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The FLT3 and PDGFR inhibitor crenolanib is a substrate of the multidrug resistance protein ABCB1 but does not inhibit transport function at pharmacologically relevant concentrations

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Abstract

Background—Crenolanib (crenolanib besylate, 4-piperidinamine, 1-[2-[5-[(3-methyl-3-oxetanyl)methoxy]-1H-benzimidazol-1-yl]-8-quinolinyl]-, monobenzenesulfonate) is a potent and specific type I inhibitor of fms-like tyrosine kinase 3 (FLT3) that targets the active kinase conformation and is effective against FLT3 with internal tandem duplication (ITD) with point mutations induced by, and conferring resistance to, type II FLT3 inhibitors in acute myeloid leukemia (AML) cells. Crenolanib is also an inhibitor of platelet-derived growth factor receptor alpha and beta and is in clinical trials in both gastrointestinal stromal tumors and gliomas.

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Conflict of interest The authors declare that they have no conflict of interest.

Methods—We tested crenolanib interactions with the multidrug resistance-associated ATP-binding cassette proteins ABCB1 (P-glycoprotein), ABCG2 (breast cancer resistance protein) and ABCC1 (multidrug resistance-associated protein 1), which are expressed on AML cells and other cancer cells and are important components of the blood-brain barrier.

Results—We found that crenolanib is a substrate of ABCB1, as evidenced by approximate five-fold resistance of ABCB1-overexpressing cells to crenolanib, reversal of this resistance by the ABCB1-specific inhibitor PSC-833 and stimulation of ABCB1 ATPase activity by crenolanib. In contrast, crenolanib was not a substrate of ABCG2 or ABCC1. Additionally, it did not inhibit substrate transport by ABCB1, ABCG2 or ABCC1, at pharmacologically relevant concentrations. Finally, incubation of the FLT3-ITD AML cell lines MV4-11 and MOLM-14 with crenolanib at a pharmacologically relevant concentration of 500 nM did not induce upregulation of ABCB1 cell surface expression.

Conclusions—Thus ABCB1 expression confers resistance to crenolanib and likely limits crenolanib penetration of the central nervous system, but crenolanib at therapeutic concentrations should not alter cellular exposure to ABC protein substrate chemotherapy drugs.

Keywords

Crenolanib; ABCB1; FLT3; Platelet-derived growth factor receptor; Acute myeloid leukemia; Glioma

Introduction

Diverse kinase inhibitors are being tested alone and in combination with chemotherapy drugs with the goal of improving treatment outcomes in a variety of malignancies. Crenolanib (crenolanib besylate, 4-piperidinamine, 1-[2-[5-[(3-methyl-3-oxetanyl)methoxy]-1H-benzimidazol-1-yl]-8-quinolinyl]-,monobenzenesulfonate) (Fig. 1) is undergoing clinical testing in acute myeloid leukemia (AML) as a potent and specific inhibitor of the class III receptor tyrosine kinase (RTK) fms-like tyrosine kinase 3 (FLT3) [1–4] and in gastrointestinal stromal tumors (GIST) [5] and gliomas [6–8] as an inhibitor of class III RTK platelet-derived growth factor receptor (PDGFR)alpha. It also has promising activity in melanoma [9].

The type 3 tyrosine kinase fms-like tyrosine kinase 3 (FLT3) is expressed on AML cells in most AML patients [10], and is mutated, most commonly by internal tandem duplication (ITD), in up to a third [11–15]. FLT3-ITD mutations result in constitutive and aberrant FLT3 signaling, promoting survival and proliferation of AML cells with these mutations [16]. FLT3-ITD is associated with a high relapse rate and short-disease free survival following both chemotherapy and allogeneic hematopoietic stem cell transplantation [11–15]. Given the high frequency of FLT3-ITD mutations in AML and the poor treatment outcomes of patients whose AML cells harbor these mutations, much work has focused on identifying and developing inhibitors of FLT3 signaling and testing them both as single agents and in conjunction with AML chemotherapy regimens.

Diverse FLT3 inhibitors have been tested preclinically and in clinical trials. First-generation FLT3 inhibitors, including midostaurin (PKC412) [17] and lestaurtinib (CEP-701) [18], did

not have optimal potency, specificity or pharmacokinetic properties. Sorafenib, a multikinase inhibitor approved for treatment of renal, hepatic and thyroid carcinomas, is an effective FLT3 inhibitor and is used off-label in AML with FLT3 mutations, but its activity is frequently lost over time, sometimes due to development of resistance-inducing point mutations in FLT3-ITD [19]. Quizartinib (AC220) is the most potent and specific FLT3 inhibitor identified to date [20], but its clinical activity is also not durable, and, like sorafenib, it induces mutations in FLT3-ITD that disrupt binding of the drug to the target and have been demonstrated to be a mechanism of acquired resistance [21, 22].

The importance of identifying new compounds that inhibit FLT3-ITD with acquired point mutations associated with resistance to current FLT3 inhibitors has become apparent. Quizartinib and sorafenib are type II FLT3 inhibitors that bind to the inactive conformation of the kinase and prevent its activation. In contrast, type I inhibitors target the active conformation of the kinase and may be effective against FLT3-ITD with point mutations conferring resistance to type II FLT3 inhibitors. Ponatinib, approved as a BCR-ABL inhibitor, is also a potent type I FLT3 inhibitor with activity against FLT3 with induced point mutations [23, 24], but it is not being developed as a FLT3 inhibitor due to increased risk of vascular adverse events. Crenolanib is a type I FLT3 inhibitor that is active at nanomolar concentrations against FLT3-ITD, FLT3 D835 mutations, which are less common, wild-type FLT3 and FLT3-ITD with resistance-conferring point mutations induced by quizartinib or sorafenib [1–4].

In addition to inhibiting FLT3, crenolanib also inhibits platelet-derived growth factor receptor alpha and beta (PDGFRA, PDGFRB), and is in clinical trials in gastrointestinal stromal cell tumors (GIST) with PDGFRA mutations associated with imatinib resistance [5] and in gliomas [6], which are characterized by PDGFRA amplification and mutations in both children and adults [7, 8]. Moreover, it may also have efficacy in other solid tumors, such as melanoma [9].

In a phase I clinical trial, the recommended dose of crenolanib was 100 mg administered orally twice daily with food, yielding a mean maximum plasma concentration of 225 ng/ml, or approximately 0.5 μM (molecular weight 443.54), on Day 1, with mean maximum plasma concentration increasing to approximately 1 μM on Day 15 [25]. These concentrations greatly exceed the predicted concentration for target inhibition. Crenolanib is being administered orally at 100 mg three times daily in a phase II clinical trial in AML due to its half-life of 8 to 9 h, and has been well tolerated and shown promising clinical activity [26]. Crenolanib exhibits little plasma protein binding [4].

Little is known yet about mechanisms of resistance to crenolanib [4]. Of note, many other kinase inhibitors in current use or in clinical trials in cancer therapy are substrates and/or inhibitors [27–29] of the ATP-binding cassette (ABC) proteins ABCB1 [P-glycoprotein (Pgp); MDR1], ABCG2 [breast cancer resistance protein (BCRP)] and ABCC1 [multidrug resistance-associated protein 1 (MRP1)], drug efflux proteins that are frequently expressed on AML cells and other cancer cells [30]. Notably, the first-generation FLT3 inhibitors midostaurin [31], lestaurtinib [32], tandutinib [33, 34], sorafenib [35–37] and sunitinib [37,

38] are all substrates and/or inhibitors of ABCB1 and ABCG2, and our group previously demonstrated that both quizartinib [39] and ponatinib [40] are ABCG2 inhibitors.

ABCB1, ABCG2 and ABCC1 are also important components of the blood-brain barrier [41]. As such, drugs that are substrates of these ABC proteins have limited penetration of the central nervous system (CNS), and drugs that inhibit the transport function of these proteins have the potential to increase CNS penetration of their substrate drugs.

We sought to characterize interactions of crenolanib with the ABC proteins ABCB1, ABCG2 and ABCC1.

Materials and methods

Cell lines

Vincristine-selected HL60/VCR cells [42], overexpressing ABCB1, were obtained from Dr. Ahmad R. Safa, Indiana University, Indianapolis, IN, doxorubicin-selected HL60/ADR cells, overexpressing ABCC1 [43], from Dr. Kapil Bhalla, University of Kansas Cancer Center, Kansas City, KS, and mitoxantrone-selected 8226/MR20 myeloma cells [44], overexpressing wild-type ABCG2 [45], from Dr. William Dalton, Moffitt Cancer Center, Tampa, FL. HL60/VCR cells were maintained in drug-free RPMI 1640 medium with 10 % fetal bovine serum (FBS) and 8226/MR20 in RPMI 1640 medium with 10 % FBS and 20 nM mitoxantrone. Transfected K562 cells stably overexpressing ABCB1 [46] or wild-type ABCG2 [47] were gifts from Dr. Michael Gottesman, National Cancer Institute, Bethesda, MD and Dr. Yoshikazu Sugimoto, Kyoritsu University of Pharmacy, Tokyo, Japan, respectively. They were cultured in RPMI 1640, pH 7.4, supplemented with 10 % FBS at 37 °C in a humidified atmosphere containing 5 % CO2. Parental HL60 and K562 cells and MV4-11 and MOLM-14 cells, with FLT3-ITD [48], were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Materials

Crenolanib was purchased from Selleck Chemicals, Houston, TX, and was stored at −80 °C as a 100 mM stock solution in dimethyl sulfoxide. Cell Proliferation Reagent WST-1 was purchased from Roche Diagnostics (Indianapolis, IN). The fluorescent ABCB1 substrate 3,3′-diethyloxacarbocyanine iodide [$DiOC_2(3)$] was purchased from Sigma-Aldrich (St Louis, MO), the fluorescent ABCG2 substrate pheophorbide A (PhA) from Frontier Scientific (Logan, VT) [40], and the ABCC1 protein substrate rhodamine 123 (RH 123) from Sigma-Aldrich [40]. The ABCB1 inhibitor PSC-833 was obtained from Novartis Pharmaceutical Corporation (East Hanover, NJ), the ABCG2 inhibitor fumitremorgin C (FTC) was purchased from Sigma-Aldrich and the ABCC1 inhibitor p-[dipropylsulfamoyl] benzoic acid (probenecid) from Sigma-Aldrich [40]. [^{125}I]-Iodoarylazidoprazosin ([^{125}I]-IAAP) (2200 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Cell viability assay

Viable cell numbers following drug treatment were measured using the WST-1 assay [40]. Briefly, 1×10^3 cells were seeded in 100 μL complete medium per well in 96-well tissue culture plates and incubated with crenolanib (0–10 μM) at 37°C in 5% CO2 for 96 h. 10 μL WST-1 reagent was then added to each well, incubation was continued for two additional hours and the color developed was quantified according to the manufacturer's instructions. Each experiment was performed in triplicate. IC_{50} concentrations were calculated by the least square fit of dose-response inhibition in a non-linear regression model using GraphPad Prism V software (GraphPad Software, Inc., La Jolla, CA).

ABCB1 ATPase assay

High-Five insect cells were infected with recombinant baculovirus encoding ABCB1-His6. Membranes were isolated using hypotonic lysis and differential centrifugation, and the ATPase activity of vanadate (Vi)-sensitive ABCB1 expressed in membrane vesicles was measured in the presence of crenolanib at serial concentrations, as previously described [49]. Briefly, crude membrane protein (100 μg protein/ml) from ABCB1-expressing High-Five insect cells was incubated at 37 °C with crenolanib in increasing concentrations with and without 0.3 mM sodium orthovanadate, and the amount of inorganic phosphate released and the Vi-sensitive ATPase activity were measured.

ABCB1 cell surface expression

To detect ABCB1 cell surface expression, cells were incubated with MRK16 antibody for 1 h, washed twice with PBS, and then incubated with phycoerythrin (PE)-conjugated anti-human antibody for 30 min. Cells were acquired on a FACSCanto II (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR), as previously described [40].

Uptake of fluorescent ABC protein substrates

To measure the effect of crenolanib on uptake of fluorescent ABC protein substrates, HL60/VCR and K562/ABCB1 cells (1×10^6), expressing ABCB1, were incubated for 30 min at 37 °C with $DiOC_2(3)$ (0.6 ng/ml) and crenolanib (0–10 μM) or PSC-883 (2.5 μM) as a positive control, 8226/MR20 and K562/ABCG2 cells, expressing ABCG2, with PhA (1 μM) and crenolanib (0–10 μM) or FTC (10 μM) as a positive control, and HL60/ADR cells, expressing ABCC1, with RH 123 (0.5 μg/mL) and crenolanib (0–10 μM) or probenecid (1 mmol/L) as a positive control, as previously described [40]. Cells were then washed twice, resuspended in phosphate-buffered saline (PBS) and kept on ice until analysis. They were then acquired on a FACSCanto II flow cytometer and analyzed using FlowJo software. Substrate content after uptake with and without modulator was compared using the Kolmogorov-Smirnov statistic, expressed as a D-value ranging from 0 (no difference) to 1 (no overlap) [50], with D-values ≥0.2 indicating significant modulation, based on previous work [51].

Photoaffinity labeling of ABCB1 and ABCG2 with [^{125}I]-Iodoarylazidoprazosin (IAAP)

High-Five insect cell membrane vesicles expressing ABCB1 (50–70 μg protein) were incubated with 0–20 μM crenolanib for 5 min at 21–23 °C in 50 mM Tris-HCl, pH 7.5. [^{125}I]-IAAP (2200 Ci/mmole), 3–6 nM, was added and incubation was continued for 5 additional minutes under subdued light. ABCB1 crosslinked with [^{125}I]-IAAP was separated on 7 % SDS-PAGE gels and the amount of [^{125}I]-IAAP incorporated into ABCB1 was quantified as previously described [52].

Results

Crenolanib resistance is conferred by ABCB1, but not ABCG2 or ABCC1

To determine whether crenolanib is a substrate for the multidrug resistance (MDR)-associated ABC proteins ABCB1, ABCC1 and ABCG2, we studied cytotoxicity of crenolanib in HL60/VCR and HL60/ADR cells, expressing ABCB1 and ABCC1, respectively, with parental HL60 as a control, as well as transfected K562/ABCB1 and K562/ABCG2 cells, with parental K562 cells as a control. HL60/VCR and K562/ABCB1 cells, overexpressing ABCB1, were 6.9- and 3.6-fold resistant to crenolanib, respectively, in relation to parental HL60 and K562 cells (Fig. 2a and b and Table 1). In contrast, K562/ABCG2 and HL60/ADR cells, overexpressing ABCG2 and ABCC1, respectively, were not resistant to crenolanib, in relation to parental K562 and HL60 cells (Fig. 2b and c and Table 1). These data are consistent with crenolanib being a substrate of ABCB1, but not ABCC1 or ABCG2. To confirm that ABCB1 mediates crenolanib resistance, we studied crenolanib cytotoxicity in HL60/VCR and K562/ABCB1 cells in the presence and absence of 2.5 μM PSC-833, a specific inhibitor of ABCB1-mediated substrate transport. PSC-833 fully reversed resistance to crenolanib in both HL60/VCR and K562/ABCB1 cells (Fig. 2d and e and Table 1).

Crenolanib stimulates ABCB1 ATPase activity in a concentration-dependent manner

To further study the interaction of crenolanib with ABCB1, we studied its effect on ATP hydrolysis mediated by ABCB1, as described in (Materials and Methods). Crenolanib was found to stimulate ABCB1 ATPase activity in a concentration-dependent manner (Fig. 3), suggesting that it interacts as a typical substrate of ABCB1 at the drug-binding pocket of the transporter.

Crenolanib treatment of FLT3-ITD cells does not upregulate ABCB1 cell surface expression

We then sought to determine whether treatment of cells with crenolanib induced expression of ABCB1. To this end, we cultured MV4-11 and MOLM-14 cells, both human cell lines with FLT3-ITD, with crenolanib at the pharmacologically relevant concentration of 500 nM [25]. Cells were counted at 48 and 96 h and tested for viability and for surface expression of ABCB1, measured by flow cytometry. Cell concentrations after 96-hour culture with 500 nM crenolanib and DMSO control were 0.73×10^5/ml and 6.75×10^5/ml, respectively. No induction of ABCB1 was seen on cells treated with crenolanib, in relation to DMSO control. It appeared that cell surface expression actually decreased on both MV4-11 (Fig. 4a) and MOLM-14 (data not shown) cells. This occurred in conjunction with a decrease in both

forward and side scatter (Fig. 4b) and morphologic changes consistent with monocytic maturation in surviving crenolanib-treated cells (Fig. 4c).

Crenolanib does not alter substrate transport by ABCB1, ABCG2 or ABCC1 at pharmacologically relevant concentration

We next sought to determine whether crenolanib alters substrate transport by ABCB1, ABCG2 or ABCC1. To this end, we measured uptake of fluorescent substrates of these transport proteins in the presence of crenolanib at concentrations of 0 to 10 μM. Cell lines studied included HL60/VCR and K562/ABCB1, overexpressing ABCB1, K562/ABCG2 and 8226/MR20 cells, overexpressing ABCG2, and HL60/ADR, overexpressing ABCC1. Significant effect on ABCB1, ABCC1 or ABCG2 substrate transport was not seen with crenolanib at concentrations up to 1 μM, but progressive effect was seen at 5 and 10 μM (Fig. 5), consistent with crenolanib inhibiting the activity of ABCB1 only at high concentrations.

Crenolanib inhibits [^{125}I]-IAAP photocrosslinking of ABCB1 only at high concentrations

We further examined the effect of crenolanib on substrate binding of ABCB1 by studying its effect on the photocrosslinking of ABCB1 with a photoactivatable substrate analog, [^{125}I]-IAAP. Crenolanib inhibited [^{125}I]-IAAP photocrosslinking of ABCB1 at high concentrations, with 50 % inhibition at 10 μM, but had little effect at lower concentrations, below 1 μM, that are pharmacologically relevant in patients (Fig. 6).

Discussion

Crenolanib is a type I FLT3 inhibitor with activity at nanomolar concentrations against FLT3-ITD, FLT3 with D835 mutation, wild-type FLT3, and FLT3-ITD with resistance-conferring point mutations induced by quizartinib or sorafenib; it is being tested in clinical trials in patients with AML with FLT3 mutations [1–4]. It is also an inhibitor of PDGFRA, as well as PDGFRB, and is being tested in clinical trials in patients with GIST [5] and gliomas [6–8]. Its efficacy in gliomas is also dependent upon its ability to cross the blood-brain barrier.

We studied interactions of crenolanib with the ABC proteins ABCB1, ABCG2 and ABCC1, which are expressed on AML cells and other cancer cells and are associated with multidrug resistance, and are also important components of the blood–brain barrier. We found that cells expressing ABCB1 were resistant to crenolanib, while cells expressing ABCG2 or ABCC1 were equally sensitive to crenolanib as parental cells not expressing these transporters. Additionally, while crenolanib had no effect on ABCB1, ABCG2 or ABCC1 substrate transport at the less than 1 μM concentrations expected in patients, concentration-dependent inhibition of substrate transport by crenolanib at higher concentrations was observed in cells expressing these transporters.

Crenolanib transport by ABCB1 was evidenced by higher IC_{50} concentrations in cells overexpressing ABCB1, in relation to parental cells, reversal of crenolanib resistance in ABCB1-expressing cells by the ABCB1-specific transport inhibitor PSC-833, and crenolanib stimulation of ABCB1 ATPase activity. Crenolanib also inhibits ABCB1

substrate transport activity, but only at concentrations in the range of 5 μM and above, which are higher than pharmacologically relevant concentrations in patients. Of note, crenolanib was previously identified as an ABCB1 inhibitor in a high-throughput screen, but concentration-dependence and relationship to pharmacologically relevant concentrations were not addressed [53]. Ambudkar et al. defined three classes of ABCB1 inhibitors based on their effects on ABCB1 ATPase activity [54]. Class I agents stimulate ATPase activity at low concentrations but inhibit it at high concentrations, while Class II compounds stimulate ATPase activity in a concentration-dependent manner without any inhibition, and Class III compounds inhibit ATPase activity. We found crenolanib to be a Class II agent.

The clinical importance of ABCB1-mediated efflux as a mechanism of resistance to crenolanib in AML with FLT3-ITD and in GIST and gliomas remains to be determined. ABCB1 is frequently overexpressed on AML cells and association of its overexpression with inferior treatment outcomes is well established [55]. Of note, however, in one report, presence of FLT3-ITD and ABCB1 overexpression were found to be adverse prognostic factors that were usually mutually exclusive in untreated AML [56]. In contrast, it is not known whether ABCB1 is upregulated in relapsed or refractory FLT3-ITD AML. ABCB1 is not infrequently overexpressed in GIST [57, 58], while data on ABCB1 expression in gliomas are inconsistent [59, 60].

The fact that crenolanib appears to be a transport substrate of ABCB1 raises concern not only about crenolanib resistance in cancer cells overexpressing ABCB1, but also induction of ABCB1 in the setting of crenolanib treatment, as ABCB1 has been shown to be induced by substrate transport [61]. Induction of ABCB1 by FLT3 inhibitors has not been studied previously. We tested for induction of ABCB1 cell surface expression in two human AML cell lines with FLT3-ITD, MV4-11 and MOLM-14, treated with crenolanib at a pharmacologically relevant concentration, and found no induction of ABCB1 cell surface expression. Of note, crenolanib actually induced a decrease in ABCB1 cell surface expression on MV4-11 and MOLM-14 cells. This was associated with, and likely caused by, cellular maturation analogous to the myeloid differentiation of AML cells described in patients treated with the FLT3 inhibitor quizartinib [62]. Patient samples were not studied here, and cellular maturation of patient cells has not been studied with crenolanib, but similar findings may be expected.

In addition to ABCB1 expression on gliomas, its expression on the intact blood-brain barrier could limit access of crenolanib to gliomas. However, in a preclinical study evaluating crenolanib penetration in a spontaneous glioblastoma murine model, crenolanib showed increased penetration in tumor-bearing brain as compared to normal brain, indicating a disrupted blood-brain barrier in tumor-bearing mice [63]. Relevance to human central nervous system tumors is unknown. A clinical trial of crenolanib in gliomas is ongoing, and results are awaited.

Since crenolanib is an ABCB1 substrate, there is a potential for competitive inhibition of transport of co-administered ABCB1 substrate medications. However we demonstrated that crenolanib does not inhibit the transport function of this multidrug transporter at the plasma concentrations that are achieved in patients, and it is therefore unlikely to alter transport of

co-administered chemotherapy drugs in cancer cells. In contrast, higher concentrations are expected to be achieved in the gastrointestinal tract, and could alter absorption of orally co-administered ABC drug transporter substrate chemotherapy drugs and other medications, as we previously showed for quizartinib [64].

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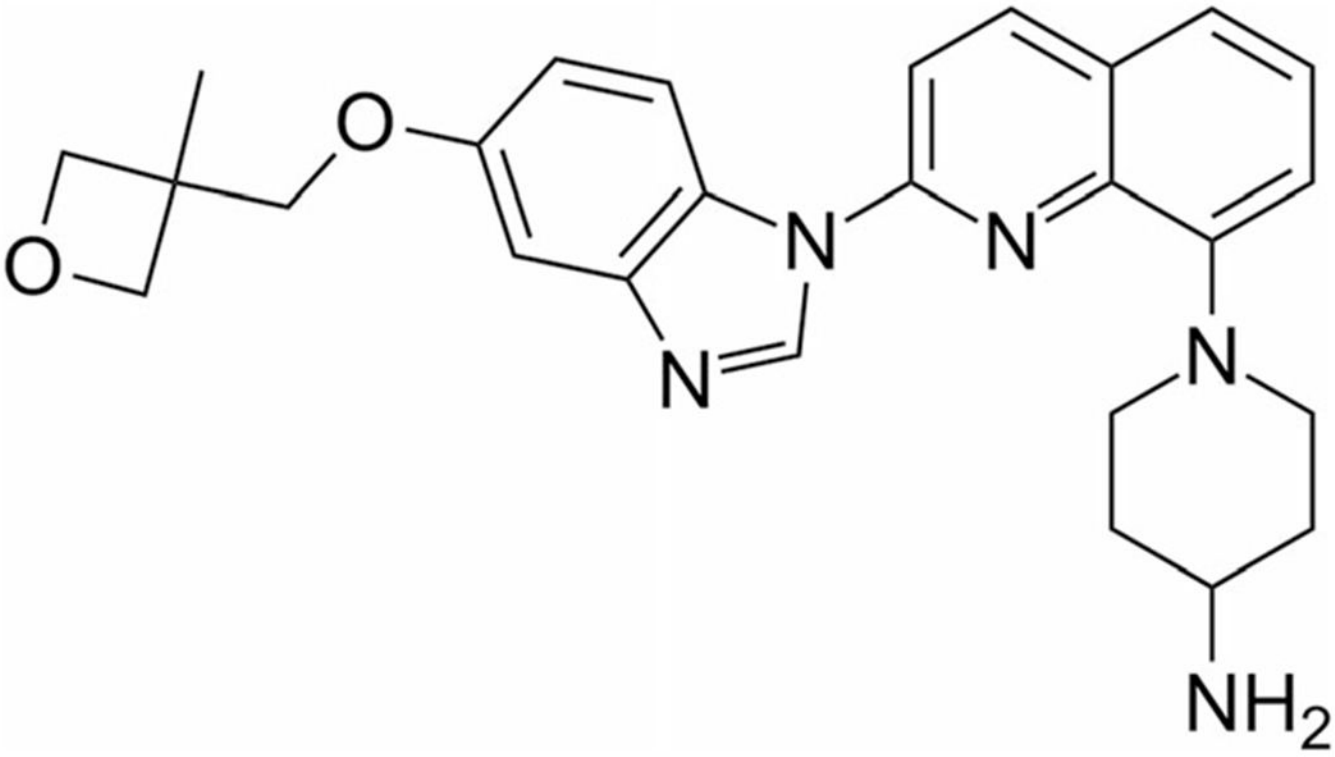

Fig. 1.
Chemical structure of crenolanib

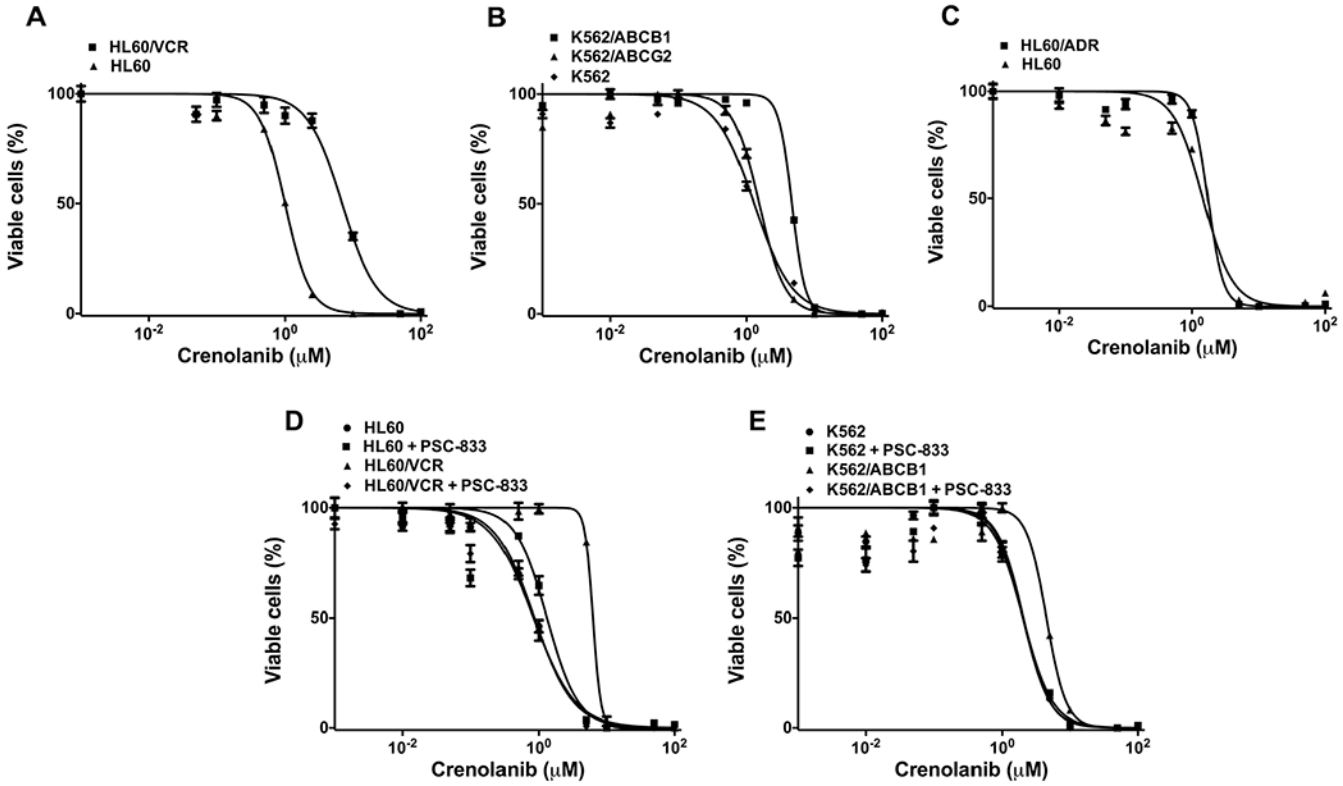

Fig. 2.

Cells overexpressing ABCB1 are approximately 5-fold resistant to crenolanib, while ABCC1 and ABCG2 do not confer crenolanib resistance. **a, b, c.** Crenolanib cytotoxicity in cells overexpressing ABCB1 (HL60/VCR, K562/ABCB1), ABCG2 (K562/ABCG2) and ABCC1 (HL60/ADR) and parental cells (HL60, K562) is shown. Multidrug resistant cells overexpressing ABCB1, ABCC1 or ABCG2 and parental cells were cultured in 96-well tissue culture plates and incubated with crenolanib (0–10 μM) at 37 °C in 5 % CO_2 for 96 h. Viability of crenolanib-treated cells was evaluated using the WST-1 assay, and IC_{50}s were calculated as described in (Materials and Methods). **d, e.** Crenolanib resistance of ABCB1-overexpressing cells is reversed by the ABCB1-specific inhibitor PSC-833. Viability of crenolanib-treated HL60/VCR and K562/ABCB1 cells, as well as HL60 and K562 parental cells, was measured in the presence and absence of the ABCB1-specific inhibitor PSC-833 at 2.5 μM. Mean and standard error values from three independent experiments are shown

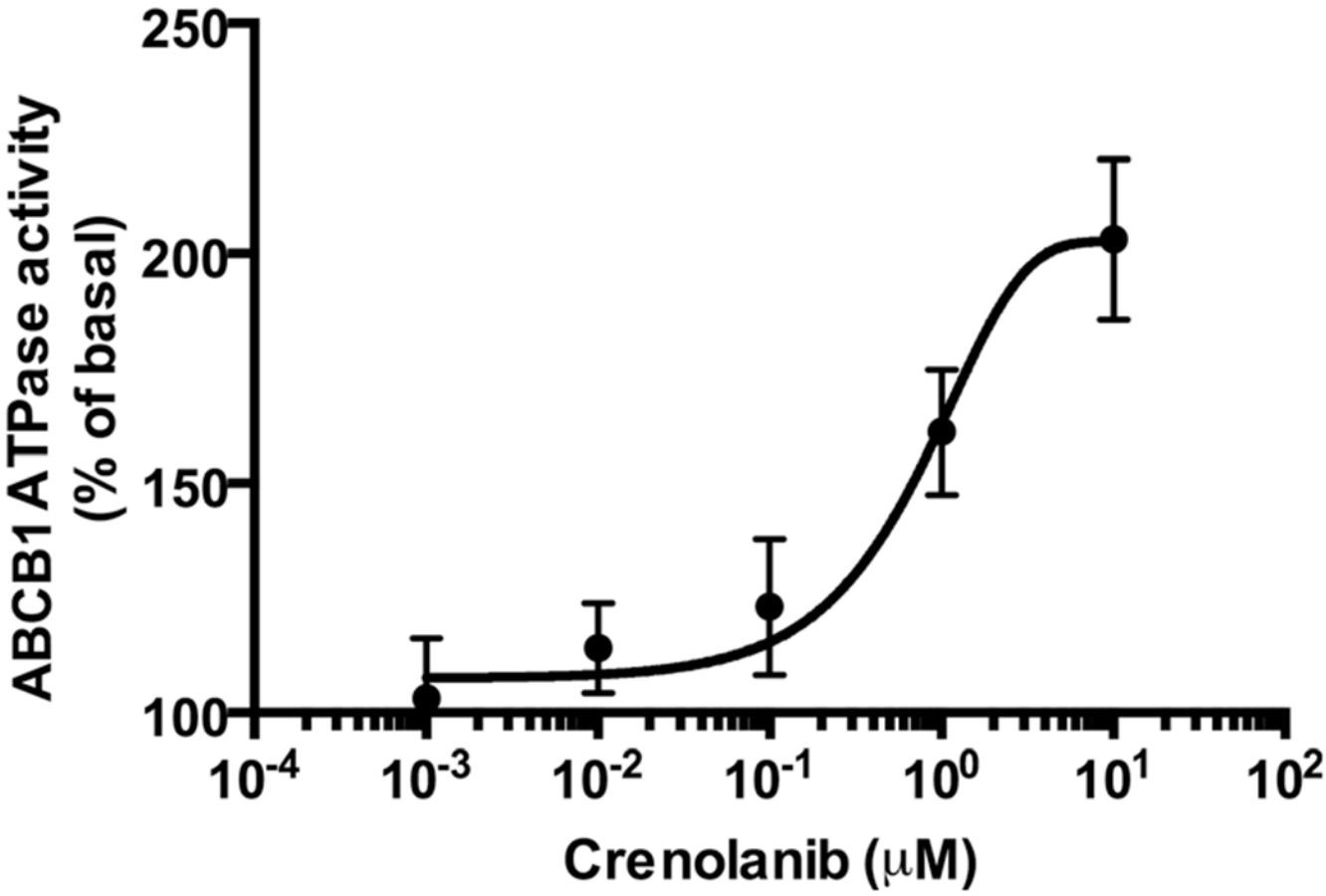

Fig. 3.

Crenolanib stimulates ABCB1 ATPase activity, consistent with transport by ABCB1. Crude membrane preparations from ABCB1-expressing High-Five insect cells were incubated with crenolanib in increasing concentrations with and without 0.3 mM sodium orthovanadate. The amount of inorganic phosphate released and the vanadate-sensitive ATPase activity were measured. The basal ATPase activity was considered as 100 % and the stimulation of ATP hydrolysis by varying concentrations of crenolanib (calculated as % increase of basal activity) (Y-axis) was plotted as a function of crenolanib concentrations (X-axis). The average from independent duplicate experiments is shown, with *error bars* representing standard error

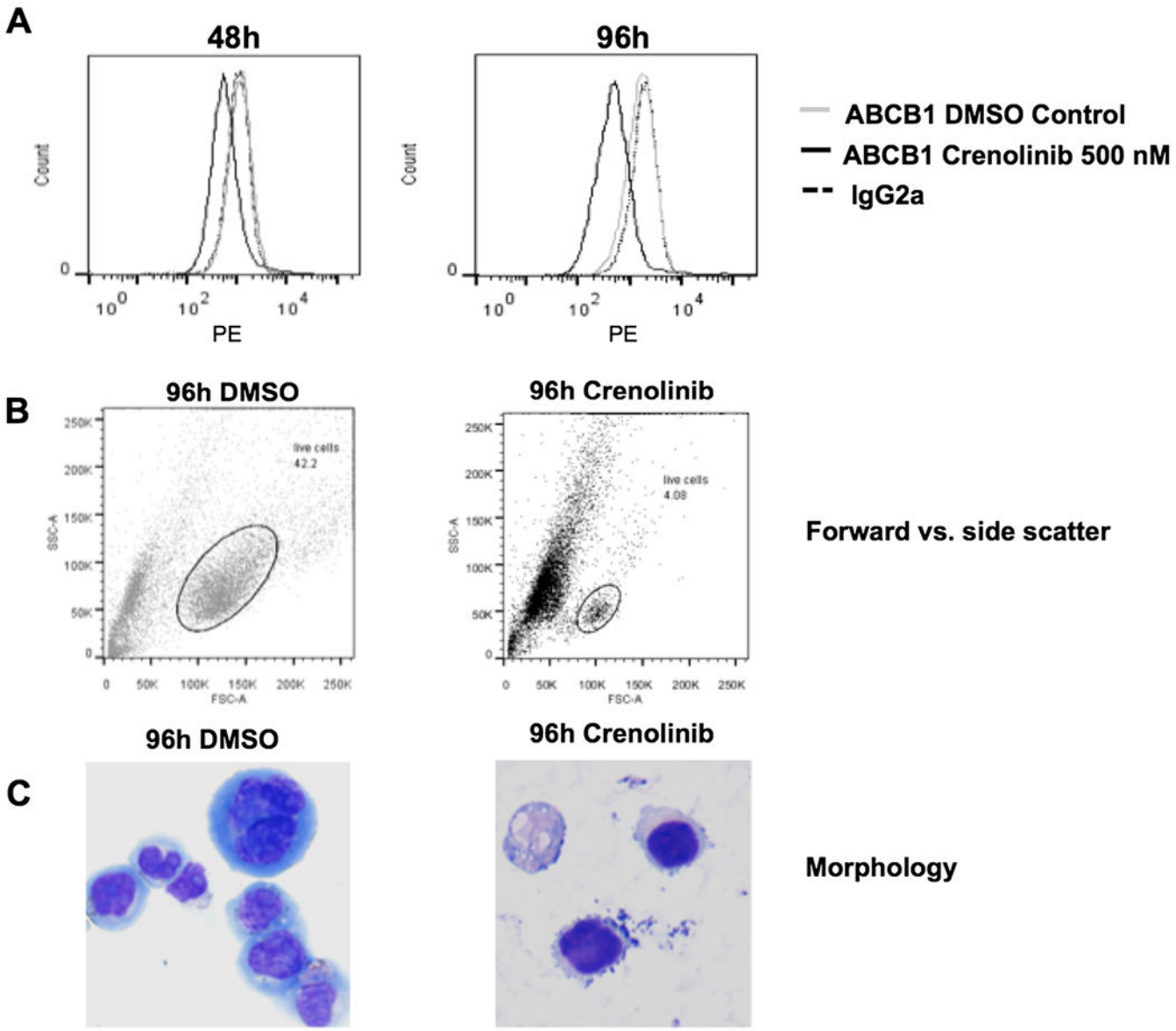

Fig. 4.

Crenolanib treatment does not increase the cell surface expression of ABCB1. MV4-11, human myeloid leukemia cells expressing FLT3-ITD, were treated with 500 nM crenolanib or DMSO control and cell surface expression of ABCB1 was measured by flow cytometry at 48 and 96 h as described in (Materials and Methods). No increase in ABCB1 cell surface expression was seen in crenolanib-treated cells, and, of note, a progressive decrease in ABCB1 cell surface expression was actually seen, in association with flow cytometric and morphologic changes indicative of cellular maturation. **a.** Decreased ABCB1 cell surface expression on MV4-11 cells treated for 48 and 96 h with crenolanib, in relation to DMSO control. **b.** Decreased forward and side scatter of MV4-11 cells treated for 96 h with crenolanib, in relation to DMSO control. **c.** Cellular maturation of MV4-11 cells treated for 96 hwith crenolanib, in relation to DMSO control. Cytospin preparations (Wright-Giemsa stain x 400) of MV4-11 cells treated with DMSO control for 96 h (*left*) shows intermediate

to large cells with a monocytoid appearance, including some multinucleate cells, with ‘blastic’ fine chromatin and prominent nucleoli. In contrast the same cells after incubation with 500 nM crenolanib for 96 h (*right*) are smaller and have dense chromatin, and their morphology more closely resembles that of mature monocytes

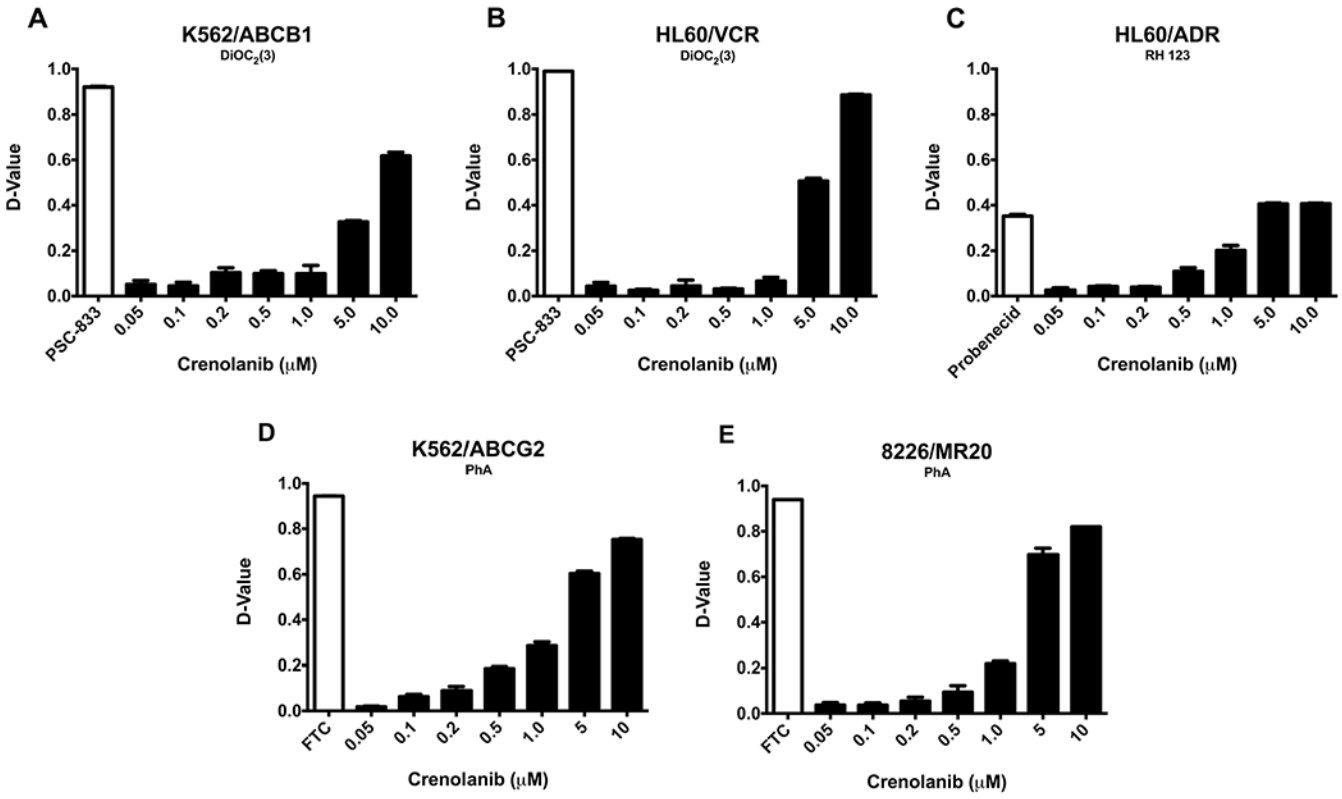

Fig. 5.

Crenolanib does not alter substrate transport by ABCB1, ABCC1 or ABCG2 at pharmacologically relevant concentrations. Uptake of the fluorescent substrates of ABCB1, ABCC1 and ABCG2 3,3′-diethyloxacarbocyanine iodide [$DiOC_2(3)$], rhodamine 123 (RH 123) and pheophorbide A (PhA), respectively, was measured in the presence of crenolanib at concentrations of 0–10 μM, as described in (Materials and Methods). Crenolanib at concentrations up to 1 μM did not affect ABCB1, ABCC1 or ABCG2 substrate transport, but a concentration-dependent decrease in transport was seen at 5 and 10 μM. Mean and standard error values from three independent experiments are shown

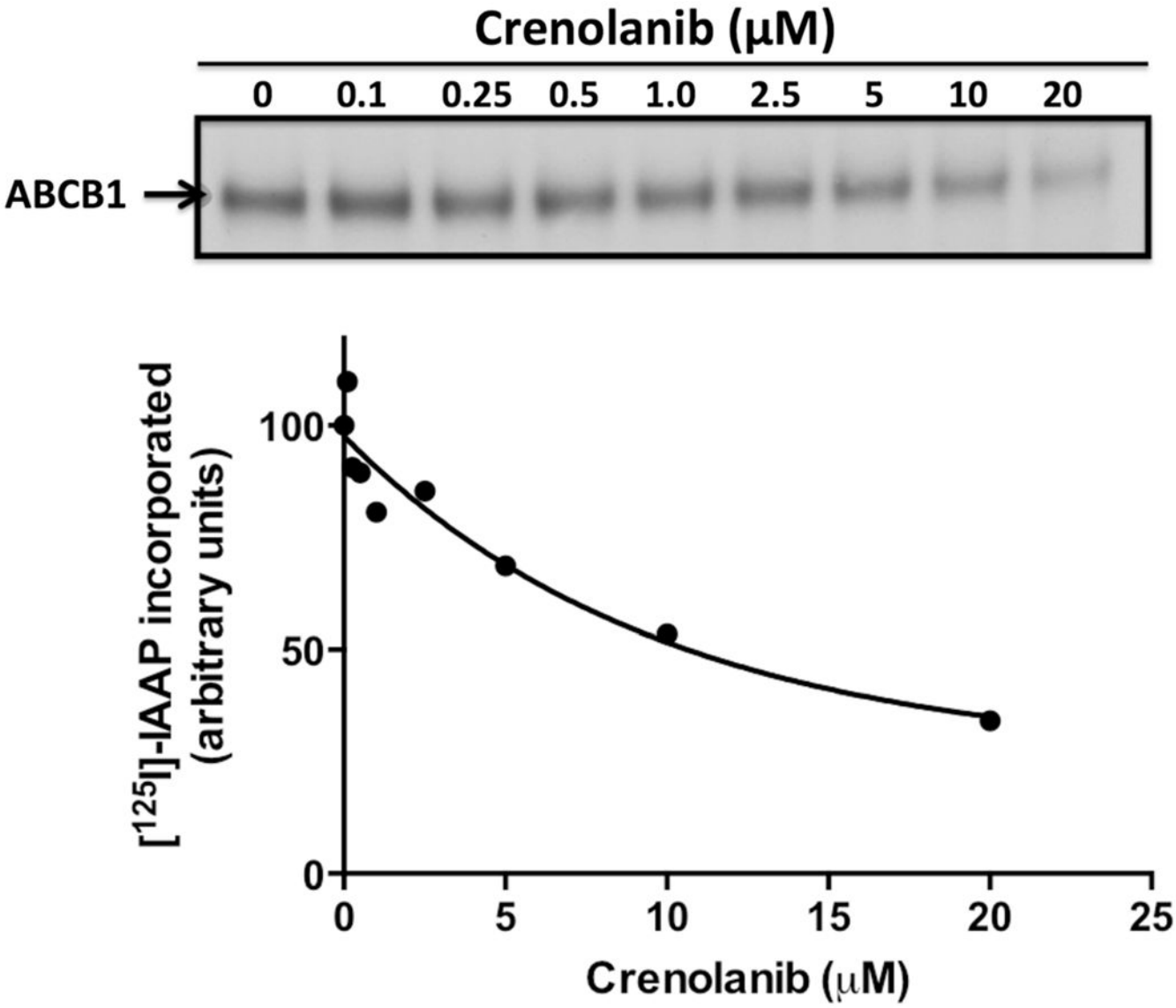

Fig. 6.

Crenolanib does not alter[^{125}I]-IAAP photocrosslinking of ABCB1 at pharmacologically relevant concentrations. The effect of crenolanib (0–20 μM) on photocrosslinking of ABCB1 with [^{125}I]-IAAP was studied as described in (Materials and Methods). The *upper panel* shows a representative autoradiogram. The average values from two independent experiments are shown in the graph. The amount of [^{125}I]-IAAP incorporated into ABCB1 in the absence of crenolanib was considered as 100 % and the percent decrease in [^{125}I]-IAAP incorporation (Y-axis) at the indicated concentrations of crenolanib was plotted as a function of crenolanib concentration used (X-axis)

Table 1

Crenolanib IC_{50} values of ABC protein-overexpressing and parental cell lines

Cell line	IC_{50} (μM)	Standard error
HL60	1.00	0.03
HL60/VCR	6.93	0.03
K562	1.30	0.03
K562/ABCB1	4.67	0.01
K562/ABCG2	1.54	0.03
HL60	1.46	0.04
HL60/ADR	1.72	0.06
HL60	0.86	0.02
HL60+PSC-833	1.32	0.06
HL60/VCR	6.27	0.02
HL60/VCR+PSC-833	0.84	0.04
K562	2.02	0.05
K562+PSC-833	2.02	0.08
K562/ABCB1	4.49	0.04
K562/ABCB1+PSC-833	2.06	0.08