The natural product, CBT-1, inhibits Pgp- and MRP1-mediated transport

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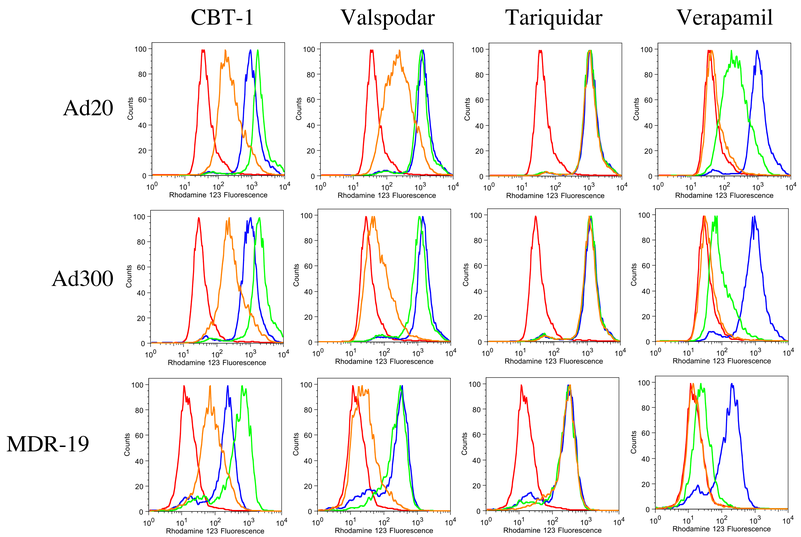
Abstract:
Drug resistance remains a significant impediment to successful cancer chemotherapy. Overexpression of ATP-binding cassette (ABC) transporters in cancer cells has been shown to confer a drug-resistance phenotype. In order to overcome resistance mediated by ABC transporters, inhibitors have been developed and are currently in clinical trials. CBT-1 is a natural product currently in clinical trials as a Pgp inhibitor. To further characterize the interactions between ABC transporters known to confer a drug resistance phenotype, we conducted several experiments with cell lines expressing varying levels of ABC transporter proteins. Methods: We used flow cytometry assays to assess the ability of CBT-1 to inhibit ABC transporters and cytotoxicity assays to to confirm the ability of CBT-1 to reverse ABC-transporter mediated drug resistance. Interaction of CBT-1 with drug binding sites was determined using [^{125}I]-iodoarylazidoprazosin. Results: In flow cytometry assays, we found 1 μM of CBT-1 completely inhibited transport of the fluorescent Pgp substrate rhodamine 123 in Pgp-overexpressing, drug-selected SW620 Ad20 and Ad300 colon carcinoma cells, as well as in MDR1-transfected human embryonic kidney cells (HEK293). In 4-day cytotoxicity assays with the known Pgp substrates vinblastine, pactlitaxel and depsipeptide, 1 μM CBT-1 completely inhibited Pgp-mediated drug resistance in SW620 Ad20 cells. CBT-1 at 1 μM was also shown to prevent [^{125}I]-iodoarylazidoprazosin labeling of Pgp, suggesting that CBT-1 acts at the drug-binding site of Pgp. To determine if CBT-1 interacted with other ABC transporters associated with cancer drug resistance, we examined the effect of CBT-1 on MRP1-mediated calcein transport. CBT-1 was found to inhibit MRP1-mediated calcein efflux at 10 μM in MRP1 overexpressing MCF-7/VP cells, suggesting that CBT-1 is able to inhibit multiple transporters. CBT-1 was not found to have an effect on ABCG2-mediated pheophorbide a transport in ABCG2-transfected HEK293 cells. Conclusions: We conclude from our studies that CBT-1 is an effective Pgp inhibitor and that it additionally inhibits the MRP1 transporter. Further clinical studies with CBT-1 are warranted.

Introduction

Cellular mechanisms of drug resistance fall generally into two classes: those that prevent drugs from reaching their target and those that cause genetic changes that affect drug sensitivity. ATP-binding cassette (ABC) transport proteins are known to induce resistance due to their ability to lower intracellular drug concentrations in an energy-dependent manner. P-glycoprotein (Pgp), the product of the *MDR1* (*ABCB1*) gene, has been studied extensively and is known to transport a wide range of chemotherapy drugs such as the anthracyclines, vincas, taxanes, etoposide, mitoxantrone, bisantrene and the histone deacetylase inhibitor depsipeptide. Subsequent to the discovery of Pgp, the multidrug resistance associated protein, MRP1 (ABCC1), was cloned from lung carcinoma cells selected in adriamycin and was found to confer resistance to etoposide, vincristine and adriamycin. The most recently reported ABC transporter associated with drug resistance, ABCG2, is a half-transporter whose substrates include mitoxantrone, topotecan, and flavopiridol.

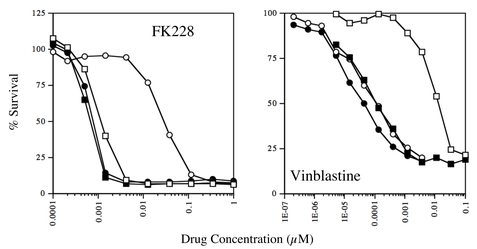
CBT-1 is an orally-administered, natural product currently being developed as a Pgp inhibitor. Phase I trials with CBT-1 and paclitaxel or doxorubicin have been completed and phase II trials are currently in progress. The initial phase I studies demonstrated that CBT-1 did not affect the pharmacokinetics of doxorubicin or taxol and no neurological toxicities were observed. As the clinical development of CBT-1 progresses, it becomes important to biochemically characterize the interactions between CBT-1 and the ABC transporters shown to transport chemotherapeutic agents. Thus, we examined the ability of CBT-1 to inhibit Pgp-mediated rhodamine transport and compete IAAP labeling of Pgp. CBT-1 was also found to block MRP1-mediated transport, but had no effect of ABCG2-mediated transport.

1 CBT-1 inhibits Pgp-mediated rhodamine efflux



Drug-selected Ad20 and Ad300 cells as well as MDR1-transfected HEK-293 cells (MDR-19) were incubated with 0.5 μg/ml rhodamine 123 in the presence or absence of 0.1, 1 or 10 μM of CBT-1, verapamil, valspodar or tariquidar for 30 min. Subsequently, cells were washed and allowed to incubate in rhodamine-free medium for 1 h continuing without (red line) or with 0.1 μM (orange line), 1 μM (green line), or 10 μM (blue line) of inhibitor. Representative histograms from at least 3 independent experiments are shown. CBT-1 was able to partially inhibit rhodamine 123 efflux even at a concentration of 100 nm (heavy solid line) in the Ad20, Ad300, and MDR-19 cell lines, while complete inhibition was observed at 1 μM (solid line). Valspodar, shown in the second column, was comparably potent. Tariquidar completely inhibited rhodamine transport at 100 nm (heavy solid line) in the Ad20, Ad300, and MDR-19 cell lines, making it the most potent inhibitor examined. Verapamil, shown in the last column, was a relatively poor inhibitor, since 10 μM (dashed line) was required to completely prevent rhodamine transport in all of the Pgp-overexpressing cell lines.

2 CBT-1 abrogates Pgp-mediated drug resistance



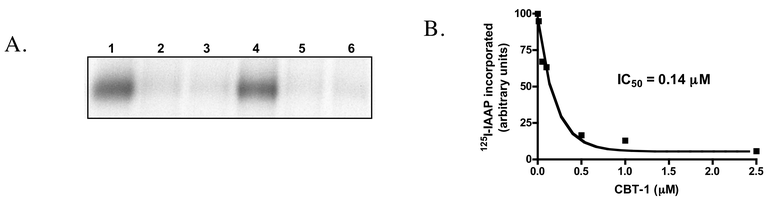
Four-day cytotoxicity assays were performed with SW620 parental (squares) or Pgp-overexpressing SW620 Ad20 cells (circles) in the presence (filled symbols) or absence (open symbols) of 1 μM CBT-1. Cells were plated (2500 cells/well) in 96-well, flat-bottom plates and allowed to attach overnight at 37°C in 5% CO2. Chemotherapeutic compounds were subsequently added to the cells at various concentrations and allowed to incubate for 96 hours. Cells were fixed with 50% (w/v) trichloroacetic acid and then stained with 0.4% sulforhdaomine B (w/v) in 1% acetic acid. After washing the plates in 1% acetic acid, the dye was solubilized in 50% Trizma base and plates were read at an absorbance of 540 nm. Addition of CBT-1 completely abrogated resistance to FK228 (depsipeptide) and vinblastine in the SW620 Ad20 cells

3 Table 1: Reversal of drug resistance by CBT-1

Drug	SW620 IC_{50}	SW620 Ad20 IC_{50}
Vinblastine	0.004±0.003	0.02±0.008
Vinblastine + 1 μM CBT-1	0.0002±0.0002	0.0005±0.0004
Paclitaxel	0.002±0.003	0.3±0.2
Paclitaxel + 1 μM CBT-1	0.002±0.002	0.002±0.002
FK228	0.001±0.0006	0.03±0.01
FK228 + 1 μM CBT-1	0.0006±0.0002	0.0006±0.002
5-FU	24±16	29±23
5-FU + 1 μM CBT-1	34±16	41±19

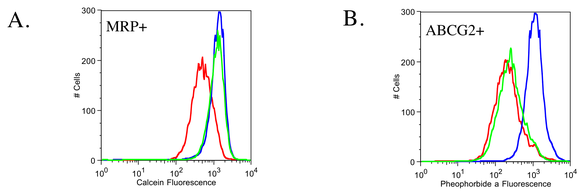
Summary of results from cytotoxicity assays performed on SW620 parental and Pgp-overexpressing SW620 Ad20 cells with the Pgp substrate drugs vinblastine, paclitaxel, and FK228, as well as the non-substrate drug 5-FU, in the presence or absence of 1 μM CBT-1. Addition of CBT-1 completely reversed resistance to vinblastine, paclitaxel and FK228 in SW620 Ad20 cells. IC_{50} values for 5-FU remain unchanged in the presence or absence of CBT-1.

4 Effect of CBT-1 on [^{125}I]-IAAP labeling of Pgp



Crude membranes isolated from Pgp-overexpressing High Five insect cells were incubated with the desired inhibitor for 10 min after which 3-6 nM [^{125}I]-IAAP (2200 Ci/mople) in 50 mM Tris-Hcl (pH 7.5) was added and the samples were incubated for an additional 5 min in the dark . Samples were then exposed to a UV light source for 10 min at room temperature. Protein a sepharose beads (100 μL, Amersham Biosciences, Piscataway, NJ) were then added and incubated for 16h at 4°C. The beads were peletted by centrifuging at 13,000 rpm for 5 min at 4°C and then washed with RIPA buffer in 1% aprotinin. SDS-PAGE sample buffer (25 μL) was then added and the samples were incubated for 1h at 37°C, followed by the addition of 25 μl of water and an additional incubation at 37°C for 30 min. Samples were separated by PAGE on a 7% Tris-acetate gel, followed by drying and exposure to film. As seen in panel A, CBT-1 at a concentration of 10 μM (land 2) readily competes [^{125}I]-IAAP labeling of Pgp (control shown in lane 1), suggesting it interacts at the drug binding site of [^{125}I]-IAAP. Other well-known Pgp inhibitors valspodar (lane 3), tariquidar (lane 5) and cyclosporine a (land 6) also prevented photolabeling of Pgp by [^{125}I]-IAAP at a concentration of 10 μM. Verapamil had no effect on [^{125}I]-IAAP labeling of Pgp at 10 μM. The IC_{50} for [^{125}I]-IAAP inhibition by CBT-1 was 0.14 μM, as seen in panel B. This compares favorably to previously published values for other known Pgp inhibitors such as biricodar (VX-710), 0.75 μM; cyclosporine a, 3.5 μM and verapamil, >100 μM

5 Inhibition of MRP1-mediated, but not ABCG2-mediated transport by CBT-1



A. To determine whether CBT-1 interacts with MRP1, the MRP1-overexpressing, etoposide-selected cell line MCF-7/VP, was incubated in 0.1 μM of the MRP1 substrate calcein AM alone or in the presence of 10 μM CBT-1 or 25 μM of the known MRP1 inhibitor, MK-571 , for 30 min. Cells were then washed and allowed to incubate in calcein-free medium for 1h, continuing with CBT-1 (green line) or MK-571 (blue line) or without inhibitor (red line). MRP1-mediated transport, denoted by the red line is inhibited by CBT-1 at a concentration of 10 μM (green line), comparable to that of MK-571 (blue line). B. ABCG2-transfected HEK-293 cells were incubated with 1 μmof the ABCG2 substrate pheophorbide a in the presence of 25 μM CBT-1 or 10 μM FTC. Cells were then washed and allowed to incubate in pheophorbide-free medium continuing with CBT-1 (green line) or FTC (blue line) or without inhibitor (red line). While FTC readily increases intracellular pheophorbide a fluorescence (blue line), CBT-1 has no effect (green line) and is comparable to cells treated without inhibitor (red line).

Summary and Conclusions

- CBT-1 is an orally administered Pgp inhibitor currently in Phase II trials.
- At a concentration of 1 μM, CBT-1 completely abrogated rhodamine transport in drug selected SW620 Ad20 and Ad300 cells as well as *MDR1*-transfected HEK-293 cells.
- Pgp-mediated resistance to vinblastine, paclitaxel and FK228 was completely reversed in SW620 Ad20 cells by 1 μM CBT-1.
- CBT-1 interacts with Pgp at the prazosin-binding site, as evidenced by competition of labeling of Pgp by [^{125}I]-IAAP.
- CBT-1 at a concentration of 10 μM also inhibits MRP1-mediated transport; no effect was observed on ABCG2-mediated transport at CBT-1 concentrations as high as 25 μM.
- Inclusion of CBT-1 into treatments strategies for diseases where Pgp or both MRP1 and Pgp are expressed may reverse drug resistance and increase response.