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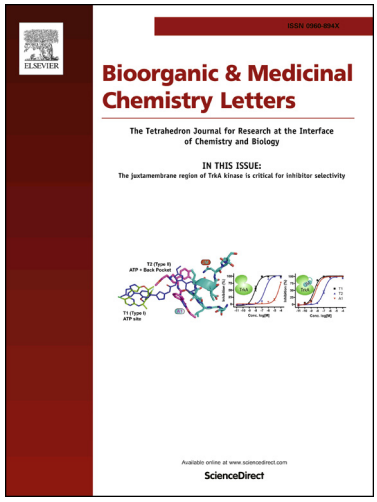

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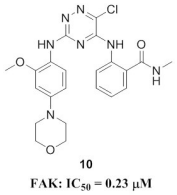

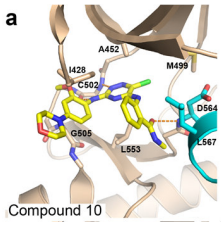

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Synthesis of novel 1,2,4-triazine scaffold as FAK inhibitors with antitumor activity

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ABSTRACT

A series of 1,3,5-triazinic inhibitors of focal adhesion kinase (FAK) has recently been shown to exert antiangiogenic activity against HUVEC cells and anticancer efficacy against several cancer cell lines. In this report, we designed and synthesized a series of new compounds containing a 1,2,4-triazine core as novel scaffold for FAK inhibitors. These compounds displayed 10^{-7} M IC_{50} values, and the best one showed IC_{50} value of 0.23 μM against FAK enzymatic activity. Among them, several inhibitors potently inhibited the proliferation of glioblastoma (U-87MG) and colon (HCT-116) cancer cell lines. Docking of compound **10** into the active site of the FAK kinase was performed to explore its potential binding mode.

1. Introduction

Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase and scaffold protein localized to focal adhesions, is an important functional regulator of cell signaling within the tumor microenvironment.[1,2] This protein is emerging as a promising therapeutic target because it is over-expressed at both the transcriptional and translational level in a large variety of cancers, including pancreatic, ovarian and cervical cancers, prostate neoplasm, head and neck squamous cell carcinoma, glioblastoma, colorectal, breast, lung and kidney cancers.[3–5] Ample evidence has indicated that FAK signaling pathways can stimulate tumor progression and metastasis formation through their regulation of cell migration, invasion and angiogenesis.[6–8] However, some of FAK's functions in tumorigenesis remain under investigation.

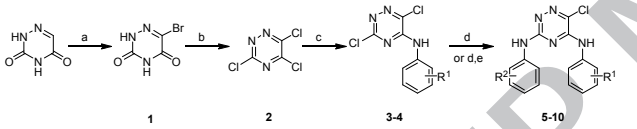
In the past few years, phase I and II clinical trials have been initiated with small FAK inhibitor molecules.[9–11] They decrease tumor growth and metastasis development in several preclinical models and possess initial clinical activity in patients, even if some adverse events have been reported.[12] The trials are studied either individually or in combination to prevent diseases related to FAK signaling or overcome resistance related to FAK inhibition. The progress in evaluation of FAK inhibitors in both preclinical models and human clinical trials holds good promise.[12,13] Thus, extensive research efforts are currently invested in the discovery of novel FAK inhibitors for developing highly specific therapeutic drugs for particular tumor phenotypes.

Crystal structures of FAK kinase domain have been published and their studies have been helpful for structure-based design efforts aimed at improving selectivity and potency of FAK inhibitors.[14–16] Structural scaffolds of FAK inhibitors described as type I inhibitors, generally bearing nitrogen heterocyclic ring, occupy the nucleotide binding pocket in a manner to bind the hinge region of the kinase domain. Nitrogen(s) in the ring can form hydrogen bonds with the carbonyl group of Cys502/Glu500 residues of the kinase hinge region. The aromatic ring makes hydrophobic contacts with Ala452 and Leu553 in the catalytic site. It was observed that pyrimidine derivatives, specifically 2,4-pyrimidine derivatives, are the major scaffolds found in compounds described by pharmaceutical companies and academic research.[11,17–21]

As part of our ongoing efforts to identify new chemical classes of FAK inhibitors associated with antiproliferative activity, we previously described a series of 1,3,5-triazinic inhibitors, which show antiangiogenic activity against HUVEC cells and antiproliferative efficacy against several cancers in cell growth studies (glioblastoma U-87MG, colon cancer HCT-116, breast cancer MDA-MB-231, and prostate cancer PC-3).[22,23] X-ray crystallographic analysis of the cocrystal structure of these compounds in the FAK kinase domain revealed that the mode of interaction is highly similar to that observed in the complex of TAE-226, a potent ATP competitive inhibitor of FAK, designed by Novartis Pharma AG.[15] As TAE-226, compound **34**[22] stabilizes an unusual helical conformation of the DFG motif (Asp-Phe-Gly) in which the φ torsion angle of Asp564 is rotated by 113° compared with the active kinase domain. Despite the

very similar interaction mode of these compounds, the *in vitro* potency of 1,3,5-triazinic compound **34**, is approximately 45-fold lower than that of TAE-226. From X-ray structure we proposed that one major reason could be due to the missing chlorine atom in our 1,3,5-triazinic compound, which in TAE226 makes van der Waals interactions with Met499. It has been shown previously that filling binding cavities to improve geometric fits and hence van der Waals contacts can significantly contribute to binding affinity.[24] The aim of the work reported herein was to develop 1,2,4-triazinic compounds as novel scaffold of FAK inhibitors, which can contain one chlorine atom in position 6, able to provide van der Waals interactions with Met499.

The synthetic route of new 1,2,4-triazinic compounds **5-10** was outlined in Scheme 1. Starting from commercially available 1,2,4-triazine-3,5(2H,4H)-dione (Scheme 1), the electrophilic bromine was introduced in position 6 of the triazinic ring at room temperature to obtain the intermediate **1** (91%), which was converted to 1,2,4-trichlorotriazine **2** in presence of N, N-diethylaniline, phosphorus pentachloride and phosphoryltrichloride at 130 °C with a high yield of 81%.[25] The first chlorine atom in position 5 of compound **2** is displaced by substituted anilines in the presence of triethylamine in THF at reflux to afford monosubstituted compounds **3-4** in good yields (64-68%). The chlorine atom in position 3 of compound **3-4** was further substituted to the compounds **5-10** in the presence of camphorsulfonic acid (CSA) in isopropanol through the agency of the corresponding arylamines in a range of yield of 48-55%, except for compound **7** which was obtained after deprotection of the amino protecting Boc group. Final products **5-10** were fully characterized by NMR and mass spectroscopy.

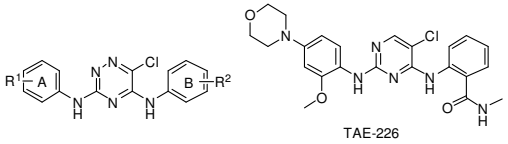


Scheme 1. a) Br_2, b) PCl_5/N,N-diethylaniline/$POCl_3$/130°C, c) arylamine/Et_3N/THF/reflux, d) arylamine/CSA/isopropanol/reflux, e) TFA/CH_2Cl_2

All compounds were evaluated for their ability to inhibit FAK kinase activity using a TR-FRET based kinase assay as described previously.[26] TAE-226 was used as a control. In our test conditions, TAE-226 inhibited the activity of FAK with IC_{50} value of 7 nM (Table 1), which was similar to previously reported data.[27]

As presented in Tables 1, tested compounds demonstrated potencies with IC_{50} values in the 10^{-7} M range. The choice of the different substituents at R^1 on the ring A and R^2 on the ring B was based on the results obtained in the previous 1,3,5-diarylaminotriazinic series.[22] We have chosen amide in the ortho position of the ring B and varied the R^2 substituents on the ring A, including 3,4,5-OCH_3, 4-CH_2NH_2 and 2-OCH_3-4-morpholino groups. These different pharmacomodulations showed effects similar to what had been obtained in the diarylamino-1,3,5-triazine series. Indeed, compound **10** with 2-OCH_3-4-morpholino group shows the best FAK inhibitory activity with IC_{50} of 0.23 µM, compared with its diaryl-1,3,5-aminotriazinic analogue with IC_{50} of 0.32 µM.[22] It seems that methoxy in the 2 position of the ring A in compound **10** can make slightly better interactions with the hinge than the compound **9** without this group, since compound **9** has a somewhat higher IC_{50} value. Unfortunately, the addition of chlorine in position 6 on the 1,2,4-triazine ring (compound **10**) failed to gain affinity, as compared to TAE226 (IC_{50} = 7 nM).

Table 1. In Vitro Enzymatic Activities of Novel 1,2,4-diarylaminotriazines compared with TAE-226.



N°	R^1	R^2	IC_{50} (µM)
5	3,4,5-OCH_3	3-$NHSO_2CH_3$	2.5 ± 0.3
6	3,4,5-OCH_3	2-$CONHCH_3$	0.64 ± 0.06
7	4-CH_2NH_2	2-$CONHCH_3$	0.56 ± 0.06
8	2-OCH_3-4-Cl-5-CH_3	2-$CONHCH_3$	0.87 ± 0.04
9	4-morpholino	2-$CONHCH_3$	0.34 ± 0.03
10	2-OCH_3-4-morpholino	2-$CONHCH_3$	0.23 ± 0.03
TAE-226			0.007 ± 0.002

In an attempt to gain insight into the putative binding mode of these inhibitors with the FAK kinase, compound **10** was docked into the apo FAK kinase structure (PDB ID 2jkk with TAE226 removed). The kinase receptor was kept rigid, whereas for the ligand 7 rotatable bonds were defined. Docking was performed with Autodock 4.2, using a standard protocol. In brief, the protein structure was screened within a cubic box with size 25 × 25 × 25 Å centered on the ATP binding site, applying a space grid of 0.375 Å. The lowest energy and most populated cluster was selected after 100 cycles of running a Lamarckian genetic algorithm with a population size of 150. A maximum of 2.5 million energy evaluations was applied, and results were clustered using a tolerance of 2.0 Å. Autodock computed a mean binding energy of -9.46 kcal/mol for the lowest energy cluster, which contained 57 of the 100 docked structures. The best pose in the cluster is shown in Fig.1 and has a calculated binding energy of -9.96 kcal/mol. The next best cluster was only singly populated with a calculated binding energy of -8.58 kcal/mol.

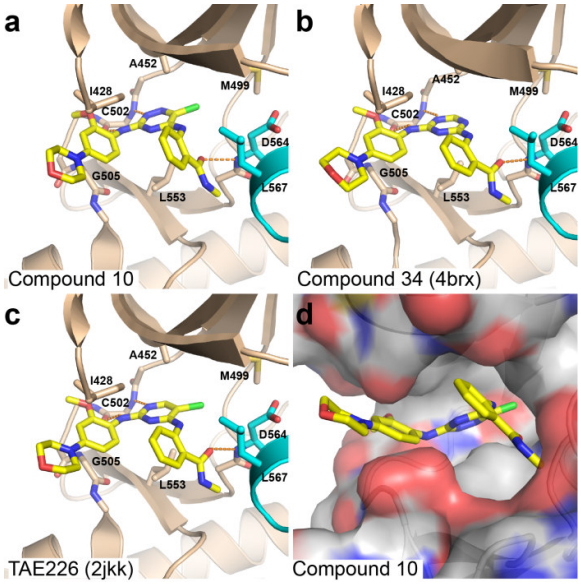


Figure 1. (a) Docking of Compound **10** into the ATP binding pocket of FAK. (b-c) Crystal structures of compound **34** (PDB ID: 4brx) and TAE226 (PDB ID: 2jkk) are shown bound to the active site of the FAK kinase. Key side chains and the inhibitors (yellow) are shown in stick representation. For clarity residues 429–431 in the P-loop are rendered transparent. Hydrogen bond interactions between receptor and ligand are shown as orange dashes lines. (d) The lowest energy docking pose of compound **10** fits well into the ATP binding pocket of FAK.

The resulting 3D binding modes of this compound to FAK is depicted in Figure 1a. Hydrogen bond interactions between receptor and ligand are shown as orange dashes lines. As shown in Fig.1d, compound **10** fits well into the ATP binding pocket. Comparison of compound **10** with TAE-226 (PDB ID 2jkk)[15] and a 1,3,5-triazine compound **34** that we reported previously (PDB ID 4brx)[22] indicates a very similar binding mode to the hinge region and the DFG motif of the FAK kinase (Figs 1a-c).

As shown in Fig 1a, two hydrogen bonds are formed between the carbonyl group of Cys502 of the kinase hinge with nitrogens in the 1,2,4-triazine and 2-methoxyaniline moieties. The carbonyl group on the ring B forms a hydrogen bond with the backbone nitrogen of Asp564 of the DFG motif. The carbon atoms in the 1,2,4-triazine ring A make hydrophobic contacts with Ala452 and Leu553, whereas carbons of the 2-methoxyaniline ring interact with Ile428 and Gly505. The chlorine atom at the C6 position of the 1,2,4-triazine ring penetrates deepest into the ATP binding pocket and is located near the gatekeeper residue Met499. Our data shows that the van der Waals interactions between the chlorine atom and Met499 did not play an important role for binding affinity. Therefore, the reason that the in vitro potency of compound **10** is lower than that of TAE-226 is likely due to the extra nitrogen in the 1,2,4-triazine ring. Sharing electrons between three electronegative nitrogens in the 1,2,4-triazine ring, compared to two nitrogens in the pyrimidine ring of TAE226, result in less electrons being available in the triazine ring for hydrogen bonding with the hinge backbone of FAK, hence weakening the interaction.

The inhibition of FAK autophosphorylation was first tested on human glioblastoma (U-87MG), human colon carcinoma (HCT-116), using FACEFAK ELISA kit (Active Motif Europe, Belgium). As shown in Figure 2, FAK autophosphorylation was significantly inhibited by treatment with the best inhibitor **10**, compared with TAE-226. Consistent with its inhibitory activity shown against the FAK kinase, compound **10** blocked tyrosine 397 phosphorylation in a dose-dependent manner in these two cancer cell lines, suggesting that these inhibitors are able to effectively inhibit cellular FAK autophosphorylation and phosphorylation of kinase targets.

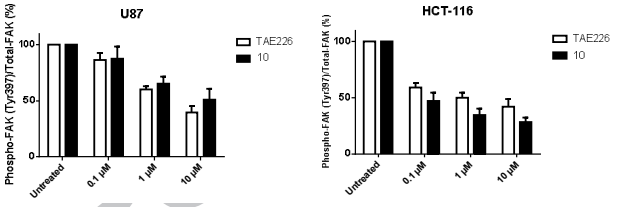
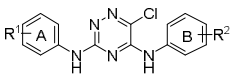


Figure 2. Cells (U-87MG and HCT-116) were treated for 48 h with 0.1, 1 and 10 µM of compound **10** or TAE-226 as control. Data obtained by the Face method are expressed as the fold decrease relative to the control and represent mean of triplicate readings of three independent experiments. A decrease of FAK tyrosine 397 phosphorylation in a dose-dependent manner is shown for **10** and for TAE-226.

Then, inhibition of cancer cell growth was investigated with the 1,2,4-triazinic compounds **5-10** using the WST-1 assay. As shown in Table 2, IC_{50} values of these compounds against two tumor cell lines (U87MG and HCT-116) ranged between 0.19 and 17 µM except for compounds **9** to which two cells are not sensitive in terms of decrease in cell survival. Among them, U-87MG cells were the most sensitive to compound **7** with an IC_{50} value of 2.4 µM, similar to that of TAE-226. HCT-116 cells showed more sensitivity to compounds **10** than to TAE-226.

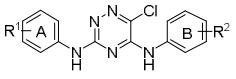
Table 2. Cytotoxicity on U-87MG and HCT-116 cell lines.



N°	R^1	R^2	U-87MG IC_{50} (µM)	HCT-116 IC_{50} (µM)
5	3,4,5-OCH_3	3-$NHSO_2CH_3$	12.5 ± 0.8	14.8 ± 0.5
6	3,4,5-OCH_3	2-$CONHCH_3$	17.0 ± 2.1	14.9 ± 1.4
7	4-CH_2NH_2	2-$CONHCH_3$	2.4 ± 0.2	1.8 ± 0.09
8	2-OCH_3-4-Cl-5-CH_3	2-$CONHCH_3$	9.2 ± 0.6	5.8 ± 0.6
9	4-morpholino	2-$CONHCH_3$	10.1 ± 0.9	0.5 ± 0.02
10	2-OCH_3-4-morpholino	2-$CONHCH_3$	13.3 ± 1.2	0.19 ± 0.03
TAE-226			1.3 ± 0.07	0.39 ± 0.04

The effects of these compounds and TAE-226 on the tumorigenicity of these cancer cell lines were also examined using an in vitro colony formation assay. These compounds inhibited colony formation of the two cancer cell lines in a dose-dependent manner. In general, comparison of table 2 and 3, shows that these compounds, like TAE-226, have less cytotoxic effects but demonstrate stronger antitumorigenic effects on the cancer cell line growth. As shown in Table 3, these compounds showed a similar effect on U-87MG cells as TAE-226, except for compounds **5** and **7**, which demonstrated a stronger antitumorigenic effect with an IC_{50} value of 70 and 14 nM, compared with TAE-226 (170 nM). Similar results were also observed for HCT-116 cells, since compound **5** showed a stronger antitumorigenic effect with an IC_{50} value of 60 nM, compared with TAE-226 (270 nM). The different cellular properties (cell permeability, intracellular stability, and distribution) or off-target effects might account for these strong growth inhibitions on the two cancer cell lines.

Table 3. Inhibition of Colony Formation on U-87MG and HCT-116 cell lines.



N°	R^1	R^2	U-87MG IC_{50} (µM)	HCT-116 IC_{50} (µM)
5	3,4,5-OCH_3	3-$NHSO_2CH_3$	0.07 ± 0.006	0,06 ± 0.004
6	3,4,5-OCH_3	2-$CONHCH_3$	1.7 ± 0.1	2.1 ± 0.2
7	4-CH_2NH_2	2-$CONHCH_3$	0.014 ± 0.001	1.1 ± 0.1
8	2-OCH_3-4-Cl-5-CH_3	2-$CONHCH_3$	0.5 ± 0.02	4.5 ± 0.4
9	4-morpholino	2-$CONHCH_3$	0.7 ± 0.03	1.7 ± 0.2
10	2-OCH_3-4-morpholino	2-$CONHCH_3$	1.5 ± 0.09	1.0 ± 0.1
TAE-226			0.17 ± 0.04	0.27 ± 0.04

In summary, we have synthesized a series of novel 1,2,4-diarylaminotriazines as FAK inhibitors. Molecular docking of the most potent inhibitor **10** into binding site of FAK was performed, and the results showed compound **10** could bind in the FAK active site as compound **34** belonging to 1,3,5-triazinic series and TAE-226 to the pyrimidine series. These results clearly show the importance of the number and location of nitrogens beared by the aromatic ring inserted in the catalytic pocket as well for H bonding as for hydrophobic interactions. Moreover, the potential effect of polarizable water molecules inside the active site may have an important role in ligand-macromolecule interactions.[28] Otherwise, the 1,2,4-triazinic inhibitors, like TAE-226, showed less cytotoxic effects but had stronger antitumorigenic effects on the cancer cell lines (U87-MG and HCT-116). Especially, compounds **5** and **7** exhibited higher antitumorigenic effects in U-87MG cells with IC_{50} values of 70 and 14 nM, respectively, compared with TAE-226. Finally, taking account of the activity data and binding information of these novel scaffolds as FAK inhibitors it should provide a reliable tool for reasonable design of FAK inhibitors in the future.

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