Biochemical and Biophysical Research Communications 437 (2013) 319–324

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of methyl violet 2B as a novel blocker of focal adhesion kinase signaling pathway in cancer cells

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ARTICLE INFO

Article history:
Received 18 June 2013
Available online 28 June 2013

Keywords:
Focal adhesion kinase
Methyl violet 2B
FAK signaling blocker
Structure-based virtual screening
FER phosphorylation inhibition
Adhesion/migration/invasion suppression

ABSTRACT

The focal adhesion kinase (FAK) signaling cascade in cancer cells was profoundly inhibited by methyl violet 2B identified with the structure-based virtual screening. Methyl violet 2B was shown to be a non-competitive inhibitor of full-length FAK enzyme vs. ATP. It turned out that methyl violet 2B possesses extremely high kinase selectivity in biochemical kinase profiling using a large panel of kinases. Anti-proliferative activity measurement against several different cancer cells and Western blot analysis showed that this substance is capable of suppressing significantly the proliferation of cancer cells and is able to strongly block FAK/AKT/MAPK signaling pathways in a dose dependent manner at low nanomolar concentration. Especially, phosphorylation of Tyr925-FAK that is required for full activation of FAK was nearly completely suppressed even with 1 nM of methyl violet 2B in A375P cancer cells. To the best of our knowledge, it has never been reported that methyl violet possesses anti-cancer effects. Moreover, methyl violet 2B significantly inhibited FER kinase phosphorylation that activates FAK in cell. In addition, methyl violet 2B was found to induce cell apoptosis and to exhibit strong inhibitory effects on the focal adhesion, invasion, and migration of A375P cancer cells at low nanomolar concentrations. Taken together, these results show that methyl violet 2B is a novel, potent and selective blocker of FAK signaling cascade, which displays strong anti-proliferative activities against a variety of human cancer cells and suppresses adhesion/migration/invasion of tumor cells.

1. Introduction

Focal adhesion kinase (FAK), a 125 kD non-receptor tyrosine kinase, plays a central role in regulating signal transduction mediated by integrin as well as functioning in the control of focal adhesion dynamics and cell mobility. FAK has been implicated in cell adhesion, spreading, invasion, differentiation and survival [1–4]. FAK is over-expressed in various late-stage human cancers including breast, colon, lung, prostate and melanoma [5–9]. Thus, FAK is a promising target for cancer therapeutics.

The FERM domain (the band 4.1 and ERM homology domain) located at the N terminus of FAK performs regulatory functions. Binding of the FERM domain to the kinase domain inactivates FAK by locking it into an auto-inhibited conformational state [10]. The engagement of integrin receptors with components of the extracellular matrix (ECM) is thought to restore the active conformation, and hence autophosphorylation of Tyr397. The autophosphorylated Tyr397 recruits Src and serves as a binding site for the SH2 domain of Src, The recruited Src sequentially phosphorylates other tyrosine residues of FAK including Tyr576/577, Tyr861, and Tyr925, which eventually results in full activation of FAK catalytic activity. This in turn promotes the AKT/MAPK signal transduction cascade [11]. Activated Src, recruited by autophosphorylated Tyr397, also phosphorylates Paxillin and p130CAS. These proteins interact with FAK and play major roles in focal adhesion.

The COOH-terminal domain of FAK is composed of two proline-rich protein domains and a focal adhesion targeting (FAT) domain that functions as a scaffold. The proline-rich protein domains (PR-I and PR-II) interact with SH3-domain containing proteins such as p130Cas. The FAT domain binds to focal adhesion proteins including paxillin, which leads to localization of FAK to the site of focal adhesion. The cytoplasmic tyrosine kinases FER and FPS/FES are known to transfer cell signaling from the cell surface to the

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http://dx.doi.org/10.1016/j.bbrc.2013.06.078

cytoskeleton like FAK [12]. Notably, FER is reported to catalyze the phosphorylation of FAK Tyr577, Tyr861 and Tyr925 residues but not the Tyr397 and Tyr576 residues [13].

Several small molecule FAK inhibitors, including TAE226 and PF-562,271, PF-04554878 and GSK2256098, have been reported and some (PF-562271, PF-04554878, GSK2256098 and BI853520) of them entered clinical investigations [5,14,15]. Most FAK inhibitors disclosed to date compete with ATP binding. Recently, the allosteric FAK inhibitors C4, INT2-31, Y11 and Y15 have been reported [9,16–18]. The inhibitor C4 interferes with the protein–protein interaction between VEGFR and FAK, while inhibitor INT2-31 blocks formation of IGFR and FAK complex. Y11 and Y15 act by inhibiting autophosphorylation of the FAK Tyr397 residue.

In an effort to identify novel small molecule FAK inhibitors that possess high kinase selectivities, we carried out docking-based in silico virtual screening. Our objective was to identify novel allosteric FAK inhibitors, which target a novel binding pocket located immediately below Glu403 in the linker region between FERM and the kinase domain [19]. This novel binding site is located close to Tyr397 of which site is targeted by the reported allosteric FAK inhibitors, Y11 and Y15 [17,18]. Methyl violet 2B was identified as a hit compound by using this virtual screening approach and the binding site of methyl violet 2B on FAK remains unconfirmed and needs to be experimentally confirmed. The results arising from experimental based characterization showed that methyl violet 2B strongly blocks FAK cellular signaling pathway as well as FAK activation/phosphorylation in cancer cell and inhibits cancer cell adhesion/invasion/migration at low nanomolar concentrations. Below, we describe these findings in the context of experimental results, which demonstrate that methyl violet 2B is a highly selective blocker of FAK signaling cascade.

2. Materials and methods

See Supplementary data.

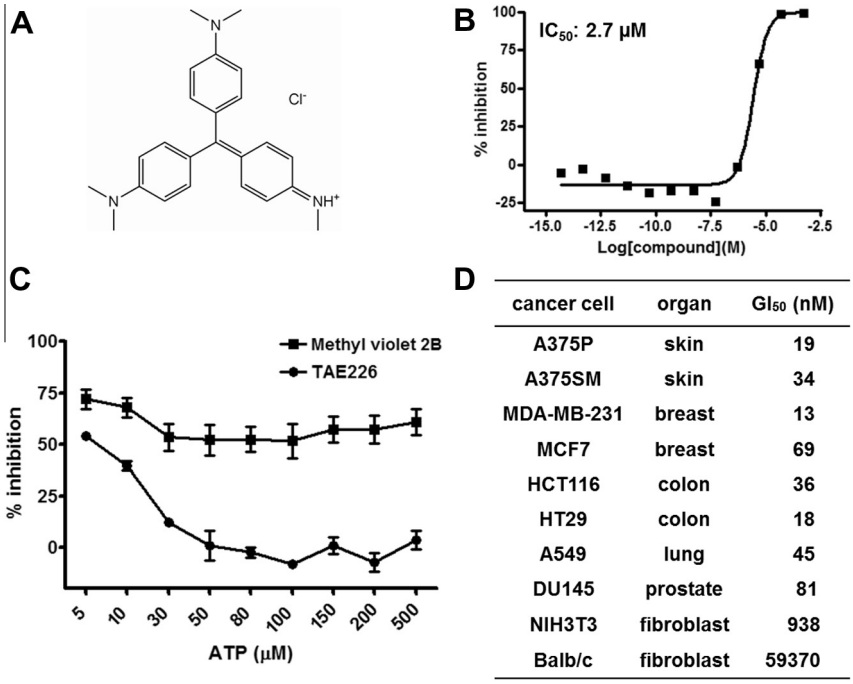
3. Results

3.1. Methyl violet 2B moderately inhibits the kinase activity of FAK in biochemical assays

Full-length FAK biochemical kinase assays were carried out to estimate the IC_{50} of methyl violet 2B. Methyl violet 2B was found to have an IC_{50} of 2.7 μM (Fig. 1B) in the fluorescence LANCE® Ultra kinase assay system. The LANCE® Ultra kinase assay utilizes ULight™-labeled poly-EY, a synthetic peptide substrate of FAK, and a europium-labeled anti-phospho antibody. The intensity of light emission, the readout of LANCE® Ultra kinase assay, is proportional to the degree of ULight™-labeled poly-EY. The level of phosphorylation of poly-EY was found to decrease by addition of methyl violet 2B in dose dependent manner in the presence of FAK, whereas the intensity of light emission was not significantly changed even at high concentrations of methyl violet 2B in the absence of FAK enzyme (Fig. S2). This result indicates that the fluorescence-based kinase assay is not significantly interfered with by fluorescence of methyl violet 2B.

3.2. Methyl violet 2B is a non-ATP competitive FAK inhibitor

Further studies revealed that the FAK inhibitor methyl violet 2B, identified by using a docking-based virtual screening protocol, does not target the ATP-binding site of the kinase. For this purpose, we used TAE226 as a reference compound which is a known ATP competitive inhibitor of FAK. The kinase-inhibitory activity of methyl violet 2B on full-length FAK was found to be independent of the concentration of ATP whereas the kinase-inhibitory activity of TAE226 is inversely proportional to the concentration of ATP (Fig. 1C). Thus, methyl violet 2B is a non-ATP competitive, which binds to a site remote from ATP-binding site of FAK.



cancer cell	organ	GI_{50} (nM)
A375P	skin	19
A375SM	skin	34
MDA-MB-231	breast	13
MCF7	breast	69
HCT116	colon	36
HT29	colon	18
A549	lung	45
DU145	prostate	81
NIH3T3	fibroblast	938
Balb/c	fibroblast	59370

Fig. 1. Methyl violet 2B identified using enzyme and cell-based screening. (A) Chemical structure of methyl violet 2B. (B) Kinase-inhibitory activity (IC_{50}) of methyl violet 2B on full-length FAK enzyme from LANCE® kinase assay. (C) ATP dependency of methyl violet 2B and TAE226 in blocking the kinase activity of full-length FAK enzyme. (D) Methyl violet 2B exhibits cancer-specific anti-proliferation.

3.3. Methyl violet 2B exhibits a remarkable cancer-specific anti-proliferative activity

Based on the kinase-inhibitory activity of methyl violet 2B on FAK, we next investigated the growth inhibitory activities of methyl violet 2B on human cancer cells. For this purpose the colorimetric cell viability assay (MTT) method, CellTiter 96® was employed. Methyl violet 2B was observed to exhibit profound anti-proliferative activities with GI_{50} values of 13–80 nM against melanoma (A375P, A375SM), breast (MDA-MB-231, MCF7), colon (HCT116, HT29), lung (A549), and prostate (DU145) cancer cell lines, but not normal fibroblast (NIH3T3 and Balb/c) cells (Fig. 1D). The proliferation of normal fibroblast cells was 10 to 1000-fold less (GI_{50} = 1–10 μM) suppressed compared to that of the cancer cells. This finding indicates that methyl violet 2B possesses a differential cytotoxicity between normal cells and cancer cells. We also compared the MTT assay (absorbance) data with viability data derived from the CellTiter-Glo® assay, a luminescent cell viability assay. No significant difference between the two sets of cell viability data was observed. It should be noted that methyl violet 2B exhibited stronger antiproliferative activities (double-digit nM of GI_{50}) on cancer cells compared with its moderate kinase-inhibitory activity (2.7 μM of IC_{50}) against FAK enzyme, which implies that other cellular targets of methyl violet 2B might exist in cancer cells.

3.4. Methyl violet 2B has an extremely high kinase selectivity profile

In order to explore the kinase selectivity profile of methyl violet 2B, inhibitory activities against a diverse panel of 336 recombinant human kinases (Kinase HotSpot℠ screen except FAK) were determined employing a radioactivity based *in vitro* assay and respective concentrations of methyl violet 2B and ATP of 1 and 10 μM (Fig. 2). The results of this study show that methyl violet 2B displays an extremely high kinase selectivity profile as reflected by the finding that it serves as an inhibitor of only three of the 336 kinases probed. Specifically, methyl violet 2B at a concentration of 1 μM inhibits the kinase activities of only BLK (B-lymphoid kinase), FER (FES and FES-related), and MELK (maternal embryonic leucine zipper kinase), with respective percent inhibitions of 59%, 43%, and 63%. The three kinases inhibited by methyl violet 2B, including BLK, FER, and MELK, are depicted as hits using large red and blue circles within the human kinase dendrogram composed of 336 kinases displayed in Fig 2. In this series, BLK (B-lymphoid kinase) is a prototypical member of the Src family of kinases (SFK) that includes Lyn, Fyn and Yes that have been implicated in renal cell carcinoma [20]. In addition, FER (FES and FES-related), ubiquitously expressed in mammalian cells, is a nuclear and cytoplasmic tyrosine kinase that regulates cell–cell adhesion [12]. Interestingly, overexpression of FER in suspended hepatocytes causes phosphorylation of specific FAK tyrosine residues Tyr577, Tyr861, or Tyr925, but not Tyr397 or Tyr576 [13]. It is important to note that no chemical inhibitors of FER have been discovered to date, to the best of our knowledge. Finally, MELK (maternal embryonic leucine zipper kinase) is overexpressed in aggressive tumors and its function has been implicated with mammary tumorigenesis and, as a result, it is a potential target in breast tumor-initiating cells [21]. Methyl violet 2B inhibitory activity against FER should contribute to some extent to its inhibitory activity against FAK phosphorylation in cancer cells because FER phosphorylates specific FAK tyrosine residues. In addition, the anti-proliferative effects of methyl violet 2B on cancer cells could result from a combination of its inhibitory effects on FAK and FER in the intracellular environment.

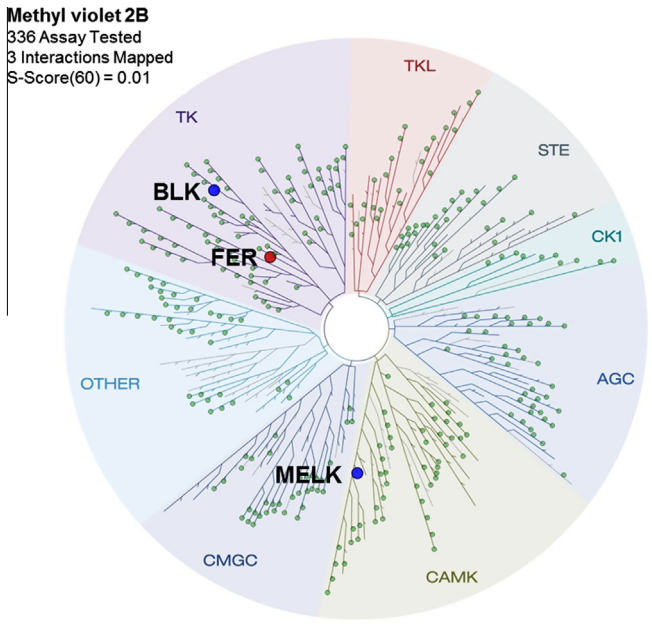


Fig. 2. Protein kinase selectivity of methyl violet 2B (1 μM) was determined against 336 human protein kinases by a Kinase HotSpotSM screen. The three main hits (BLK, FER, and MELK) were represented within the human kinase dendrogram. Weak or no hits are labeled with small green circles (below 40% inhibition).

3.5. Methyl violet 2B strongly inhibits FAK/AKT/MAPK signaling and induces apoptosis in cancer cells

It has been reported that the FAK signaling pathway is correlated with cell survival and proliferation [22,23]. Consequently, we investigated the effect of methyl violet 2B on the intracellular FAK signaling pathway as well as FER phosphorylation of the A375P human melanoma cell line. We determined the concentrations of methyl violet 2B that are required to inhibit phosphorylation of specific FAK tyrosine residues (Tyr397, Tyr576, Tyr577, Tyr861, and Tyr925) in A375P cells. The results showed that the inhibitory effect of methyl violet 2B on phosphorylation of Tyr925, Tyr861, and Tyr577 in A375P cells is much greater than that on FAK enzyme (2.7 μM of IC_{50}) in the biochemical kinase assay (Fig. 3A). In particular, phosphorylation of Tyr925 is nearly completely suppressed when an extremely low concentration (1 nM) of methyl violet 2B is used. In contrast, phosphorylation of Tyr397 and Tyr576 is inhibited to a lesser extent by this substance than is that of Tyr925, Tyr861, and Tyr577. Phosphorylation of Tyr397 is significantly or completely suppressed using 500 nM or 1000 nM, respectively, of methyl violet 2B but phosphorylation of Tyr576 is not completely inhibited even at a concentration of 1000 nM (Fig. 3D). It is of note that the degree of autophosphorylation (p-Tyr397) inhibition is relatively correlated to biochemical kinase assay data (2.7 μM of IC_{50}) in contrast to that of p-Tyr925 inhibition. The results of the kinase inhibition assay described above suggest that FER phosphorylation would be attenuated in the presence of this substance. Indeed, we observed that methyl violet 2B significantly inhibits phosphorylation of FER Tyr402 in A375P even at very low concentrations (10 nM) and that the level of FER Tyr402 phosphorylation decreases in dose-dependent manner (Fig. 3A). The intracellular inhibitory effect of methyl violet 2B on FER Tyr402 phosphorylation is significantly stronger than that seen in the *in vitro* FER kinase inhibition experiment (43% inhibition at 1 μM of methyl violet 2B).

It is worth recalling that FER regulates FAK through phosphorylation of Tyr577, Tyr861, and Tyr925 but not Tyr397 and Tyr576 [13]. Thus, the suppression of FER Tyr402 phosphorylation seems

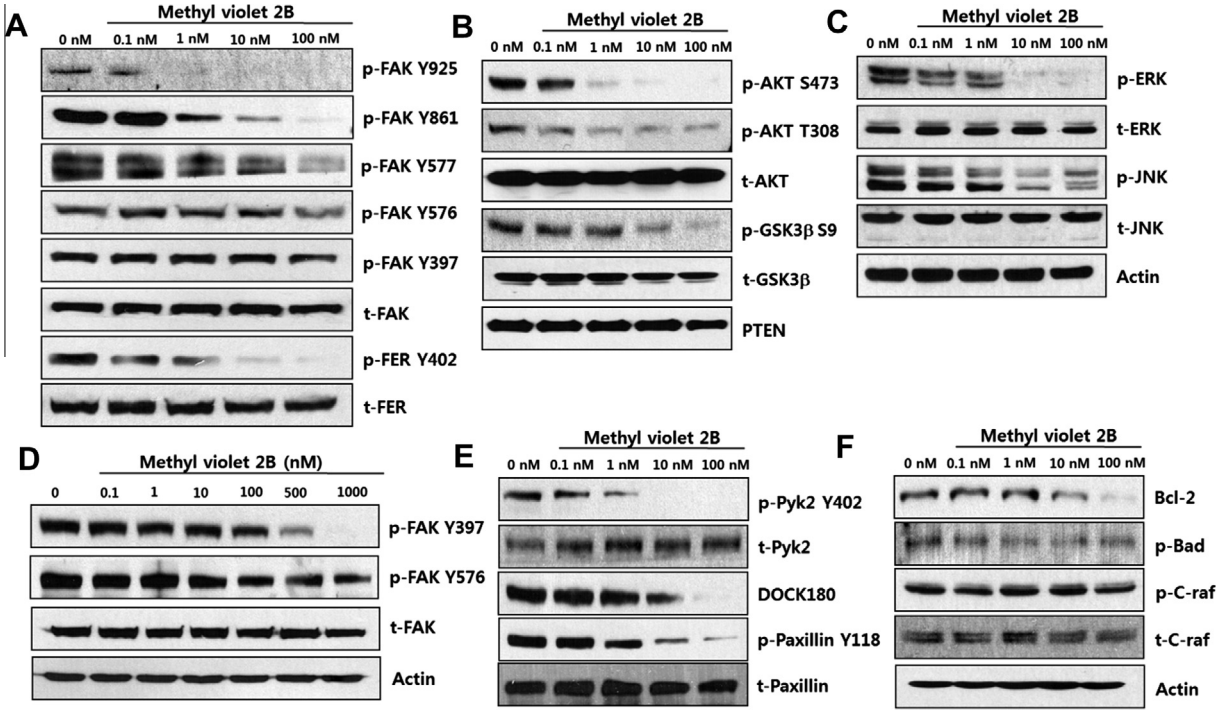


Fig. 3. Methyl violet 2B disrupts signaling of FAK/AKT/MAPK and induces apoptosis in cancer cells. A375P cells were treated with indicated concentration of methyl violet 2B for 48 h. Expression levels of both phosphorylated and total FER and FAK were measured by using Western blot analysis (A). Methyl violet 2B decreases the signaling of AKT (B) and MAPK (C) in dose-dependent manner. Inhibition of p-Y397 and p-Y576 was assessed with higher concentration of Methyl violet 2B (D). Downstream and co-factor signaling of FAK were determined under the same experimental condition (E).To investigate the state of apoptosis in A375P cells, the expression of Bcl-2 and p-Bad was assessed by using Western blot analysis (F). The signaling of C-raf was determined under the same experimental conditions. Actin was measured to confirm that equal amounts of protein were loaded.

to be consistent with selective inhibition of phosphorylation of specific FAK tyrosine residues. We next investigated the effect of methyl violet 2B on DOCK180 (Dedicator of cytokinesis), Pyk2 (proline-rich tyrosine kinase 2), which is related to FAK, and Paxillin, which is a focal adhesion-associated adaptor protein [24–26]. p130CAS/Crk/DOCK180 is a downstream complex of FAK. The results demonstrate that methyl violet 2B promotes a significant decrease in the level of DOCK180, phospho-Pyk2, and phospho-Paxillin at low concentrations and in a dose-dependent manner (Fig. 3E). It is important to note that phosphorylation of Tyr402, the autophosphorylation site of Pyk2, is completely blocked at very low concentrations (10 nM) of methyl violet 2B while the phosphorylation of Tyr397, the autophosphorylation site of FAK, is completely inhibited at only much higher (1000 nM) concentrations of this substance.

The effect of methyl violet 2B on AKT phosphorylation in A375P cells was evaluated next. The findings show that this substance dramatically inhibits p-AKT Ser473 and p-AKT Thr308 even at a concentration of 1 nM (Fig. 3B). In addition, phosphorylation of GSK3βS9, the AKT downstream substrate [27], is significantly blocked by methyl violet 2B in a dose-dependent manner but the level of PTEN is not altered by this substance. Also, an investigation of the effect on MAPK signaling in A375P shows that methyl violet 2B displays a strong inhibitory effect on p-ERK and p-JNK even at 10 nM (Fig. 3C). The expression of p38 is not detected in A375P cells (data not shown).

A study of the apoptotic effects of methyl violet 2B in A375P cells reveals that this substance promotes a significant decrease in the level of the anti-apoptotic molecule Bcl-2 (Fig. 3F). However, compared to that of Bcl-2, phosphorylation of Bad is less affected and methyl violet 2B does not affect phosphorylation of C-raf, which is known to be a Bcl-2 regulator. Overall, the observations made in this study show that methyl violet 2B is capable of blocking the FAK/AKT/MAPK signaling pathway that is related to the cell survival and proliferation. This effect results in the suppression of the growth and progression of tumor cells. Moreover, methyl violet 2B induces programmed cell death through inhibition of the Bcl-2 anti-apoptotic molecule.

3.6. Methyl violet 2B significantly inhibits focal adhesion in cancer cells

FAK-dependent cellular focal adhesion is a highly dynamic progression of events that involves cell survival, cell cycling, and malignancy through integrin signaling [28,29]. In order to explore the effects of methyl violet 2B on FAK-dependent cellular focal adhesion, perturbations of vinculin and F-actin levels were determined. For this purpose, A375P human melanoma cells were treated with different concentrations of methyl violet 2B for 48 h and then immunostained with vinculin antibody and the secondary antibody conjugated with Alexa Fluor 488 (green fluorescence), for determining vinculin levels as well as Alexa Fluor 594 conjugated Phalloidin for determining F-actin levels (Fig. 4A and B). Treatment with methyl violet 2B was found to result in a dose-dependent decrease of both vinculin and F-actin levels. Inspection of fluorescent images of the cells shows that focal adhesion and actin stress fibers are not well-assembled when A375P cells are treated with more than 10 nM of methyl violet 2B.

Talin is large cytoplasmic cytoskeletal protein whose C-terminal region includes three actin binding sites and vinculin binding sites and whose N-terminal region contains a band 4.1, ezrin, radixin, moesin homology (FERM) domain [30]. A study of the effect of methyl violet 2B on phosphorylation of ezrin, radixin, moesin (ERM family) in A375P cells showed that the level of the p-ERM is significantly decreased even when very low (1 nM) doses of methyl violet 2B are utilized (Fig. S3A). Overall, the results demon-

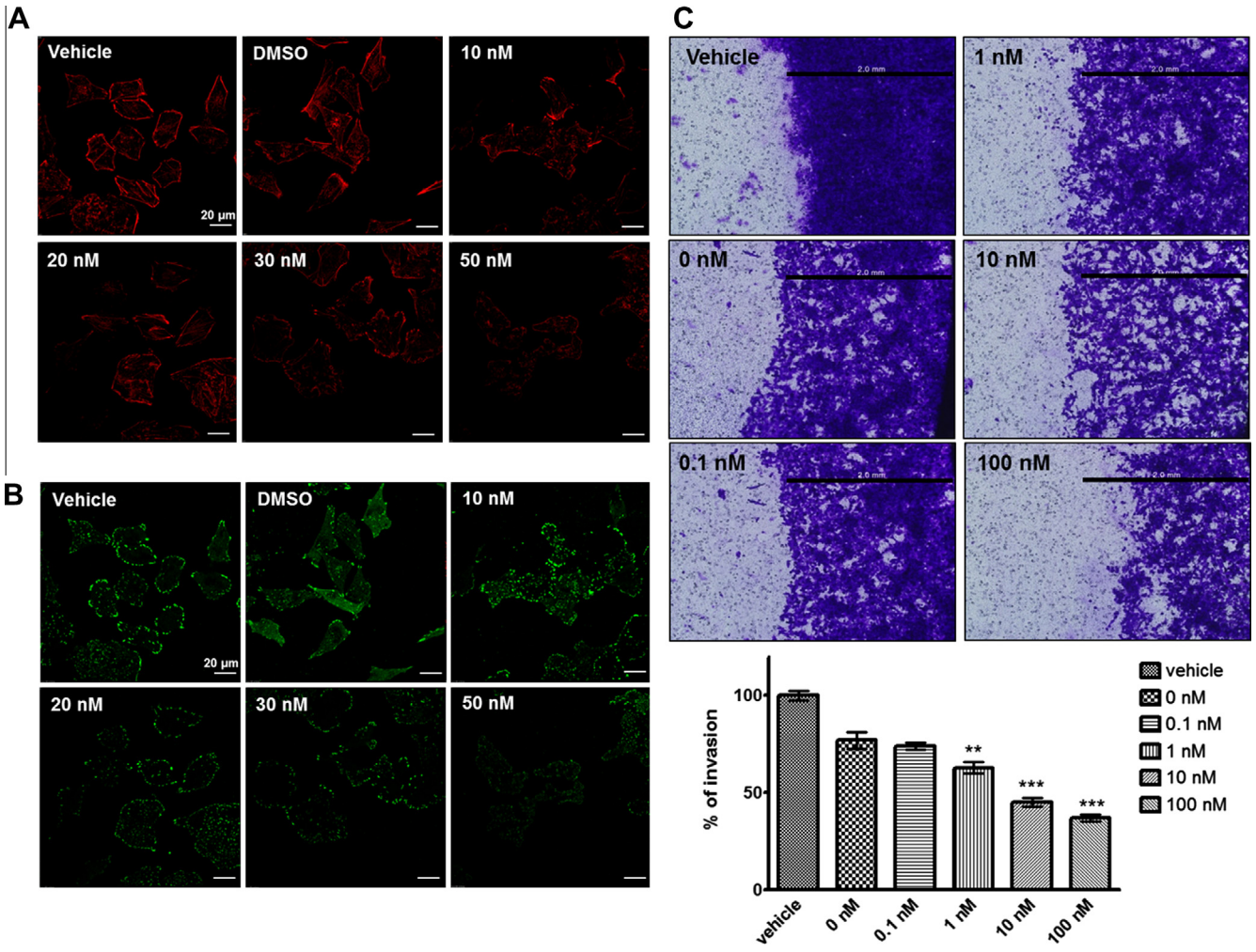


Fig. 4. Methyl violet 2B significantly inhibits focal adhesion and invasion. A375P cells were treated with indicated concentration of methyl violet 2B for 48 h. Focal adhesion was analyzed by immunostaining with vinculin and F-actin antibody (A, B). Invasion assay was performed using a QCM ECMatrix Cell Invasion Assay, and the percentage of invasion was quantitated by dissolving stained invasive cells. $^{**}p < 0.001$; $^{***}p < 0.0001$; significantly different from vehicle control (C).

strate that FAK-dependent cellular focal adhesion is significantly blocked even by very low concentrations of methyl violet 2B.

3.7. Methyl violet 2B potently inhibits invasion and migration

Malignancy of cancer cells is recognized to be a consequence of cell growth, survival, invasion and metastasis [31,32]. We have already shown that methyl violet 2B (1) inhibits the FAK kinase activity in an ATP non-competitive manner (Fig. 1C), (2) displays a specific anti-proliferative effect on various cancer cells (Fig. 1D), and (3) promotes a decrease in the level of focal adhesion molecules (Fig. 3). Our investigation next focused on the effects of methyl violet 2B on the invasion and migration of cancer cells in the A375P melanoma cell line. The results of a QCM ECMatrix Cell Invasion Assay reveal that this substance (1 nM) causes a dose dependent decrease in invasion of A375P cells (Fig. 4C). Also, the results of a wound healing assay using the Ibidi Culture-Insert show that methyl violet 2B suppresses cancer cell (A375P) motility in a dose-dependent manner (Fig. S3B).

4. Conclusion

In the study described above, we demonstrated that methyl violet 2B is a novel, potent and highly selective blocker of FAK signaling pathway that very significantly suppresses FAK phosphorylation in cancer cells. This substance was identified by using docking-based virtual screening of a previously unidentified FAK binding site. This proposed binding site of methyl violet 2B remains undetermined and needs to be confirmed by X-ray crystallography and/or NMR studies. In addition, we observed that methyl violet 2B selectively inhibits the activities of three kinases, including FER, BLK, and MELK among 336 recombinant kinases tested. Moreover, we found that this substance displays extremely potent anti-proliferative activities against various human cancer cell lines while having a much lower effect on normal cells. In addition, methyl violet 2B strongly inhibits focal adhesion, invasion, and migration of cancer cells, making it a potentially important substance for chemical biology studies related to cancer therapies. Taken together, methyl violet 2B strongly and specifically suppresses the proliferation, focal adhesion, invasiveness, and motility of cancer cells and may serve as a unique template to develop anticancer therapeutic agents which block FAK signaling cascade.

Acknowledgments

This research was supported by Korea Institute of Science and Technology and a grant (2011-0028676) from the creative/challenging research program of National Research Foundation of Korea funded by the Ministry of Education, Science and Technology, Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.078.

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