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Target fishing and docking studies of the novel derivatives of aryl-aminopyridines with potential anticancer activity

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ABSTRACT

A set of 16 previously synthesized aryl-aminopyridine and aryl-aminoquinoline derivatives have been evaluated for cytotoxic activity against three cancer cell lines (human cervical cancer—HeLa; human chronic myeloid leukemia—K562; human melanoma—Fem-x) and two types of normal peripheral blood mononuclear cells, with and without phytohemaglutinin (PBMC − PHA; PBMC + PHA). Twelve of the studied compounds showed moderate cytotoxicity, with selectivity against K562 but not the remaining two cancer cell lines. Four compounds were not active in cytotoxicity assays, presumably due to high predicted lipophilicity and low solubility. To rationalize the observed cytotoxic effects, structure-based virtual screening was carried out against a pool of potential targets constructed using the inverse docking program Tarfisdock and bibliographical references. The putative targets were identified on the basis of the best correlation between docking scores and *in vitro* cytotoxicity. It is proposed that the mechanism of action of the studied aminopyridines involves the disruption of signaling pathways and cancer cell cycle through the inhibition of cyclin-dependent kinases and several tyrosine kinases, namely Bcr-Abl kinase and KIT receptor kinase. The obtained results can guide further structural modifications of the studied compounds aimed at developing selective agents targeting proteins involved in cancer cell survival and proliferation.

1. Introduction

In recent years, a number of newly synthesized aminopyridine derivatives have been biologically evaluated and determined to posses the potential to interact with therapeutically relevant targets, ranging from androgen receptors to various cell cycle regulatory proteins.[1–5] The antiproliferative effects of several aminopyridine derivatives[6], as well as some structurally related aminopyrimidines[7], have strongly been attributed to inhibition of cyclin-dependent kinases. This presents a particularly promising aspect of this compound class, as it can be the basis for development of novel selectively-acting anticancer agents.

Our co-worker's research group recently designed and synthesized a series of aryl-aminopyridine and aminoquinoline derivatives.[8] They employed a strategy of mimicking the planarity and hydrogen-bonding properties of the adenosine triphosphate (ATP) 6-aminopurine core to design compounds which could potentially be of medicinal interest. Directed by their core structural features and previous reports that both aminopyridines and aminoquinolines[9] can exhibit potent cytotoxic effects, possibly through interfering with cell cycle regulation, the cytotoxic effects of these newly synthesized compounds were preliminarily evaluated using tumour cell lines, revealing some antiproliferative potential.

Although such in vitro testing using cancer cells can offer initial insights into the biological profile of novel compounds, the observed cytotoxic effects can be highly non-specific in nature and can involve several molecular targets. Rationalizing the observed effects, thus, remains a prerequisite for improving the selectivity and potency of the investigated compounds. High-throughput screening, using commercially available assay kits, is one way of achieving this goal. Alternatively, however, the process of identifying molecular targets of novel chemical entities can also be streamlined utilizing resolved three-dimensional structures of biologically relevant macromolecules which have become available over the past decade. These structures are used together with various computational methods in computer-aided drug design (CADD).[10]

In silico target fishing[11] can enable the discovery of a number of putative targets for a given set of small molecules with known

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biological effects. One of the programs offering this possibility is Tarfisdock.[12] Tarfisdock relies on exploring the interaction of studied ligands with a predetermined set of potential targets using a reverse molecular docking approach. Docking a small molecule to a bio-active macromolecule to measure the interaction and visualize the optimal pose of this ligand–protein complex is one of the widely used techniques in CADD. Molecular docking involves the implementation of a search algorithm, which generates plausible ligand-target structures; and a scoring function, which is used to calculate the binding energies between ligand and target to identify which of the plausible complex structures are of most interest. Tarfisdock results can identify macromolecules with which the studied ligands can potentially interact.

While the use of Tarfisdock can help generate a small pool of putative targets, obtained results need to be carefully considered and further refined. As in other areas of structure-based virtual screening, false positives can be an issue[13] as docking scores and actual binding affinities may differ considerably.[11,14] Therefore, information on putative targets should always be integrated with available experimental data, previously published studies and additional docking experiments with select targets.

In this work, a set of 16 previously synthesized aryl-aminopyridines have been evaluated for cytotoxic activity at three different cancer cell lines and two types of normal cells. To rationalize the results of in vitro testing and elucidate the mechanism of action of these compounds, Tarfisdock was used to identify a preliminary pool of putative targets. This pool was refined and subsequently manually expanded. Interaction of aryl-aminopyridines with the expanded target pool was investigated using the docking program AutoDock[15] to gain an insight into possible interactions of this scaffold with different cell cycle regulatory proteins. Based on the obtained results of virtual screening, a potential mechanism of action of these newly synthesized compounds is proposed and ways for further developing this compound group into novel anticancer agents are discussed.

2. Experimental

2.1. Biological tests

2.1.1. Preparation of stock solutions

Stock solutions of the test compounds were prepared in dimethyl sulfoxide (DMSO) at 10 mM, filtered through Millipore filters (0.22 μm), and diluted for use in the nutrient medium to the relevant working concentrations. For all of the cells used, the nutrient medium was RPMI 1640 without phenol red, supplemented (final concentrations) with L-glutamine (3 mM), streptomycin (100 μg/mL), and penicillin (100 IU/mL), fetal bovine serum (10%; FBS; 56 °C heat-inactivated) and HEPES (25 mM), adjusted to pH 7.2 (bicarbonate solution). For cell survival determinations, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in phosphate buffered saline, pH 7.2 (to 5 mg/mL), and filtered through Millipore filters (0.22 μm) before use.

2.1.2. Cell culture

The HeLa (human cervix adenocarcinoma) and Fem-x (human malignant melanoma), cells were cultured as monolayers in the nutrient medium (see above), while the K562 (human myelogenous leukemia) cells were maintained as suspension cultures in the same nutrient medium. All of these cells were grown at 37 °C in 5% CO_2 and a humidified air atmosphere. The PBMCs (peripheral blood mononuclear cells) were separated from whole heparinized blood of healthy volunteers by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation. The interface cells were washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g/L gelatin polymers, pH 7.4), counted and resuspended in nutrient medium to the required cell concentration.

2.1.3. Cell sensitivity analysis

The HeLa and Fem-x cells were seeded (2000 cells per well) into 96-well microtiter plates, and 20 h later, after cell adherence, five different concentrations of the test compounds were added to the wells. The final test compound concentrations were from 12.5 to 200 μM. Only nutrient medium was added to the cells in the control wells. For the K562 cells the test compounds were added to cell suspensions (3000 cells per well) 2 h after cell seeding, to the same final concentrations applied to the HeLa and Fem-x cells. All of the experiments were carried out in triplicate. Nutrient medium with the corresponding concentrations of the test compounds, but void of cells, was used as the blank. PBMCs were seeded (150,000 cells per well) into nutrient medium without or with 5 μg/mL PHA (Welcome Diagnostics, England) in 96-well microtiter plates, and 2 h later the test compounds were added to the wells, in triplicates, to the five final concentrations of: 12.5, 25.0, 50.0, 100 and 200 μM, except to the control wells where only nutrient medium was added. Nutrient medium with the corresponding concentrations of the test compounds, but void of cells, was used as the blank.

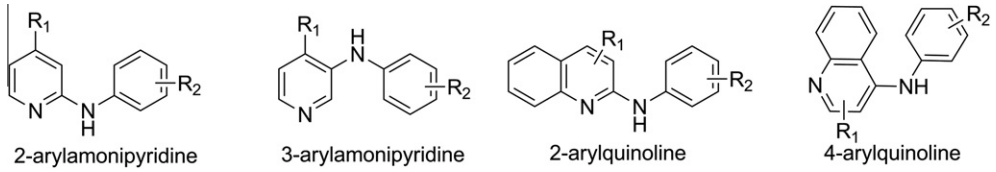
2.1.4. Determination of target-cell survival

Cell survival was determined by the MTT test according to the method of Mosmann et al.[16] and modified by Ohno and Abe[17], at 72 h after the test compound additions. Briefly, 20 μL MTT solution (5 mg/mL in phosphate buffered saline) was added to each well. The samples were incubated for a further 4 h at 37 °C in 5% CO_2 with a humidified atmosphere. Then, 100 μL 10% sodium dodecyl sulfate (SDS) was added to each of the wells, and the absorbance of the cell medium from each well was measured at 570 nm the next day. To calculate the cell survivals (%), the absorbance at 570 nm of each of the samples with cells grown in the presence of the test compounds were divided by the absorbance of the control sample (the absorbance of cells grown in nutrient medium only), after the subtraction of the blank sample absorbance.

2.2. Docking studies

2.2.1. Preparation of the ligand molecules

The structures of all 16 aryl-aminopyridines synthesized in our previous work (summarized in Scheme 1 and Table 1.) were drawn with ChemBioDraw, converted into 3D structures and saved as .mol2 files in ChemBio3D. These files were imported into Avogadro[18] (© http://avogadro.openmolecules.net) and the protonation states of ionizable groups were adjusted to correspond to pH 7.4. Since all 16 molecules are weak bases (predicted $pK_a(b) \sim 3.9$), no additional hydrogens were added and molecule charges were not altered at pH 7.4. The structures were opened with VegaZZ and minimized after assigning Gasteiger charges[19], using AMMP and conjugate gradients (Force field: SP4, Minimization steps: 3000, Dielectric constant: 80.0000, Toler: 1.0000 and Graphic update: (1). Conformational search was carried out by AMMP (Force field: SP4, Method: random, Steps: 2000; Minimize all conformations: Steps: 500, Toler: 0.01; Dielectric constant: 80.0 and Graphic update: 1; all flexible torsions were selected for conformational search) and the structure's trajectory, output and energy were saved. The best (lowest energy) conformation was saved and further optimized using the semi-empirical Molecular Orbital calculations package MOPAC[20] (theory type: PM3). The resulting structure was saved as a new .mol2 file for docking simulations.



Scheme 1. Parent scaffolds of compounds investigated in this study.

Table 1
Structures of novel compounds investigated in this study

Aminopyridine scaffold				Aminoquinoline scaffold			
Name	Type	R_1	R_2	Name	Type	R_1	R_2
SP24	2-Aryl–	*p*-Me-Phenyl	*p*-MeO	SBJ31	2-Aryl–	4-H	*p*-MeO
SP28	2-Aryl–	*m*-CF3-Phenyl	*p*-MeO	SBJ32	2-Aryl–	4-H	*p*-F
SP29	2-Aryl–	*m*-CF3-Phenyl	*p*-Et	MPA1	2-Aryl–	3-*p*-Me-Phenyl	*p*-MeO
SP31	2-Aryl–	*p*-Me-Phenyl	2-Naphthyl	MPA10	4-Aryl–	3-*p*-MeO-Phenyl	*p*-MeO
SP32	2-Aryl–	*m*-CF3-Phenyl	2-Naphthyl	MPA11	4-Aryl–	3-Benzene	*p*-MeO
SP34	2-aryl–	*m*-CF3-Phenyl	*m*-MeO	MPA12	2-Aryl–	3-Benzene	*m*-MeO
SBJ26	2-Aryl–	H	*p*-MeO	MPA13	2-Aryl–	3-Benzene	*p*-Et
SBJ27	3-aryl–	H	*p*-MeO				
SBJ29	2-Aryl–	*p*-Me-Phenyl	*p*-F				

Synthesis described in Ref. 8.

2.2.2. Defining a virtual screening pool

Searching for targets and defining a virtual screening pool was carried out in two consecutive steps.

Firstly, Tarfisdock[12] was applied with PDTD (Potential Drug Target Database).[21] The online reverse docking server Tarfisdock with PDTD includes 1207 entries of 841 known and potential drug targets from PDB, divided into 15 therapeutic areas and 13 biochemical criteria, respectively.

The active site of each protein was defined as all residues within 6.5 Å of the ligand bound in the crystal structure. A sphere file for the active site was generated using the SPHGEN program.[22] DOCK scoring function and fragment-based incremental addition searching algorithm (DOCK 4.0,[23]) is used in Tarfisdock. In general, the top 2%, 5% or 10% of the ranking list (interaction energy value (kcal/mol)) of each therapeutic area and the whole database were allowed to be generated automatically by Tarfisdock, from which the user may select protein candidates for further biological and docking study.

The optimized structures of 16 compounds were uploaded onto an online reverse dock server Tarfisdock and neoplastic disease category was manually selected to identify potential cancer related targets. Top 10% output of the targets ranked according to energy scores of each minimized molecule were adopted initially (11 targets). The results were compared to the list of targets described in the PDTD.[21]

Secondly, to refine and expand the target pool identified by Tarfisdock, SCIENCEDIRECT online bibliography database was searched using keywords 'Hela (Fem-x, K562)' AND 'docking' AND 'pdb'; The ligand category in the protein data bank (PDB)[24] (http://www.pdb.org/pdb/home/home.do) was queried for 'aminopyridines' as a keyword, and the kinase related PDB entries were selected if they contained ligands that have structural similarity to novel aminopyridine compounds investigated in this study (14 targets).

2.2.3. Virtual screening

The docking studies were carried out using GriDock, software based on AutoDock 4 and developed for virtual screening utilizing multi-core architectures. PDB structures of all 24 protein targets were prepared for screening using VegaZZ 2.3.2.38[25,26] by removing water molecules, adding missing hydrogens, setting correct bond types and assigning Gasteiger charges. If a PDB file contained more than one subunit, only one of the monomers was taken into consideration.

The residues within the 15 Å radius around the ligand molecule were selected, the ligand was removed prior to running AutoGrid to create the atom maps .map, grid parameter .gpf and receptor .pdbqt file. The *database.sdf* containing 16 molecules as ligands and *receptor.pdbqt* as a receptor for each protein in the screening pool were used to run virtual screening using GriDock. Each ligand was flexibly docked into the generated binding sites using the AutoDock's Lamarckian algorithm. The default settings for AutoDock and GriDock were used for docking, namely genetic-based algorithm was used with 10 runs with 100,000 energy evaluations and a maximum number of generations of 27,000, with crossover rate set to 0.8. This resulted in 10 solutions evaluated by binding scores and clustered in groups of conformations with a root mean square deviation (RMSD) lower than 2 Å. Mean binding energies values (standard error less than 0.5 kcal/mol) varied in a similar way as the highest binding energies for all substrates. Since the latter reflect the most favourable interactions between a substrate and a target, these docking scores are reported and were used in subsequent calculations. The automatically generated *.dlg* files were used for visualization of the best conformations of each molecule in interaction with the receptor.

2.2.4. Correlation of $\log IC_{50}$ with active aryl-aminopyridines binding energy scores

After virtual screening, a data mining software, Gretl 1.9.1[27], was used to generate correlations (5% critical value (two-tailed) = 0.5760 for $n = 12$) between experimental $\log IC_{50}$ and binding energy scores of active molecules (compounds No. 1, 2 and 7 to 16, as numbered in Table 2), in order to identify the potential targets of these aryl-aminopyridine analogues. Compounds **3**–**6** were omitted for all cell lines because of lack of cytotoxicity, while compound **15** was additionally omitted for HeLa cell line.

Table 2
Cytotoxic activity of aryl-aminopyridine derivatives against HeLa, Fem-x, K562, PBMC − PHA and PBMC + PHA cells

No.	Name	Cytotoxicity (IC_{50} ± SD, μM)[a]				
		HeLa	Fem-x	K562	PBMC − PHA	PBMC + PHA
1	SP24	43 ± 3	33 ± 2	19 ± 1	52 ± 4	34 ± 3
2	SP28	140 ± 8	68 ± 3	78 ± 2	132 ± 5	105 ± 5
3	SP29	>200	>200	nd[b]	nd	nd
4	SP31	>200	>200	nd	nd	nd
5	SP32	>200	>200	nd	nd	nd
6	SP34	>200	>200	nd	nd	nd
7	SBJ26	117 ± 11	123 ± 14	63 ± 18	46 ± 4	42 ± 5
8	SBJ27	145 ± 26	118 ± 20	68 ± 1	96 ± 6	183 ± 7
9	SBJ29	146 ± 15	79 ± 8	54 ± 11	127 ± 9	146 ± 7
10	SBJ31	51 ± 5	43 ± 2	30 ± 7	91 ± 4	70 ± 6
11	SBJ32	33 ± 6	35 ± 4	36 ± 3	90 ± 6	65 ± 4
12	MPA1	102 ± 2	65 ± 6	75 ± 6	137 ± 2	118 ± 10
13	MPA10	104 ± 0	56 ± 3	25 ± 2	122 ± 6	105 ± 4
14	MPA11	64 ± 4	35 ± 1	25 ± 3	72 ± 2	62 ± 2
15	MPA12	act[c]	129 ± 7	76 ± 5	119 ± 7	81 ± 9
16	MP13	140 ± 3	92 ± 6	99 ± 7	73 ± 1	66 ± 1

[a] Results are presented as mean IC_{50} ± standard deviation (IM).
[b] nd—not determined.
[c] act—some cytotoxicity was observed but the results of IC_{50} calculations were at near the limit of the tested range.

3. Results and discussion

3.1. Cytotoxicity evaluation

The 16 aryl-aminopyridine and aryl-aminoquinoline derivatives investigated in this study share a degree of structural similarity with other compounds based on the same scaffold that have previously been evaluated for their biological effects.[6,9] As these compounds possessed cytotoxic properties, it was presumed that the newly synthesized aminopyridine derivatives could also have similar biological activity. To explore this, a cytotoxicity evaluation was carried out using three different cancer cell lines and two types of normal peripheral blood mononuclear cells. Results of these cytotoxicity assays are summarized in Table 2.

A moderate degree of cytotoxicity was observed for 12 of the tested compounds, while compounds SP29, SP31, SP32 and SP34 displayed no significant effects on cell death. Except for compounds SBJ26 and MP13, selectivity of cytotoxic effects towards cancer cells as opposed to normal blood mononuclear cells was observed with respect to K562 cell line only, which was significantly more susceptible to the tested compounds than either PBMC − PHA (p <0.01) or PBMC + PHA cells (p <0.05).

Lack of in vitro activity of 4 of the assayed compounds was then considered. Core structural features of the tested aminopyridine and aminoquinoline derivatives were designed to mimic the 6-aminopurine core of ATP, potentially allowing these compounds to competitively bind to ATP sites within their putative targets. The variable substituents attached to the aminopyridine core are of lipophilic character, which could facilitate the formation of hydrophobic interactions with corresponding pockets within the target proteins. However, the highly lipophilic character of naphthyl and three fluoromethylphenyl substituents found in 4 inactive compounds is likely responsible for their lack of activity, as the high lipophilicity and low solubility of these compounds will tend to prevent them from efficiently permeating into the intracellular environment. These 4 compounds could possibly form favourable interactions and were not discarded as structures with potential for further modifications, and were not excluded from subsequent docking studies.

Although moderate, the cytotoxic activity of the studied aryl-aminopyridine derivatives seems to present a promising starting point for further structural modifications of this class of compounds, which requires the rationalization of their mechanism of action. As was previously discussed, the cytotoxic effects of similar compounds have mostly been attributed to interaction with cyclin-dependent and checkpoint kinases, but aminopyridines are also known to interact with a variety of different targets. A non-specific mode of action might be responsible for the low to moderate cytotoxic activity of the investigated compounds. Accordingly, we next decided to construct a pool of potential targets rather than attempting to immediately limit our target search to one or more cell cycle regulatory proteins previously implicated in the effects of similar compounds.

3.2. Target pool construction

Identifying and validating a target from hundreds of candidate macromolecules is a challenging task. To rationalize the cytotoxic effects of the studied aryl-aminopyridines, a pool of potential targets was constructed in two steps. First, a reverse docking procedure was carried out using Tarfisdock. Tarfisdock can automatically select a potential target pool according to the energy score rankings by docking the studied set of ligands to all targets in the neoplastic related category of PDTD. Eleven targets in total for each molecule of aryl-aminopyridines (top 10% of neoplastic related targets category) were selected. Out of all 16 identified potential targets, those that occur in more than five compounds were considered, yielding a preliminary pool of 14 targets.

While reverse docking strategy has been implemented with success in identifying potential protein targets for small molecules with known biological activity[28–30], there are some generally recognized limitations of this approach. Mainly, the limited accuracy of the scoring function used may lead to the occurrence of false positives, while the underrepresentation of rare proteins or those for which three-dimensional structures are not available can cause false negatives.[31,32] Furthermore, Tarfisdock is based on DOCK 4.0 method whose scoring function only accounts for van der Waals and electrostatic interactions but not for hydrogen bonding and solvation free energy. The DOCK 4.0 method is, thus, less computationally intensive, making it suitable for fast screening at the expense of reduced accuracy. The neoplastic related category of the online Tarfisdock PDTD database is also limited in size to 95 entries, and was last updated on 2008-02-21.[21] Any cancer-related 3D structures that were resolved or modified after this date will not be available in this database.

For these reasons, the target pool of 14 proteins generated by Tarfisdock was treated only as a working hypothesis. Notably, aminopyridines seemed to show a high affinity for several target proteins with large binding cavities (e.g. Holo-Neocarzinostatin, Cytohrome P450 reductase). Considering the scoring function limitations, this was attributable to non-specific interactions between small ligand molecules and the large binding pockets, and such targets were not included in the final pool.

To complete the target pool for virtual screening, a second, knowledge-based approach was used. A bibliographic search yielded several targets which are a part of cell signalling pathways and could potentially account for the antiproliferative action of the studied aminopyridines. The final target pool is summarized in Table 3 and consisted of 16 potential targets and 24 PDB entries.

Consequently, the target pool that was constructed presents a comprehensive set of enzymes and regulatory proteins which could be implicated in the antiproliferative effects of the studied aminopyridines. As such, it was considered as a good set of targets for virtual screening.

Table 3
Selection of the protein targets for aryl-aminopyridines, found by the reverse docking Tarfisdock and search of Science Direct and Protein Data Bank

Targets	PDB	Source
Neutrophil collagenase 8 (MMP8)	1I76,1BZS	Tarfisdock
Adenosine deaminase (ADA)	2ADA	Tarfisdock
Aspartate carbamoylchangease (ATCase)	1ACM	Tarfisdock
Carboxypeptidase A (CPA)	6CPA,1CPS	Tarfisdock
CDK/CYCLIN	1GII,1H08,1OIU	Tarfisdock, Bibliography search
Elongation factor Tu (EF-Tu)	1HA3	Tarfisdock
Human histone deacetylase (HDAC)	1C3R	Bibliography search
BCR-ABL	3K5V,1OPJ,1FPU	Bibliography search
Topoisermerase	1SC7	Bibliography search
Tubulin	1SA0	Bibliography search
C-jun N-terminal kinase (JNKs)	2GMX,2NO3	Bibliography search
Focal adhesion kinase (FAK)	2JKO	Bibliography search
Actin-like kinase (ALK)	3GXL	Bibliography search
Aurora kinase	2C6D,2C6E	Bibliography search
KIT receptor	1T46	Bibliography search
cAMP-dependent protein kinase (PKA)	1JLU	Tarfisdock

3.3. Virtual target screening

Virtual screening was carried out using AutoDock 4.0. As opposed to DOCK 4 used in Tarfisdock, AutoDock 4 features a more comprehensive five-term empirical scoring function for energy evaluation, accounting for dispersion/repulsion, hydrogen bonding, electrostatics, and desolvation.[33]

The binding energies for 16 studied compounds against each of the 24 protein targets alone did not give a clear indication as to which of the proteins presents a target responsible for the observed cytotoxic effects. It should be noted that the resolution at which the respective structures have been resolved is not uniform (1.20–3.58 Å) which can affect the degree to which obtained docking scores are directly comparable between targets. Thus, binding energies were subsequently correlated to the experimentally determined IC_{50} values using data mining techniques. The matrix of binding energies of all ligands into 24 protein targets was analysed using Gretl 1.9.1 to obtain a correlation table with the in vitro measured cytotoxicity. Since no definitive measure of cytotoxic activity was established for 4 of the studied compounds (compounds 3–6 in Table 2), a correlation matrix was generated for 12 active molecules (5% critical value (two-tailed) = 0.5760 for $n = 12$). The obtained results revealed a number of targets which could be implicated in the mechanism of action of the studied compounds. Targets for which the highest correlation was observed, together with the corresponding correlation coefficients are shown in Table 4. All of the relevant binding energies are summarized in Table 5.

The correlation varied considerably between a given target and the cytotoxicity determined in different cells (Table 4). The affinity for kinases CDK, ABL and KIT selected by Tarfisdock and knowledge -based approaches correlated more strongly to cytotoxicity in HeLa, Fem-x and K562 cancer cells than normal cells. Same can be observed, though less consistently, for Aurora Kinase A and Topoisomerase 1. In particular, the cytotoxicity towards human malignant melanoma Fem-x is consistently correlated with binding energies in all targets except MMP8. Correlations for Matrix Metalloproteinase 8 (Neutrophil Collagenase-2) were similar in

Table 4
The correlation coefficients (R) between cytotoxicity of investigated compounds ($-\log IC_{50}$) and energy scores for the docking of active molecules against selected targets[a]

	CDK			ABL	KIT	TOPO 1	AURORA A	MMP8	
	1gii	1h08	1oiu	1fpu	1t46	1sc7	2c6e	1i76	1bzs
log HeLa	−0.340	−0.2429	−0.5676	−0.3771	−0.6410	−0.5090	-0.4209	0.0921	−0,4198
log Fem−x	−0.5362	−0.5007	−0.6107	−0.6223	−0.6296	−0.6021	−0.5738	−0.0859	−0.2733
log K562	−0.297	−0.2303	−0.2953	−0.4279	−0.4541	−0.269	−0.1949	0.093	−0.0891
log PBMC + PHA	0.248	0.2356	0.1625	−0.2191	−0.2095	0.1606	−0.0641	−0.1027	0.4192
log PBMC − PHA	−0.1626	−0.1371	−0.3407	−0.463	−0.4424	−0.3284	−0.3731	−0.0307	−0.1039

The docking was carried out using GriDock and AutoDock.
[a] Correlation coefficients were calculated for sets of 12 molecules for all cell lines except for HeLa cell line for which 11 molecules exhibited measurable activity.

Table 5
The binding energy scores in kcal/mol obtained by GriDock/AutoDock 4 for all 16 ligands against the most probable targets and corresponding PDB entries

	CDK			ABL	KIT	TOPO 1	AURORA A	MMP8	
	1gii	1h08	1oiu	1fpu	1t46	1sc7	2c6e	1i76	1bzs
SP24	−7.4	−7.13	−7.35	−8.04	−7.41	−7.77	−6.96	−6.65	−7.55
SP28	−6.9	−7.03	−7.05	−7.21	−5.79	−6.93	−6.62	−6.48	−7.62
SP29	−7.12	−7.33	−7.53	−6.52	−6.5	−6.49	−7.51	−7.59	−7.56
SP31	−8.89	−7.64	−8.64	−8.47	−7.56	−8.53	−8.03	−9.36	−8.95
SP32	−7.46	−7.85	−8.86	−7.14	−6.57	−8.49	−7.92	−7.18	−6.79
SP34	−6.29	−6.44	−6.19	−6.5	−6.22	−7.08	−5.53	−6.54	−7.45
SBJ26	−5.1	−5.7	−5.84	−6.26	−5.78	−5.45	−5.25	−6.42	−6.14
SBJ27	−5.26	−4.91	−5.36	−5.67	−5.65	−5.06	−5.32	−6.41	−5.77
SBJ29	−7.16	−7.82	−6.95	−7.59	−6.82	−6.71	−6.06	−8.1	−7.23
SBJ31	−6.76	−6.79	−6.94	−6.93	−6.95	−7.16	−5.8	−7.56	−6.9
SBJ32	−6.79	−6.74	−7.51	−7.12	−6.96	−7.31	−6.8	−7.12	−7.42
MPA1	−6.85	−7.33	−7.02	−6.47	−6.48	−7.64	−6.23	−6.15	−7.53
MPA10	−6.79	−6.41	−6.67	−6.64	−6.12	−6.76	−5.77	−6.57	−6.88
MPA11	−7.56	−7.88	−7.34	−8.03	−6.41	−7.18	−7.02	−6.76	−6.95
MPA12	−7.26	-6.95	−7.32	−6.73	−6.26	−7.24	−6.29	−6.04	−8.29
MP13	−7.2	−6.78	−7.03	−7.53	−6.86	−7.37	−6.91	−8.84	−6.43

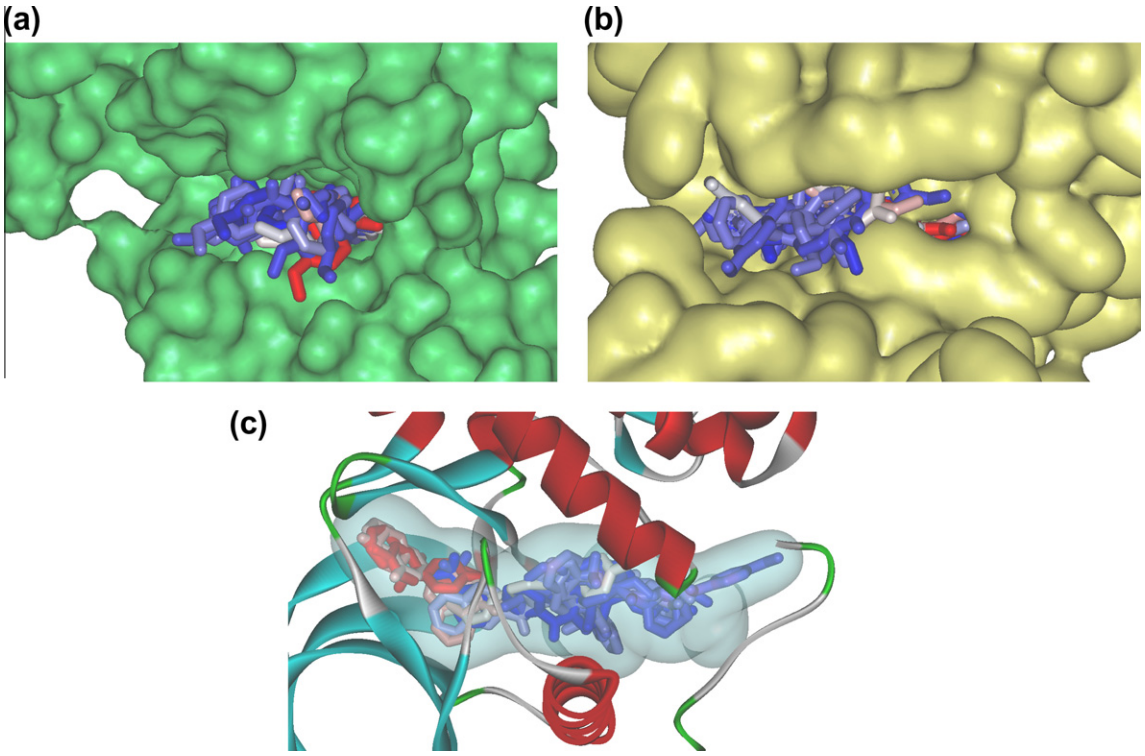


Figure 1. Visualization of binding modes within active sites of selected potential targets. All 16 ligands are shown in stick representation in the protein cavities of (a) CDK2 (pdb entry 1GII), (b) Aurora Kinase (pdb entry 2C6E) and (c) KIT (pdb entry 1T64). Substrates are represented as sticks, targets are shown either in a surface representation or as a ribbon with a binding pocket surface.

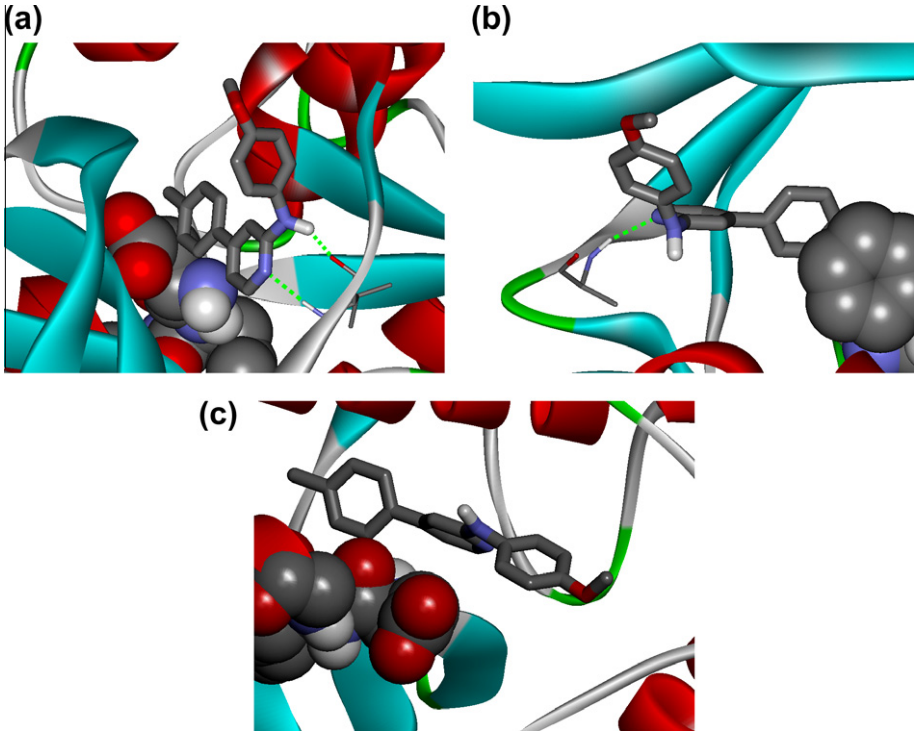


Figure 2. Visualization of binding modes of SP24 within the active sites of (a) CDK2 (pdb entry 1GII), (b) Aurora Kinase (pdb entry 2C6E) and (c) KIT (pdb entry 1T64).Proteins are represented as ribbons with the DFG loop shown in a CPK representation and interacting residues are shown as thin sticks. The hydrogen bonds are shown as green lines.

all cell lines and comparatively smaller. Conversely, affinity for ABL kinase and KIT Receptor kinase was well correlated to cytotoxicity in each of the cell lines.

Surprisingly, in Table 5, where binding energy scores are presented, the values for in vitro inactive compounds SP29, SP31, SP32 and SP34 are in fact higher than the values for the active

ligands in the series. However, the high lipophilicity and low solubility of these compounds (SP29: log*P* = 6.29, log*S* = −5.90; SP31: log*P* = 6.48, log*S* = −6.01; SP32: log*P* = 7.24, log*S* = −6.44; SP34: log*P* = 6.11, log*S* = −5.50) clearly indicate that the physicochemical properties of these compounds could be a reason for the lack of cytotoxicity due to inability of these ligands to reach the site of interaction in cells. Further optimization of lipophilic properties in the series using standard approaches should alleviate this problem.

Figure 1 illustrates the binding of these aryl-aminopyridines into the active sites of three kinases identified as their potential targets (CDK2, Aurora Kinase and KIT). The studied molecules are relatively small compared to binding sites of these three proteins, resulting in their diverse but partially overlapping binding modes. This is also consistent with the results of cluster analysis carried out after the docking simulations, whereby most clusters were modestly populated. These results clearly suggest that the interaction between the studied aminopyridines and their putative targets is non-specific in nature, which can account for their moderate potency and lack of observable selectivity.

Further analysis of the obtained binding poses also revealed possible interaction between substrates and targets, and the potential to improve the selectivity and potency of these compounds. SP24 was chosen as a representative molecule with the highest relative binding affinity and good in vitro activity, and its interactions with the active sites of three kinases are shown in Figure 2. This aryl-aminopyridine analogue binds in a close proximity of the ASP-PHE-GLY (DFG) loop which is essential for activity in many tyrosine kinases.[34] SP24 can be seen to form two hydrogen bonds with binding site residues of CKD2 (Fig. 2a), exemplifying its ATP-mimicking characteristics. Different mode of binding is observed for two other kinases (Fig. 2b and c), allowing a possibility to structurally optimize studied substrates and achieve target specificity.

3.4. Summary interpretation of results and proposed mechanism of action

The targets identified through virtual screening are known to be implicated in the pathogenesis or progression of cancer. Cyclin-dependent kinases are now well established targets in cancer treatment, with compounds such as *R*-roscovitine, which inhibits CDK2, shown to exhibit potent cytotoxic effects against proliferating cells selectively.[35] Similar can be said for ABL kinase and KIT receptor kinase, which are targets for clinically established tyrosine kinase inhibitors imatinib, dasatinib and nilotinib. Inhibition of Aurora A kinase, which is overexpressed in certain types of cancer, is also documented to have apoptotic effects.[36] Inhibition of topoisomerase I is the established mechanism of action for a number of anticancer therapeutics, including camptothecin and irinotecan.

The role of certain matrix metalloproteinase enzymes in cancer is less evident. The role of these enzymes is no longer perceived to be of importance only in the metastatic and invasive processes, but also in early stages of tumor development.[37] In specific, matrix metalloproteinase 8, which was identified as a target for the studied aminopyridines, is increasingly seen as an anti-target in cancer treatment, as it is believed to play a protective role in carcinogenesis.[38] Thus, the potential of these studied compounds to act as substrates for MMP8 seems to be an unwanted property.

The results of virtual screening and subsequent data mining suggest that the studied aminopyridines display affinity for a range of tyrosine and serine kinases and topoisomerase I. We propose the inhibition of these kinases presents the basis for cytotoxic effects observed *in vitro*. Binding energy scores of all 16 studied aminopyridines support the initial hypothesis that the lack of activity in 4 of the studied compounds can be attributed to their physicochemical properties (i.e. high lipophilicity and low solubility) rather than lack of activity at target sites. The non-specific nature of interaction with various kinases possibly presents the basis for the moderate nature of cytotoxic effects of these compounds. Another factor which could contribute to lack of potency is the unwanted interaction with MMP8, which could offset some of the antiproliferative effects of the studied aminopyridines.

Although selectivity of action was observed with respect to the K562 cell line, the cytotoxicity of the evaluated compounds was otherwise fairly non-selective. This lack of selectivity for proliferating as opposed to predominantly non-cycling cells can potentially be explained by a broad-spectrum inhibition of CDKs. Acute toxicity towards peripheral blood cells, not related to any bone marrow effects, has been documented for several broad-spectrum CDK inhibitors.[39] This should point further investigations towards profiling the CDK selectivity of the studied aminopyridines in greater detail.

3.5. Further development of the studied compounds

The measured activities of these aminopyridine derivatives are moderate. However, taken together with literature data on similar compounds and the target profile determined in this study, the consistent observation of cytotoxic effects in vitro can be seen as a confirmation of the potential of the underlying design strategy and the value of the aryl-aminopyridine scaffold.

In comparison with other drug candidates these aryl-aminopyridine analogues have lower molecular weight (average MW: 300 g/mol), which makes them good candidates for modifications to achieve higher activity and specificity. The presence of hydrogen bond forming groups also contributes to target affinity (binding energy scores between −10 and −5 kcal/mol) and appropriate modification could improve selectivity. Compounds **3**–**6** show particularly lower cytotoxicity against all cell lines, possibly due to their high lipophilic nature and low solubility.

As outlined in the previous section, further development of the studied compounds should address several issues: (i) to improve cytotoxic activity, selectivity should be improved to reduce the number of proteins targeted and achieve greater affinity for select kinases; (ii) the affinity for MMP8, an anti-target in cancer treatment, should be designed out; (iii) physicochemical profile of some of the compounds should be optimized to assure adequate distribution into the intracellular environment.

Although the investigated compounds seem to show affinity for a number of therapeutically important targets, developing novel cancer treatments should focus on assuring selectivity of action.

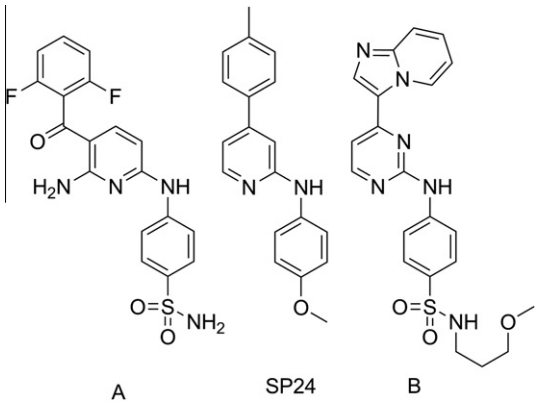


Figure 3. Comparison between SP24, one of the aminopyridines evaluated in this study, and CDK inhibitors reported in the literature.

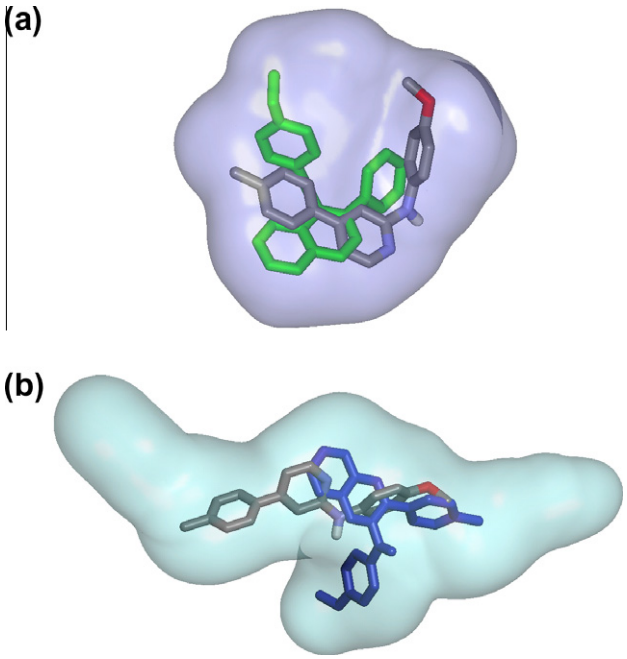


Figure 4. Overlap of binding poses SP24 (sticks in CPK colours) and MPA11 (green sticks) structures within (a) CDK2 and (b) KIT kinase binding sites represented by transparent surfaces.

Thus, further developing these aminopyridines should focus on specifically targeting CDK or tyrosine kinases, such as ABL and KIT receptor kinase. A particularly promising direction could be the development of these compounds into selective CDK inhibitors. Figure 3 illustrates structural similarity between SP24, synthesized by our co-workers, and compounds reported in the literature to present potent CDK inhibitors.

Compound A shown in Figure 3 is a 3-acyl-2,6-diaminopyridine derivative reported by Lin et al.[6] to inhibit CDK1 and CDK2 in the submicromolar range, while not interacting with other kinases such as VEGF, HER2 and EGFR. Compound B was reported by Byth et al.[40] as a CDK2 inhibitor in the nanomolar range, with selectivity over CDK1 and CDK4, as well as 12 other kinases. SAR studies conducted by Byth et al. indicated that the presence of sulfonamide moiety in Compound B contributes to selectivity over other kinases as well as reduced toxicity towards non-proliferating cells. Thus, *p*-substitution of the *N*-aryl moiety of the studied aminopyridines with a sulfonamide group could be an important direction to take in modifying these structures. Such a modification would also improve the physicochemical characteristics of these compounds.

Our docking results could guide rational design of novel derivatives of the studied scaffold by analyzing the observed interactions with the protein binding sites. This is exemplified by Figure 4, where the overlaps of best binding poses of two analogues (SP24 and MPA11) are shown for two targets. While these two share an overlapping binding site, it is possible to observe that their interactions with target proteins are dominated by different interactions. This differential mode of interaction can present a basis for subsequent tailored structure modifications. The interactions of designed analogues should be explored by means of molecular dynamics simulations to ensure that flexibility of proteins is considered and the optimal interaction and selectivity are achieved.

In terms of designing out the interaction with MMP8, most studied inhibitors of this enzyme are based on a tetrahydroisoquinoline[41,42] and malonic acid scaffolds.[43] Key features of these inhibitors include the presence of zinc chelating groups and a substructure that fits into the S1' specificity pocket of the enzyme, which allows for high affinity binding. While structurally seemingly unrelated to these scaffolds, aminopyridines also have chelating abilities and their N-aryl substituents could potentially form hydrophobic interaction with the specificity pocket. Subsequent modification efforts of the studied aminopyridines should rely on three-dimensional structures of MMP8 complexed with specific inhibitors to visualize and evaluate the best way of hindering this interaction.

Finally, drugs targeting specific signal transduction pathway proteins are known to exhibit a large variance in in vitro cytotoxicity depending on the cell line chosen for testing.[44] Further investigation of these aminopyridines and any newly synthesized derivatives should take into account the preliminary findings on their target profile when choosing the cell lines. This could help obtain a better measure of their antiproliferative potential and contribute to validating the proposed mechanism of action.

4. Conclusion

A set of 16 newly synthesized aryl-aminopyridine and aryl-aminoquinoline derivatives have been evaluated in cytotoxicity assays and moderate activity was observed. To rationalize their mechanism of action, a pool of potential targets was generated and virtual target screening was carried out. Docking results indicated that the studied compounds interact with a number of kinases included in cell cycle regulation. It is proposed that non-specific inhibition of several cyclin-dependent and tyrosine kinases is responsible for the observed cytotoxic effects. This presents a promising starting point for further structural modifications aimed at improving selectivity for CDKs in particular, while eliminating unwanted interactions with MMP8. Modifications of the studied aminopyridines could lead to the development of specific cyclin-dependent kinase inhibitors with highly selective action against cancer cells.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.06.051. These data include MOL files and InChiKeys of the most important compounds described in this article.

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