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Synthesis of novel diarylamino-1,3,5-triazine derivatives as FAK inhibitors with anti-angiogenic activity

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ABSTRACT

We report herein the synthesis of novel diarylamino-1,3,5-triazine derivatives as FAK (focal adhesion kinase) inhibitors and the evaluation of their anti-angiogenic activity on HUVEC cells. Generally, the effects of these compounds on endothelial cells could be correlated with their kinase inhibitory activity. The most efficient compounds displayed inhibition of viability against HUVEC cells in the micromolar range, as observed with TAE-226, which was designed by Novartis Pharma AG. X-ray crystallographic analysis of the co-crystal structure for compound **34** revealed that the mode of interaction with the FAK kinase domain is highly similar to that observed in the complex of TAE-226.

Angiogenesis, which is regulated by the highly coordinated function of various proteins with pro- and anti-angiogenic functions is the process of new blood vessel growth from preexisting vessels. The deregulation of angiogenesis contributes to numerous disorders such as inflammatory, ischemic and immune diseases, as well as tumor growth and metastasis formation.[1] Numerous agents targeting VEGF ligands or their receptors (VEGFR), which represent one of the best validated signaling pathways in angiogenesis, have been successfully developed and tested as anti-cancer therapies.[2] Thus far, clinical benefits achieved with VEGF- and VEGFR-targeted drugs are limited by their modest efficacy and the development of resistance.[3] Therefore, other targets involved in angiogenesis need to be examined to realize the full benefits of anti-angiogenic therapy.

Focal adhesion kinase (FAK) is an ubiquitous non-receptor tyrosine–protein kinase highly conserved and localized in focal adhesions, which is activated following binding of integrins to the extracellular matrix (ECM) or upon growth factor stimulation including that mediated by VEGF. FAK has been involved in angiogenesis as an important modulator during development evidenced by the early embryonic lethality of mice engineered to harbor an endothelial specific deletion of FAK.[4] It was reported that FAK expression in endothelial cells is necessary for the formation of new blood vessels, for the stability of the vascular network and for the survival of endothelial cells.[5] Endothelial FAK-deletion in adult mice inhibited tumor growth and reduced tumor angiogenesis.[6] Furthermore, integrin–FAK signaling has been shown to activate a number of biological processes through phosphorylation and protein–protein interactions to promote tumorigenesis. FAK also plays a prominent role in tumor progression and metastasis through its regulation of both cancer cells and their microenvironments including cancer cell migration, invasion, epithelial to mesenchymal transition. Overexpression and/or increased activity of FAK is common in a wide variety of human cancers.[7] Therefore, FAK was recently proposed as a potential target in the development of anti-cancer drugs. Some FAK inhibitors have been successfully developed, which inhibited glioma, neuroblastoma and ovarian tumor growth in vivo.[8–10] Their efficacy in tumor models may be a result of their ability to potently inhibit tumor growth and tumor-associated angiogenesis.

On another side, 1,3,5-triazine ring has been often reported as an important scaffold in many chemotherapeutic agents. For example, 1,3,5-triazine derivatives containing various amino

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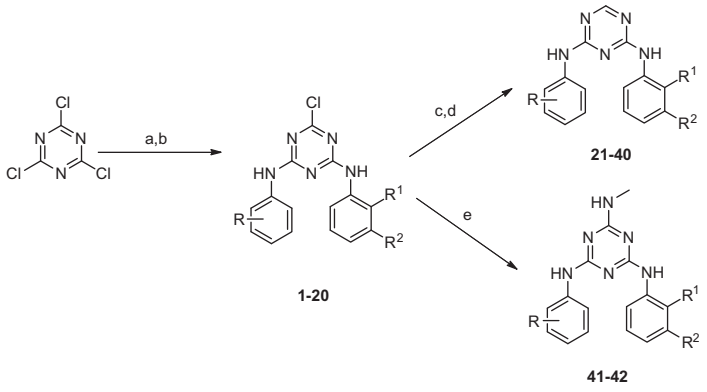
groups on the position 2, 4 or 6, such as tretamine, furazil and dioxadet, have been reported as anticancer agents.[11] Diarylaminotriazines have been claimed as ALK kinase inhibitors,[12] which may represent an effective and innovative therapy for ALCL, NSCLC, and neuroblastoma patients whose tumors harbor ALK genetic alterations.[13] Moreover, an anti-gastric ulcer agent that is commonly used in Japan, isogladine (2,4-diamino-6-(2,5-dichlorophenyl)-1,3,5-triazine), was shown to possess antiangiogenic properties in connexion with an anticancer effect.[14] The appeal of the 1,3,5-triazine core in medicinal chemistry is largely due to the ease of successive substitutions of chlorine atoms of commercially available cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) with nucleophilic groups to generate a large variety of substitutions. As a part of our research program aimed at the development for new inhibitors of FAK, a series of novel diarylamino-1,3,5-triazine derivatives was prepared, according to Schemes 1 and 2.

Starting from cyanuric chloride (Scheme 1), the first chlorine was displaced by nucleophilic substitution with arylamines at −10 °C to produce the mono substituted intermediates. These were further converted to the compounds **1**–**20** through the agency of the corresponding arylamines at room temperature. These two steps could also be performed in a one pot procedure without isolating intermediates. The displacement of the last chlorine by methylamino group was more difficult and realized under heating conditions or was made by hydrogen under catalytic hydrogenation, affording the compounds **41**–**42** and **21**–**35**, respectively, in good yields. Compounds **36**–**40** were finally obtained by cleavage of their protective group.

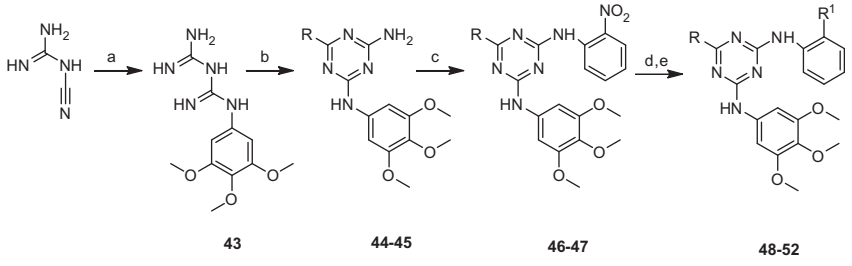
The synthesis of compounds **48**–**52** was accomplished by catalytic hydrogenation and further substitution by acetic anhydride or methanesulfonyl chloride or methyl chloroformate or dimethylcarbamoyl chloride as described in Scheme 2, from the precursors **46**–**47**, which were obtained in three steps from the microwave-assisted (MW) reaction of cyanoguanidine with arylamines and with esters and further with 1-bromo-2-nitrobenzene, using Pd-catalyzed heteroarylamination procedure.[15,16]

All compounds were evaluated for their ability to inhibit FAK kinase activity using a TR-FRET based kinase assay.[17] For detecting FAK phosphorylation activity by TR-FRET, a recombinant full length FAK protein pre-activated by Src was used with ATP and an ULight-labeled substrate poly(Glu/Tyr). Phosphorylation of the substrate was detected using an Europium-labeled phospho-specific antibody (W1024-PY20). One reported inhibitor of FAK, TAE-226, designed by Novartis Pharma AG, was included to validate the screening conditions. Under the experimental conditions, TAE-226 inhibited the activity of FAK with IC_{50} value of 7 nM (Table 1), which was similar to previously reported data.[18] As presented in Tables 1 and 2, the compounds tested demonstrated a range of potencies, clearly showing the contributions of the diarylaminotriazinic structure in terms of structure–activity relationships.

As shown in Table 1, we firstly introduced 3,4,5-trimethoxyphenylamino group on the triazine ring and a comparison of different substitutions at the position R on the triazinic ring (compounds **1**, **21**, **41** and **48**) indicated that replacement of the chlorine atom with a methylamino group for compound **41** resulted in a marginal decrease in inhibitory potency on FAK kinase activity. In contrast, removing the chlorine atom from the triazinic ring in **1** for compound **21**, displayed a about eightfold increase in inhibitory activity. Similar results were also observed for compound **22** as compared with compounds **2** and **42**. Moreover, replacement of the chlorine atom by a methyl group in compound **48** resulted in a substantial improvement in inhibitory potency as compared with **1**, but it was in the same range of **21**. This could be due to the fact that the groups R is too close to the CO of the backbone amide group of Glu-500 in the hinge (Fig. 2a), leading to steric clashes with this residue. Decreased FAK inhibitory activity might result from predisposed conformation of inhibitors less favorable to binding to the hinge region.

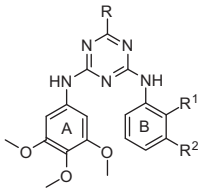


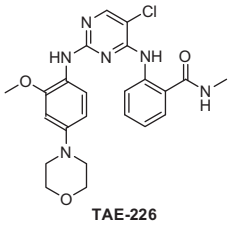
Scheme 1. Reagents and conditions: (a) $ArNH_2$/THF/DIEA/−10 °C; (b) $RPhNH_2$/THF/DIEA/rt; (c) H_2/Pd/THF/MeOH; (d) TFA/CH_2Cl_2; (e) CH_3NH_2/reflux.



Scheme 2. Reagents and conditions: (a) 3,4,5-trimethoxy $PhNH_2$/dioxane/MW, 90 °C, 15 min; (b) RCO_2Et/MeONa/THF/MW, 70 °C, 20 min; (c) 1-bromo-2-nitrobenzene/dioxane/$Pd(OAc)_2$/xantphos/Cs_2CO_3/MW, 150 °C, 15–30 min; (d) H_2/Pd/MeOH; (e) acetic anhydride or CH_3SO_2Cl or RCOCl/pyridine.

Table 1
In vitro enzymatic activities and cell viability on HUVEC of synthesized 2-(3,4,5-trimethoxyphenylamino)-1,3,5-triazine derivatives compared to TAE-226

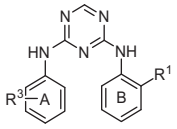




TAE-226

No.	R^1	R^2	R	FRET IC_{50} (μM)	Cell viability IC_{50} (μM)
1	$NHSO_2CH_3$	H	Cl	41.9 ± 4.6	9.5 ± 1.0
21	$NHSO_2CH_3$	H	H	5.1 ± 0,6	6.1 ± 0.9
41	$NHSO_2CH_3$	H	$NHCH_3$	65.9 ± 9.6	34.2 ± 7.6
2	H	$NHSO_2CH_3$	Cl	57.8 ± 7.6	13.3 ± 1.3
22	H	$NHSO_2CH_3$	H	9.2 ± 0.8	7.2 ± 0.8
42	H	$NHSO_2CH_3$	$NHCH_3$	41.3 ± 3.6	10.0 ± 1.2
48	$NHSO_2CH_3$	H	CH_3	7.9 ± 0.9	8.5 ± 0.4
49	$NHCOCH_3$	H	CH_3	50.8 ± 6.6	18.8 ± 2.3
50	$NHCO_2CH_3$	H	CH_3	35.9 ± 2.6	15.8 ± 1.7
51	$NHCON(CH_3)_2$	H	CH_3	54.3 ± 4.1	27.3 ± 2.1
52	$NHCOCH_3$	H	CF_3	125.6 ± 10.6	33.3 ± 3.2
23	$CONHCH_3$	H	H	0.4 ± 0.1	2.5 ± 0.1
24	$CONHCH(CH_3)_2$	H	H	2.2 ± 0.2	4.0 ± 0.9
25	$CONHCH(CH_2)_2$	H	H	6.1 ± 0.8	5.9 ± 0.7
26	$CONHCH(CH_2CH_3)_2$	H	H	6.0 ± 0.7	5.3 ± 0.9
27	$CONHCH(CH_2CH_2)_2$	H	H	5.2 ± 0.6	3.9 ± 0.6
28	$CONH(CH_2)_2OH$	H	H	23.8 ± 2.6	17.5 ± 2.1
29	$CONH(CH_2)_2N(CH_3)_2$	H	H	10.2 ± 1.5	9.4 ± 1.0
30	CO_2CH_3	H	H	17.9 ± 2.2	23.2 ± 3.1
TAE-226				0.007 ± 0.002	1.0 ± 0.1

Table 2
In vitro enzymatic activities and cell viability on HUVEC of synthesized diarylamino-1,3,5-triazine derivatives compared to TAE-226



No.	R^1	R^3	FRET IC_{50} (μM)	Cell viability IC_{50} (μM)
31	$NHSO_2CH_3$	2,4-Dimethoxy	6.1 ± 0.9	2.7 ± 0.3
32	$CONHCH_3$	2,4-Dimethoxy	1.5 ± 0.2	6.5 ± 0.8
33	$NHCOCH_3$	2,4-Dimethoxy	21.1 ± 5.2	11.2 ± 1.7
34	$CONHCH_3$	2-OCH_3-4-morpholine	0.32 ± 0.09	1.5 ± 0.2
35	$CONHCH_3$	3,4-Dimethoxy-5-$(CH_2)_2CONH_2$	0.3 ± 0.07	1.3 ± 0.2
36	$NHSO_2CH_3$	4-CH_2NH_2	2.3 ± 0.5	3.9 ± 0.9
37	$CONHCH_3$	4-CH_2NH_2	0.16 ± 0.08	2.1 ± 0.1
38	CONHEt	4-CH_2NH_2	0.44 ± 0.1	2.8 ± 0.5
39	$CONHCH(CH_3)_2$	4-CH_2NH_2	1.0 ± 0.2	3.2 ± 0.8
40	$CONHCH(CH_2CH_3)_2$	4-CH_2NH_2	1.9 ± 0.4	1.4 ± 0.7

The displacement of the sulfonamide group of position 1(R^1 in the ring B for compounds **1**, **21** and **41**) to position 2 in the second arylamine on the triazinic ring (R^2 in the ring B for compounds **2**, **22** and **42**) was less tolerated and led to decreased binding affinity toward FAK. Replacing the retro-sulfonamide group on the second arylamine (ring B for compound **48**) with a retro-amide moiety (compound **49**) or their analogues (compounds **50** and **51**) did not demonstrate any improved affinity for FAK.

Replacing the retro-sulfonamide group on the second arylamine (ring B for compound **21**) with an amide moiety (compound **23**) resulted in the last compound with a 13-fold improvement in inhibitory activity. Thus we prepared a range of amide analogues (compounds **24**–**29**) based on this compound. Consistent with what was observed for related pyrimidine inhibitors,[19] these analogs did not exhibit any improved inhibitory potency. Among these analogs, only the isopropylamide (compound **24**) showed IC_{50} values with 2.2 μM.

In an attempt to design compounds with inhibitory potency enhancement toward FAK kinase activity, we investigated the replacement of 3,4,5-trimethoxy groups on the phenyl ring (ring A) by different groups (Table 2). When the 3,4,5-trimethoxyphenyl group is replaced by 2,4-dimethoxyphenyl moiety, the corresponding compounds **31**–**33** did not display any relevant inhibitory activity changes. In contrast, increased inhibitory potencies are observed when the phenyl ring (ring A) is substituted by a CH_2NH_2 moiety in para position (compounds **36**, **37**, **39** and **40** compared

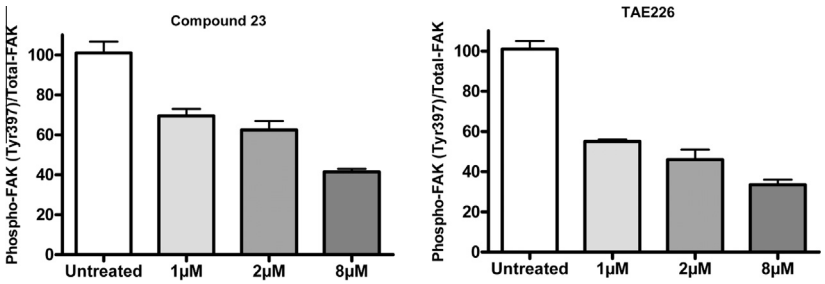


Figure 1. The cells were treated for 48 h with 1, 2 and 8 μM of compound **23** and TAE-226 as control. Data obtained by Face™ method are expressed as the fold increase relative to the control and represent mean of triplicate reading of three independent experiments. A decrease of tyrosine 397 phosphorylation of FAK in a dose-dependent manner is shown either for compound **23** and TAE-226 (p <0.001).

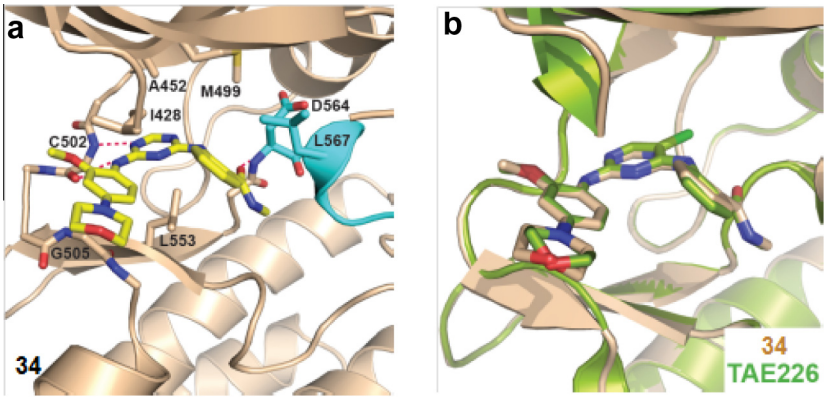


Figure 2. Structural characterization of the binding of diarylaminotriazines to kinase. (a) compound **34** is shown bound to the active site of the FAK kinase (beige ribbon with activation loopin cyan, PDB ID: 4brx). Key side chains and the inhibitors (yellow) are shown in stick representation. (b) Superposition of the two structures of **34** (beige) and TAE-226 (green) bound to FAK.

with **21**, **23**, **32**, **24** and **26**), which led to a potent inhibitor of FAK (IC_{50} = 160 nM for compound **37**). This may be attributed to an additional interaction with the Glu506 residue of the enzymatic site. It is interesting to note that a slight increased binding affinity is observed when 2-methoxy-4-morpholino groups are introduced on the phenyl ring (ring A for compound **34**), which structure is approaching to that of TAE-226, but is even showing 45-fold less potency than those of TAE-226. This might be caused by the absence of a hydrophobic interaction with Met-499 and weaken hydrogen bonding potential with the hinge region of FAK compared to TAE-226. In addition, when the phenyl ring (ring A) was substituted by 3,4-dimethoxy-5-$(CH_2)_2CONH_2$ moiety leading to the derivative **35**, this resulted in a slight improvement in inhibitory potency.

To determine if FAK activity was blocked in human umbilical vein endothelial cells (HUVEC) by these compounds, we thus assessed their ability to block endothelial drived FAK activity using FACE™FAK ELISA kit (Active Motif Europe, Belgium).[20] As shown in Figure 1, FAK autophosphorylation was significantly inhibited by treatment with compound **23** as compared to TAE-226. Consistent with the inhibitory activity of compound **23** shown against FAK kinase, compound **23** blocked tyrosine 397 phosphorylation of kinase targets in a dose-dependent manner in HUVEC cells, suggesting that these inhibitors are able to effectively inhibit endothelial cell-derived FAK autophosphorylation and phosphorylation of kinase targets at low concentrations.

Then, we were interested in examining the direct anti-angiogenic effects of these compounds on endothelial cell viability. We tested the ability of each compound to inhibit the VEGF-stimulated proliferation of HUVEC cells, by exposing cells to various concentrations of FAK inhibitors or equivalent amounts of DMSO as a vehicle control for 72 h, at which time cell viability was assessed using WST-1 colorimetric assay.[21]

As can be seen from the data reported in Tables 1 and 2, FAK inhibitors impaired VEGF-induced proliferation in a dose-dependent manner. HUVEC were sensitive to these inhibitors at relatively low concentrations, with inhibitory activity of cell viability (IC_{50}) at doses from 33 to 1.3 μM. In general, the effects of these compounds on endothelial cells could be correlated with their inhibitory activity of FAK. Cellular activity was mostly better than expected from enzymatic activity. Different cellular properties (cell permeability, intracellular stability and distribution) and/or off-target effects may account for this effect.

Furthermore, another interesting aspect was the observation that the best of our inhibitors showed similar effects on endothelial cells as compared with TAE226, although they were far from being the most potent FAK inhibitors. Then, compound **23** has been tested on a small panel of kinases and showed a strong inhibition against Fibroblast growth factor receptor 2 (FGFR2), which is abundant in endothelial cells and play an important role in the angiogenesis[22,23] (82% inhibition at 1 μM, unpublished result). The fact that this inhibitor also targets FGFR2, complicates the interpretation of the direct role of FAK inhibition in the observed angiogenic phenotype. Nevertheless, this may account for rather good effects of our inhibitors on endothelial cells by a gain of dual-specificity against both FAK and FGFR2 kinase activities.

For the purpose of analyzing interactions of our inhibitors with the FAK kinase domain, the crystal structure of **34** in the FAK kinase domain was resolved (Fig. 2a, PDB ID: 4brx). Diffraction data were collected at beamline ID14-4 at ESRF (Grenoble, France) and processed with XDS.[24] The molecular replacement protocol in Phaser was used to provide an initial set of phases using the FAK model

from PDB 2JKO.[25] This yielded a better Rfree than a simple transfer of the model to the native data set followed by rigid body and restrained refinement. Refinement was performed using the program Refmac and manual rebuilding was carried out with Coot.[26,27] The Dundee PRODRG2 Server was used to create the model for inhibitor **34**.[28] Final R-factors are 20.9/24.2 (Rwork/Rfree) for FAK/**34**.

As shown in Figure 2a, compound **34** occupies the nucleotide binding pocket, with the triazinic ring located in the adenine pocket. X-ray crystallographic analysis of the co-crystal structure revealed several hydrogen bonds and hydrophobic interactions. The nitrogens in the triazinic ring and 2-methoxyaniline moiety (ring A) form hydrogen bonds with the carbonyl group and amid of Cys502 of the kinase hinge region. The CO of carboxamide group of **34** is located near the DFG (D564-F565-G566) motif of the activation loop of the kinase domain and forms a hydrogen bond with the backbone nitrogen of Asp564 of the DFG motif. The triazinic ring shows hydrophobic contacts with Ala452 and Leu553, whereas carbons of the 2-methoxyaniline ring (ring A) interact with Ile428 and Gly505.

This mode of interaction with FAK kinase domain is highly similar to that observed in the complex of TAE-226, that stabilizes an unusual helical conformation of the DFG motif in which the phi torsion angle of Asp564 is rotated by 113° compared to the active kinase domain.[29] Such conformation is distinct from the conformation in both the active and inactive states of the kinase and could be exploited for designing inhibitor with higher specificity. The structures of these two inhibitor/protein complexes overlay perfectly at the protein and inhibitor levels (Fig. 2b).

Notably, despite the highly similar interaction mode of **34** and TAE226, the in vitro potency of **34** is approximately 45-fold lower than TAE226. On the one hand, this can be attributed to the missing chlorine atom in **34**, which in TAE226 makes van der Waals interactions with Met499. It has been shown previously that filling binding cavitiesto improve geometric fits and hence van der Waals contacts can significantly contribute to binding affinity.[30] On the other hand it is likely that the extra nitrogen in the triazine ring of **34** reduces electron density available for hydrogen bonding with the hinge backbone of FAK, hence weakening the interactions.

In summary, we have synthesized a series of novel diarylamino-1,3,5-triazine derivatives as FAK inhibitors. These inhibitors at significantly low concentration show substantial deleterious effects on endothelial cell viability and the best of our inhibitors showed a similar potency on cell viability as compared with TAE-226. Compound **23** showed significant decrease of autophosphorylation of FAK in HUVEC cells, suggesting that these compounds could effectively block a key event of FAK signaling pathway in living cells. Further experiments will be carried out in order to study other effects of these compounds on endothelial cell migration and tube formation as well as in tumor cells for the development of novel anticancer agents.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.06.038.

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20. Briefly, cells are cultured in 96-well plates at 3 × 103 cells per well and treated with the compounds at different concentrations. Following treatment, the cells are fixed using 4% formaldehyde in PBS. Each well is then incubated with a primary antibody that recognizes either phosphorylated FAK or total FAK as suggested by the manufacturer. The phospho-FAK antibody recognizes FAK only when phosphorylated at Tyr397. The total-FAK antibody recognizes FAK regardless of its phosphorylation state. Subsequent incubation with secondary HRP-conjugated antibody and developing solution provides an easily quantified colorimetric at 450 nm. The relative cell number in each well is then determined using the provided Crystal Violet solution and quantified at 595 nm. The levels of FAK phosphorylation were normalized by both the levels of total FAK protein and total cell number in each well.
21. The exponentially growing cells were seeded at 3 × 103 cells per well in 96-well plates. After 24 h, they were treated with different compounds dissolved in DMSO. Following 48 h, the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) from Roche® was added, and cells were incubated at 37 °C for 1–2 h. The Optical Density was analyzed with a microplate reader (Bio-Rad) at 490 nm to determine the cell viability. The results are expressed as the mean of three independent experiments with three determinations per tested concentration and per experiment. For each compound, the IC_{50} value was determined from a sigmoid dose–response curve using Graph-Pad Prism (GraphPad Software, San Diego, CA, USA).
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