Cube for preparative scale reductions (>2 mL), the syringe injector port was bypassed and azide solution was introduced directly into the reactor via the solvent inlet.[14] As shown in Table 2, the mass recovery using MeOH or THF was high for all preparative scale (1.1−5.5 mmol) reductions with the exception of the 2-aminopyrimidine analogue (entry **8**) in which case recovery was improved using 0.02 M AcOH/MeOH as the carrier solvent. The same 10% Pt/C CatCart was reused for all sequential reductions until conversion was observed to deteriorate, presumably because of catalyst poisoning. Performance was regained by heating the CatCart to 50 °C during the reduction or by replacing with a fresh CatCart. When incomplete reduction occurred, the recovered product mixture could be resubjected to the reaction conditions to provide desired product without over-reduction. The arylpiperidinyl amines were isolated by evaporation of the solvent in vacuo and the products carried through the subsequent library synthesis step without purification.

For the final *N*-functionalization of amines **6**{1−8}, the reactions were performed on a 50 mg scale in parallel in 1-dram glass vials held in 24-position reactor blocks. The amines were added as solutions to the acid chlorides **2**{1−5}, isocyanates **2**{6−10}, sulfonyl chlorides **2**{11−15} which had been preweighed in the glass vials by FSSI, Inc. After sealing the vials, the blocks were agitated at ambient temperature on an orbital shaker. Workup consisted of simply quenching excess reagent by the addition of methanol, and the reaction mixtures were concentrated to dryness in the reactor block using a centrifugal evaporator. In most cases, the crude products could be purified using Amgen's dedicated HT mass-triggered reverse-phase HPLC purification and reformatting platform (HTPP).[16] The HTPP at Amgen is the only component of the library production platform that requires a dedicated specialist. The flexibility of our modular library production platform is highlighted by the fact that those compounds that failed to progress through HTPP because of insufficient solubility or because of poor ionization by MS were simply redirected to the HT flash silica gel chromatography systems. The final purified compounds could then be directed back again to HTPP for reformatting into the appropriate vials for storage or further evaluation. Examples purified by each of these purification methods are shown in Table 3 (see Supporting Information for full set of final yields). Each library member was analyzed by LC-MS and HT-NMR.[17] Typically, library purification, reformatting, LC-MS and ^{1}H NMR analysis, and registration of library members for submission to the screening collection are completed within 6−10 business days. A 100% success rate was achieved for this library, with all 120 compounds obtained in sufficient quantity and purity (>90% by LC at 215 nm) for submission to the compound screening collection for further testing.

Library Analysis. The library members were tested for rat and human liver microsomal stability (RLM, HLM) and the results compared with predicted ones. The in silico models[18] were constructed and validated based on in house data generated from over 40,000 proprietary compounds. The molecular descriptors used in the models were selected from a pool of 820 different molecular descriptors covering charged surface areas, shape and Chi molecular indices, hydrophobicity, solvation properties, E-state indices, intermolecular H-bond features, and types of H-bond counts. The most important molecular properties with regard to microsomal stability were as follows. (1) Hydrophobicity: compounds with higher clogP in general have higher clearance. (2) Number of acid groups: acidic groups like COOH decrease clearance. (3) H bonds: increasing number of H bonds decreases clearance. (4) Aromaticity: aromatic compounds in general have higher clearance. Regarding the performance of the models used, typically ∼70% of new compounds are predicted within two folds, and ∼80% of new compounds are within three folds of experimental values. Of course, the predictive performance of the in silico model is highly dependent on the chemical space coverage of new compounds, that is, properties for new compounds similar to compounds already represented in the model are better predicted.

As expected, for the array described herein the accuracy of the prediction varied based on structural type (see Supporting Information for full data set). For example, amides (60% within 2-folds) and sulfonamides (61% within 2-folds) were significantly better predicted than ureas (21% within 2-folds) in the RLM assay, while amides (68% within 2-folds) and ureas (77% within 2-folds) were better predicted than sulfonamides (28% within 2-folds) in the HLM assay. Overall, the aminopyrimidines **9**{8, 1−15} (Table 4) were the best predicted structural subtype and exhibited the most desirable drug-like properties of the array, with 100% of these compounds demonstrating microsomal clearance <100 μL/min/mg and 87% with aqueous solubility >100 μM.

This subset was further evaluated for cell permeability and efflux in LLC-PK1 cells stably overexpressing human P-glycoproteins. Cell permeability (P_{app}) from the apical to basolateral surface (A2B) as well as efflux ratio (B2A/A2B) is shown in Table 4. While within the predicted range of values, the experimentally determined permeability was generally higher than predicted. The measured efflux ratios were in many cases much lower than predicted because of the higher actual A2B permeability. Based on these P_{app} values and efflux ratios, as well as high solubility, this chemset would be expected to have good intestinal absorption.

■ CONCLUSION

By allowing the chemistry to guide the selection of appropriate accelerating technologies, as opposed to forcing the chemistry to work on an existing fully integrated platform, a 100% success rate in the preparation of all library members can be achieved with minimal process development time. This library was added to the corporate compound collection for further HT screening. Computational tools utilized in the design of this library proved useful in determining the relative "drug-likeness" of the target compounds. The techniques and computational tools described herein have been adopted by medicinal chemists across Amgen's Drug Discovery organization.

■ EXPERIMENTAL PROCEDURES

Caution! *While the authors experienced no adverse events in the preparation of the aliphatic azides described herein, we advise the use of appropriate personal protective equipment and blast-shielding when repeating these procedures.*

General Remarks. All reagents were obtained from commercial suppliers and used without further purification. Solvents were obtained from EM Science and used directly. Silica gel chromatography was performed on an Isolera-4 flash purification system with prepacked silica gel cartridges (Biotage,

dx.doi.org/10.1021/co400078r | ACS Comb. Sci. 2013, 15, 503−511

Uppsala, Sweden). The H-Cube and CatCarts were purchased from ThalesNano (Budapest, Hungary). Preweighed reagents for final *N*-functionalization were obtained in 1-dram glass screw-cap vials from Frontier Scientific Services (Newark, DE). OptiBlock 24-position reactor blocks were obtained from ChemGlass (Vineland, NJ). Reactor blocks were agitated on a MaxQ 2000 orbital shaker (J-Kem Scientific, St. Louis, MO). Polypropylene 24-well plates were obtained from Thomson Instrument Co. (Oceanside, CA) and 24-well filter plates were obtained from The Automation Partnership (Hertfordshire, U.K.). Samples in 24-well plates were concentrated in vacuo on an EZ-2 Evaporator (Genevac Inc., NY). All final compounds were purified to >90% purity (at 215 nm) as determined by high-performance liquid chromatography (HPLC) on an Agilent 1100 series analytical system with tandem LC-MSD quadrupole ESI-MS. NMR spectra were determined with a Bruker DRX 400 MHz spectrometer.

*4-Azidopiperidine Hydrochloride (**4**).* A solution of *tert*-butyl 4-azidopiperidine-1-carboxylate (**3**, 1.2 g, 5.2 mmol) in 1,4-dioxane (10 mL) was treated with 1 M HCl in 1,4-dioxane (10 mL) and stirred at 60 °C. After deprotection was complete, the reaction was allowed to cool to 23 °C and concentrated in vacuo to give the desired product as the HCl salt. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.80−3.91 (m, 1 H), 3.15−3.25 (m, 2 H), 2.97 (ddd, J = 3.43, 9.68, 13.04 Hz, 2 H), 1.96−2.09 (m, 2 H), 1.61−1.76 (m, 2 H). MS (ESI, pos. ion) m/z: 127.1 [M+H].

General Procedure for the Preparation of 1-Aryl 4-Azidopiperidines 5{1−8}. A mixture of **4** (0.5 M in DMSO), aryl halide **1**{1−8} (1 equiv), and K_2CO_3 (2.5 equiv) was heated at 90 °C with stirring. After 18 h, the reaction was allowed to cool to 23 °C, diluted with water, and extracted with DCM (2×). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The crude material was adsorbed onto a plug of silica gel and purified by silica gel chromatography, eluting with a gradient of EtOAc in hexanes to provide the desired products.

***5**{1} 1-(2-(4-Azidopiperidin-1-yl)phenyl)ethanone.* Purification gradient: 5−50% EtOAc in hexanes; 61% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 7.38−7.47 (m, 2H), 7.06−7.15 (m, 2 H), 3.55−3.67 (m, 1 H), 3.18−3.30 (m, 2 H), 2.92 (t, J = 9.87 Hz, 2 H), 2.66 (s, 3 H), 2.01−2.14 (m, 2 H), 1.75−1.90 (m, 2 H). MS (ESI, pos. ion) m/z: 245.0 [M+H].

***5**{2} 2-(4-Azidopiperidin-1-yl)benzonitrile.* Purification gradient: 0−30% EtOAc in hexanes; 67% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 7.58 (dd, J = 1.61, 8.18 Hz, 1 H), 7.45−7.53 (m, 1 H), 6.99−7.06 (m, 2 H), 3.66 (tt, J = 4.07, 8.28 Hz, 1 H), 3.40−3.51 (m, 2 H), 3.06 (ddd, J = 3.14, 8.62, 12.06 Hz, 2 H), 2.11 (tdd, J = 3.34, 6.50, 12.83 Hz, 2 H), 1.84−1.99 (m, 2 H). MS (ESI, pos. ion) m/z: 228.0 [M+H].

***5**{3} 4-(4-Azidopiperidin-1-yl)benzonitrile.* Purification gradient: 10−45% EtOAc in hexanes; 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 7.49−7.55 (m, 2 H), 6.93 (d, J = 9.06 Hz, 2 H), 3.62−3.76 (m, 3 H), 3.13−3.23 (m, 2 H), 2.00−2.12 (m, 2 H), 1.70−1.83 (m, 2 H). MS (ESI, pos. ion) m/z: 228.1 [M+H].

***5**{4} Methyl 2-(4-azidopiperidin-1-yl)benzoate.* Purification gradient: 5−50% EtOAc in hexanes; 23% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 7.75 (dd, J = 1.61, 7.75 Hz, 1 H), 7.38−7.46 (m, 1 H), 6.97−7.10 (m, 2 H), 3.91 (s, 3 H), 3.58 (qd, J = 4.40, 8.73 Hz, 1 H), 3.22−3.35 (m, 2 H), 2.85−2.96 (m, 2 H), 2.06 (d, J = 10.67 Hz, 2 H), 1.78−1.92 (m, 2 H). MS (ESI, pos. ion) m/z: 261.0 [M+H].

***5**{5} Methyl 4-(4-azidopiperidin-1-yl)benzoate.* Purification gradient: 5−30% EtOAc in hexanes; 47% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 7.89−7.97 (m, 2 H), 6.91 (d, J = 8.33 Hz, 2 H), 3.88 (s, 3H), 3.62−3.76 (m, 3 H), 3.15 (ddd, J = 3.22, 9.32, 12.90 Hz, 2 H), 2.04 (br. s., 2 H), 1.69−1.84 (m, 2 H). MS (ESI, pos. ion) m/z: 261.0 [M+H].

***5**{6} 2-(4-Azidopiperidin-1-yl)pyridine.* Purification gradient: 5−50% EtOAc in hexanes; 47% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 8.19 (td, J = 1.06, 4.02 Hz, 1 H), 7.48 (ddd, J = 1.97, 7.02, 8.70 Hz, 1 H), 6.68 (d, J = 8.62 Hz, 1 H), 6.62 (ddd, J = 0.66, 4.93, 7.13 Hz, 1 H), 4.05 (td, J = 4.28, 13.52 Hz, 2 H), 3.60−3.72 (m, 1 H), 3.21 (ddd, J = 3.22, 9.79, 13.30 Hz, 2 H), 1.93−2.06 (m, 2 H), 1.61−1.77 (m, 2 H). MS (ESI, pos. ion) m/z: 204.0 [M+H].

***5**{7} 2-(4-Azidopiperidin-1-yl)pyrimidine.* Purification gradient: 5−50% EtOAc in hexanes; 72% yield. ^{1}H NMR (300 MHz, $CDCl_3$-d) δ 8.33 (d, J = 4.82 Hz, 2 H), 6.52 (t, J = 4.82 Hz, 1 H), 4.39 (td, J = 4.71, 13.67 Hz, 2 H), 3.65−3.76 (m, 1 H), 3.38−3.50 (m, 2 H), 1.92−2.04 (m, 2 H), 1.58−1.73 (m, 2 H). MS (ESI, pos. ion) m/z: 205.0 [M+H].

***5**{8} 4-(4-Azidopiperidin-1-yl)pyrimidin-2-amine.* Purification gradient: 90−100% EtOAc in hexanes; 70% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.03 (br. s., 1 H), 7.64−7.86 (m, 3 H), 6.56 (d, J = 7.60 Hz, 1 H), 3.94 (dt, J = 4.31, 8.66 Hz, 2 H), 3.46 (t, J = 10.38 Hz, 2 H), 1.84−2.08 (m, 2 H), 1.42−1.60 (m, 2 H). MS (ESI, pos. ion) m/z: 219.9 [M+H].

Screening-Scale Reduction of Azides 5{1−8}. An H-Cube hydrogenation flow reactor (ThalesNano Technology, Inc., Budapest, Hungary) was adapted to allow for fixed-loop injections (2 mL) as previously described.[14]

For each reaction screen, a 30 × 4 mm catalyst cartridge (CatCart, ThalesNano, Inc.) was placed into the CatCart holder. The temperature was set to 30 °C, flow rate to 1 mL/min, and hydrogen production to full H_2 mode. The catalyst was fully reduced and the system primed by running under stable H_2 in full mode for 10 min with solvent alone. With the system stabilized, azide solution (2 mL, 0.04 M) was introduced via the manual injector. The eluent was collected over 10 min and product purity was determined by HPLC.

Preparative-Scale Reduction of Azides 5{1−8} to Amines 6{1−8}. The injector port was bypassed on the H-Cube for the preparative scale reductions. A 10% Pt/C CatCart (30 × 4 mm, ThalesNano, Inc.) was placed into the CatCart holder. The temperature was set to 30 °C, flow rate to 1 mL/min, and the hydrogen to full H_2 mode. A solution of azide **5** in solvent (0.04 M) was then introduced directly into the H-Cube through the Knauer pump via the solvent inlet. Once the volume of azide solution was reduced to 1−2 mL, additional solvent was added to the flask while pumping was continued for 10 min. The combined eluent was concentrated in vacuo to give the products **6**{1−7} (90−98% recovered yield), which were derivatized without further purification. In the case of **6**{8}, after concentrating in vacuo, the crude product was dissolved in water and washed with dichloromethane (DCM) (2×). The aqueous phase was basified with 5 N NaOH and extracted with DCM. The DCM extract was concentrated in vacuo to afford **6**{8} in 84% yield.

*2-(4-((4-Hydroxybutyl)amino)piperidin-1-yl)benzonitrile (**8**).* Upon reduction of **5**{2} in preservative-free THF using a 10% Pt/C CatCart, the crude material was purified by reverse-phase preparative HPLC using a Phenomenex Gemini-NX, 10 μm, C-18, 110 Å, 100 × 50 mm column, A = 0.1% TFA in H_2O, B = 0.1% TFA in CH_3CN, gradient 10% to 90% B over

dx.doi.org/10.1021/co400078r | *ACS Comb. Sci.* 2013, 15, 503−511

15 min to provide **8**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.02 (br. s., 2 H), 7.42−7.61 (m, 2 H), 6.91−7.11 (m, 2 H), 5.58 (s, 4 H), 3.68 (t, J = 5.41 Hz, 1 H), 3.60 (d, J = 12.28 Hz, 2 H), 3.20 (br. s., 1 H), 3.02−3.14 (m, 2 H), 2.86 (t, J = 11.55 Hz, 2 H), 2.22 (d, J = 10.96 Hz, 2 H), 1.80−2.00 (m, 4 H), 1.69 (quin, J = 5.88 Hz, 2 H). ^{13}C NMR (300 MHz, $CDCl_3$) δ 155.19, 134.10, 134.02, 122.43, 119.12, 118.08, 106.14, 61.37, 55.15, 50.22, 45.09, 29.32, 28.73, 23.86. MS (ESI, pos. ion) m/z: 274.1 [M+H].

General Procedure for *N*-functionalization. A stock solution of each amine **6**{1−8} (0.1 M in DCM) was aliquotted (50 mg per reaction) into 1 dram screw-cap glass vials containing reagents **2**{1−15} (1.5 equiv). Triethylamine (3 equiv) was added to each vial via micropipet, the vials were tightly capped, placed in a 24-well block, and agitated on an orbital shaker at ambient temperature. Reaction progress was monitored by LC-MS. Upon completion, a small amount of methanol was added to each vial to quench excess reagent and dissolve any precipitate that may have formed. The solutions were then transferred to a 24-well polypropylene plate and solvents removed in vacuo. The residues were redissolved in dimethyl sulfoxide (DMSO) (1−2 mL) and filtered through a 24-well filter plate prior to HTPP. For the crude products that were poorly soluble in DMSO, purification by silica gel column chromatography was performed, eluting with a gradient of 0−5% or 0−6% A to B where A = 2.0 M NH_3 in MeOH and B = DCM.

Solubility Assay. Compounds as 10 mM DMSO stock solutions were charged to a 96 deep-well plate at a volume of 10 μL per well. The DMSO was removed in vacuo (Genevac Evaporator, 2.5 h, 40 °C, under full vacuum). After dry down was complete, a Tecan Evo Liquid Handler was used to transfer 200 μL PBS (1×), pH 7.4 to each well, with a comparison solution in DMSO prepared for each compound. The plates were sealed and centrifuged at 1,000 rpm for 1 min to push all liquid from the walls to the bottom of the wells. The plates were shaken at 1,500 rpm on a 3 mm radius orbital shaker for 1 h. The samples were equilibrated at room temperature for 72 h. The plates were centrifuged at 4,000 rpm for 30 min. The supernatant was analyzed by LC (215 nm, 2 μL injection volume). Peak area in PBS was compared to DMSO standard to determine solubility, accurate within the range of 1−500 μM.

Microsomal Clearance Assay. Pooled human or rat liver microsomes (0.25 mg/mL) were mixed with test compounds (1 μM) in phosphate buffer (67 mM, pH 7.4) and preincubated for 5 min at 37 °C. Subsequently, the test samples were treated with or without 1 mM NADPH and incubated at 37 °C for 30 min in a total volume of 0.2 mL. Reactions were stopped by the addition of 200 μL of ice-cold acetonitrile containing 0.5% formic acid and temazepam (500 ng/mL) as the internal standard. The quenched test samples were centrifuged at approximately 1,650g for 20 min, and the supernatants were analyzed directly for unchanged test compound using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Intrinsic clearance was calculated based on substrate disappearance rate assuming first order elimination of compound over the 30 min incubation.

Cell Permeability Assay. The parental LLC-PK1 cell line (porcine renal epithelial cells, LLC-PK1) was purchased from American Type Culture Collection (ATCC, Manassass, VA). Transfections of LLC-PK1 cells with human MDR1 gene (hMDR1-LLC-PK1) were generated. Cells were grown as previously described[19] in Medium 199 (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% (v/v) fetal bovine serum (Invitrogen, Grand Island, NY). Cells were seeded onto transwell filter membranes at a density of 90,000 cells/well in the presence of 1 μg/mL human fibronectin (BD Biosciences, Bedford, MA). Media change was performed on day 3. Compound incubations were performed 6 days post seeding. All cultures were incubated at 37 °C in a humidified (95% relative humidity) atmosphere of 5% CO_2/95% air.

Prior to the transport experiment, culture medium was aspirated from both apical and basolateral wells, cells were rinsed with warmed (37 °C) Hank's balanced salt solution supplemented with 10 mM Hepes at pH 7.4 (HHBSS, Invitrogen) as previously described.[20] HHBSS was removed from wells prior to dosing with test drugs at 5 μM in transport buffer (HHBSS containing 0.1% bovine serum albumin). One hundred-fifty microliters of transport buffer were added to receiver chambers prior to dosing in triplicate to apical or basolateral chambers. Transport studies were incubated for 2 h at 37 °C on a shaking platform. At the end of the incubation period, 100 μL samples were collected from receiver reservoirs and analyzed by LC-MS/MS on an API4000 (Applied Biosystem, Foster City, CA) triple quadruple mass spectrometer interfaced with turbo IonSpray operated in positive mode using Analyst 1.6.1 software.

The apparent permeability coefficient (P_{app}) of all tested agents was estimated from the slope of a plot of cumulative amount of the agent versus time based on the following equation:

$$P_{app} = (dQ/dt)/(A \times C_0)$$

where dQ/dt is the penetration rate of the agent (ng/s), A is the surface area of the cell layer on the Transwell (0.11 cm^2), and C_0 is the initial concentration of the test compound (ng/mL). Efflux ratio (ER) was calculated from the basolateral-to-apical permeability divided by the apical-to-basolateral permeability: ER = P_{app} B2A/P_{app} A2B.

■ ASSOCIATED CONTENT

Supporting Information

Tables of calculated physical properties, predicted RLM and HLM clearance, experimental RLM/HLM clearance, experimental solubility data, final yields, and characterization data for all library members. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: edoherty@amgen.com.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank John Eschelbach, James Petersen, Olindo Lammawin Jr., Kyung Gahm, and Dean Hickman for technical support and guidance. We also thank Les Miranda and Randy Hungate for supporting this research.

■ REFERENCES

(1) (a) Wildonger, R. A.; Deegan, T. L.; Lee, J. W. Is combinatorial chemistry on the right track for drug discovery? *J. Autom. Methods Manage. Chem.* **2003**, *25*, 57−61. (b) Kennedy, J. P.; Williams, L.;

Bridges, T. M.; Daniels, R. N.; Weaver, D.; Lindsley, C. W. Application of combinatorial chemistry science on modern drug discovery. *J. Comb. Chem.* **2008**, *10*, 345−354.
(2) (a) Sadler, S.; Moeller, A. R.; Jones, G. B. Microwave and continuous flow technologies in drug discovery. *Expert Opin. Drug Discovery* **2012**, *7* (12), 1107−1128. (b) Malet-Sanz, L.; Susanne, F. Continuous Flow Synthesis. A Pharma Perspective. *J. Med. Chem.* **2012**, *55*, 4062−4098.
(3) (a) Baumann, M.; Baxendale, I. R.; Kuratli, C.; Ley, S. V.; Martin, R. E.; Schneider, J. Synthesis of a Drug-Like Focused Library of Trisubstituted Pyrrolidines Using Integrated Flow Chemistry and Batch Methods. *ACS Comb. Sci.* **2011**, *13*, 405−413. (b) Battilocchio, C.; Baumann, M.; Baxendale, I. R.; Biava, M.; Kitching, M. O.; Ley, S. V.; Martin, R. E.; Ohnmacht, S. A.; Tappin, N. D. C. Scale-Up of Flow-Assisted Synthesis of C2-Symmetric Chiral PyBox Ligands. *Synthesis* **2012**, 635−647. (c) Lange, P.; James, K. Rapid Access to Compound Libraries through Flow Technology: Fully Automated Synthesis of a 3-Aminoindolizine Library via Orthogonal Diversification. *ACS Comb. Sci.* **2012**, *14*, 570−578.
(4) Hochlowski, J. E.; Searle, P. A.; Tu, N. P.; Pan, J. Y.; Spanton, S. G.; Djuric, S. W. An Integrated Synthesis−Purification System to Accelerate the Generation of Compounds in Pharmaceutical Discovery. *J. Flow Chem.* **2011**, *1*, 56−61.
(5) (a) Guetzoyan, L.; Nikbin, N.; Baxendale, I. R.; Ley, S. V. Flow chemistry synthesis of zolpidem, alpidem and other GABAA agonists and their biological evaluation through the use of in-line frontal affinity chromatography. *Chem. Sci.* **2013**, *4*, 764−769. (b) Desai, B.; Dixon, K.; Farrant, E.; Feng, Q.; Gibson, K. R.; van Hoorn, W. P.; Mills, J.; Morgan, T.; Parry, D. M.; Ramjee, M. K.; Selway, C. N.; Tarver, G. J.; Whitlock, G.; Wright, A. G. Rapid Discovery of a Novel Series of Abl Kinase Inhibitors by Application of an Integrated Microfluidic Synthesis and Screening Platform. *J. Med. Chem.* **2013**, *56* (7), 3033−3047. (c) Czechtizky, W.; Dedio, J.; Desai, B.; Dixon, K.; Farrant, E.; Feng, Q.; Morgan, T.; Parry, D. M.; K. Ramjee, M. K.; Selway, C. N.; Schmidt, T.; Tarver, G. J.; Wright, A. G. Integrated Synthesis and Testing of Substituted Xanthine Based DPP4 Inhibitors: Application to Drug Discovery. *ACS Med. Chem. Lett.* **2013**, *4* (8), 768−772.
(6) (a) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **2001**, *46*, 3−26. (b) Veber, D. F.; Johnson, S. R.; Cheng, H. Y.; Smith, B. R.; Ward, K. W.; Kopple, K. D. Molecular properties that influence the oral bioavailability of drug candidates. *J. Med. Chem.* **2002**, *45*, 2615−2623.
(7) Nadin, A.; Hattotuwagama, C.; Churcher, I. Lead-Oriented Synthesis: A New Opportunity for Synthetic Chemistry. *Angew. Chem., Int. Ed.* **2012**, *51*, 1114−1122.
(8) Welsch, M. E.; Snyder, S. A.; Stockwell, B. R. Privileged scaffolds for library design and drug discovery. *Curr. Opin. Chem. Biol.* **2010**, *14*, 347−361.
(9) Alabaster, C. T.; Blackburn, K. J.; Joice, J. R.; Massingham, R.; Scholfield, P. C. UK-14,275, a novel orally-active cardiac stimulant. *Br. J. Pharmacol.* **1977**, *60* (2), 284−285.
(10) Madar, D. J.; Kopecka, H.; Pireh, D.; Yong, H.; Pei, Z.; Li, X.; Wiedeman, P. E.; Djuric, S. W.; Von Geldern, T. W.; Fickes, M. G. Discovery of 2-[4-{{2-(2*S*, 5*R*)-2-Cyano-5-ethynyl-1-pyrrolidinyl]-2-oxoethyl] amino]-4-methyl-1-piperidinyl]-4-pyridinecarboxylic Acid (ABT-279): A Very Potent, Selective, Effective, and Well-Tolerated Inhibitor of Dipeptidyl Peptidase-IV, Useful for the Treatment of Diabetes. *J. Med. Chem.* **2006**, *49*, 6416−6420.
(11) *Pipeline Pilot*, Version 7.5.2; Accelrys, Inc.: San Diego, CA.
(12) Bräse, S.; Gil, C.; Knepper, K.; Zimmermann, V. Organic azides: An exploding diversity of a unique class of compounds. *Angew. Chem., Int. Ed.* **2005**, *44*, 5188−5240.
(13) Eschelbach, J. W.; Wernick, D.; Bryan, M. C.; Doherty, E. M. Characterization of dispersion effects on reaction optimization and scale-up for a packed bed flow hydrogenation reactor. *Aus. J. Chem.* **2012**, *66* (2), 165−171.
(14) Bryan, M. C.; Wernick, D.; Hein, C. D.; Petersen, J. V.; Eschelbach, J. W.; Doherty, E. M. Evaluation of a commercial packed bed flow hydrogenator for reaction screening, optimization, and synthesis. *Beilstein J. Org. Chem.* **2011**, *7*, 1141−1149.
(15) Russell, H. F.; Bremner, J. B.; Bushelle-Edghill, J.; Lewis, M. R.; Thomas, S. R.; Bates, F. A new palladium-mediated approach to 4-N-arylamino-1-butanols from peroxidic tetrahydrofuran and primary aromatic amines. *Tetrahedron Lett.* **2007**, *48*, 1637−1639.
(16) Thomas, S.; Notari, S.; Semin, D.; Cheetham, J.; Woo, G.; Bence, J.; Schulz, C.; Provchy, J. Streamlined Approach to the Crude Compound Purification to Assay Process. *J. Liq. Chromatogr. Relat. Technol.* **2006**, *29*, 699−715.
(17) For HT-NMR, samples were dissolved in d_6-DMSO in 96-well plates and transferred to 1.7 mm capillary NMR tubes using a Gilson automated liquid handler. NMR was performed on a Bruker 400 MHz NMR spectrometer with SampleJet sample changer.
(18) Lee, P. H.; Cucurull-Sanchez, L.; Lu, J.; Du, Y. J. Development of in silico models for human liver microsomal stability. *J. Comput. Aided Mol. Des.* **2007**, *21*, 665−673.
(19) Schinkel, A. H.; Wagenaar, E.; van Deemter, L.; Mol, C. A. A. M.; Borst, P. Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J. Clin. Invest.* **1995**, *96*, 1698−1705.
(20) Booth-Genthe, C. L.; Louie, S. W.; Carlini, E. J.; Li, B.; Leake, B. F.; Eisenhandler, R.; Hochman, J. H.; Mei, Q.; Kim, R. B.; Rushmore, T. H.; Yamazaki, M. Development and characterization of LLC-PK1 cells containing Sprague-Dawley rat Abcb1a (Mdr1a): Comparison of rat P-glycoprotein transport to human and mouse. *J. Pharmacol. Toxicol. Methods* **2006**, *54*, 78−89.