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

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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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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.

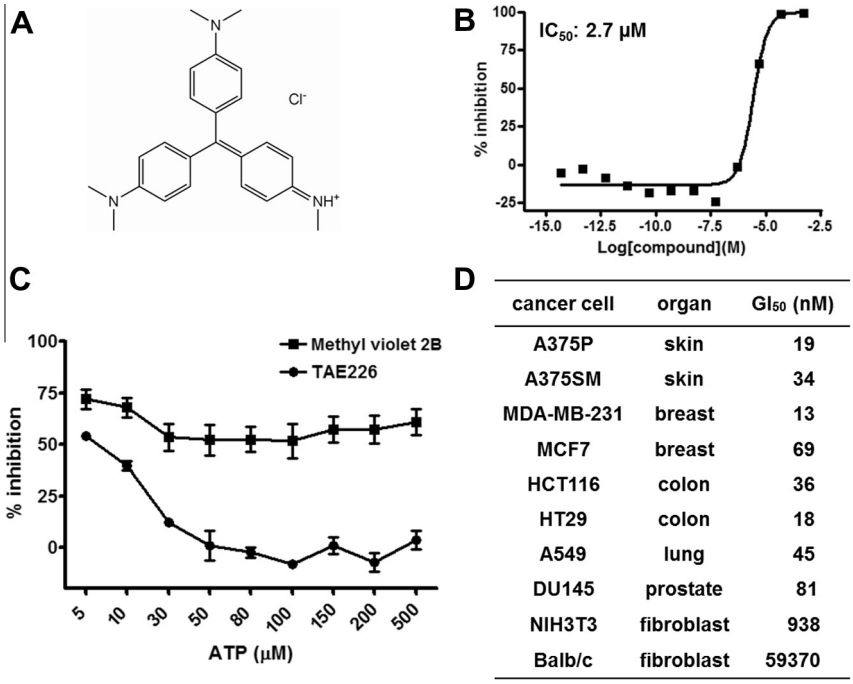
3. Results

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.