Human Organic Cation Transporter 1 Is Expressed in Lymphoma Cells and Increases Susceptibility to Irinotecan and Paclitaxel

Shivangi Gupta, Gerald Wulf, Maja Henjakovic, Hermann Koepsell, Gerhard Burckhardt, and Yohannes Hagos

Introduction

The efficacy of antineoplastic drugs is determined by the concentrations of these drugs in cancer cells (Huang, 2007). Although lipophilic antineoplastic agents may enter the tumor cell by passive diffusion, the uptake of charged, hydrophilic agents relies on the presence of transporters. By mediation of the efflux of chemotherapeutic drugs, a variety of ABC efflux transporters in drug-resistant cancer cells. These transporters have been implicated in the chemoresistance of cancer cells (Tsuij, 2002). Various factors may result in the increased expression of ABC efflux transporters in drug-resistant cancer cells. These include the altered activity of transcriptional factors, gene polymorphisms, and changes in the methylation status of promoters (Kim et al., 2006). In contrast to ABC transporters, uptake transporters may increase the intracellular concentrations of selected antineoplastic drugs and elevate the chemosensitivities of tumor cells (Shnitsar et al., 2009).

The solute carrier (SLC) superfamily is a major group of membrane transporters responsible for the uptake of nutrients, metabolites, drugs, and toxins. At present, there are 51 known SLC families with 378 genes, and these are organized on the basis of alignment similarities and substrate specificities (Hediger et al., 2004). SLC transporters undergo sequential conformational changes during the transport cycle, and some of these proteins are capable of transporting more than one substrate simultaneously (Bergeron et al., 2008). The transport of antineoplastics by SLC transporters has been reported previously for organic cation transporter 1 (OCT1) (SLC22A1) as a transporter of imatinib, oxaliplatin, and paclitaxel compared with those of OCT1-negative controls. We hypothesize that OCT1 can contribute to the susceptibility of cancer cells to selected antineoplastic drugs. In the future, an expression analysis of the transporters and the application of transporter-specific antineoplastic agents could help to tailor cancer therapy.

ABSTRACT

Antineoplastic agents directed at nuclear and cytoplasmic targets in tumor cells represent the current mainstay of treatment for patients with disseminated malignant diseases. Cellular uptake of antineoplastics is a prerequisite for their efficacy. Five of six lymphoma cell lines as well as primary samples from chronic lymphocytic leukemia patients demonstrated significant expression of SLC22A1 mRNA coding for organic cation transporter 1 (OCT1). Functionally, the antineoplastic agents irinotecan, mitoxantrone, and paclitaxel inhibited the uptake of the organic cation [3H]1-methyl-4-pyridinium iodide into OCT1-transfected Chinese hamster ovary model cells, with Kᵢ values of 1.7, 85, and 50 μM, respectively. Correspondingly, OCT1-positive cell lines and transfectants exhibited significantly higher susceptibilities to the cytotoxic effects of irinotecan and paclitaxel compared with those of OCT1-negative controls. We hypothesize that OCT1 can contribute to the susceptibility of cancer cells to selected antineoplastic drugs. The future, an expression analysis of the transporters and the application of transporter-specific antineoplastic agents could help to tailor cancer therapy.

SUPPLEMENTAL MATERIAL

The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: ABC, ATP-binding cassette; CHO, Chinese hamster ovary; CLL, chronic lymphocytic leukemia; Ct, cycle at threshold; DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDR, multidrug resistant; MPP, 1-methyl-4-phenylpyridinium; MRP, multidrug-resistance protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; LG, low glucose; MuLV, mouse leukocyte virus; OCT, organic cation transporter; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RFC, reduced folate carrier; RT, reverse transcription; SLC, solute carrier; TEA, tetraethylammonium.

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and oxaliplatin (Zhang et al., 2006), whereas OCT3 (SLC22A3) mediated the uptake of irinotecan, vincristine, and melphalan (Shnitsar et al., 2009).

The aims of this study were 3-fold: first, examine the expression of OCT1 (SLC22A1) in lymphoma cell lines and in patients with chronic lymphocytic leukemia (CLL); second, determine the potential interaction of various antineoplasics with OCT1; last, investigate the transporter-mediated cytoxicities of irinotecan and paclitaxel in cell lines and tumor cells expressing OCT1.

Materials and Methods

Reagents. Cell culture materials, including fetal calf serum, phosphate-buffered saline (PBS), RPMI 1640 medium, and Dulbecco’s modified Eagle’s medium (DMEM)-low glucose (LG), were purchased from Invitrogen (Carlsbad, CA). TRIZol reagent was obtained from Invitrogen, and mouse leukocyte virus (MuLV) reverse transcriptase was obtained from Promega (Madison, WI). Chemical reagents (analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO). Hygromycin was purchased from AppliChem (Darmstadt, Germany). [3H]-Methyl-4-pyridinium iodide (MPP) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Real-time TaqMan primers and TaqMan buffer were purchased from Applied Biosystems (Foster City, CA).

Semiquantitative Reverse-Transcription Polymerase Chain Reaction. B and T lymphoma cell lines and patient samples were used to obtain total RNA using the TRIZol reagent following the manufacturer’s guidelines. The concentration and quality of RNA were measured using a GeneQuant II spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Total RNA was isolated using the TRIzol reagent following the manufacturer’s instructions. The purity of total RNA was determined using an equal volume of acidified isopropanol. The amount of reduced MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) to purple formazan) was determined by adding 750 μl of 1 M NaOH solution to each well, followed by an incubation of 2 h at 37°C. Purple formazan crystals then were eluted in acidic isopropanol (96% isopropanol + 4% 1 N HCl). Absorbance was measured at 595 nm.

For the suspension of B lymphoma cells, 2.5 × 10^6 cells/well were seeded in round-bottom 96-well plates (Sarstedt, Nürenbrecht-Rommelsdorf, Germany) and incubated overnight. Cells were treated with substances as detailed described above and incubated at 37°C. After 15 min of incubation, cells were centrifuged (500 rpm for 10 min) and incubated in fresh medium. After 24 h of incubation, cells were pelleted again and incubated in MTT solution (750 μg/ml) in each well. Cells were incubated for 2 h at 37°C, and the reaction was stopped by adding an equal volume of acidic isopropanol. The amount of reduced MTT was assayed by measuring the absorbance at 595 nm using a Microplate Manager (model 680; Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis. All of the results are presented as mean ± S.E.M. Statistical analyses were carried out by two-way analysis of variance, and all pairwise multiple comparison procedures were
performed by the Tukey’s test as well as by the Bonferroni test. The results of the significance analysis of both tests were identical. Figures 3, 5, and 6 are generated from the two-way analysis of variance.

Results

Expression of SLC Transporter OCT1 mRNA in Lymphoma Cells. To detect OCT1 (SLC22A1) mRNA expression, we performed quantitative real-time PCR with various lymphoma cells. GAPDH expression (cycles at threshold, Ct) was used for normalization. After the standardization of the cDNA quantity by the ΔCt of GAPDH, the expression of OCT1 in lymphoma cells was subtracted from the expression of OCT1 in lymphocytes of healthy donors, yielding ΔΔCt. Figure 1A shows that OCT1 (SLC22A1) mRNA is expressed in all six lymphoma cell lines, comprising three B cell lymphoma cell lines, Karpas-422, Raji, SUDHL-4, and two T lymphoma cell lines, Jurkat and HUT-78, and one Hodgkin lymphoma cell line, L-428. Compared with that of the control lymphocytes, the expression level of OCT1 in the lymphoma cell lines, except in SUDHL-4, increased by 1.5 to 6 cycles.

In leukemia cell samples from patients with untreated CLL, we found OCT1 mRNA to exceed the expression in normal lymphocytes by an up to 4-fold difference in Ct (Fig. 1B). No expression of OCT2 (SLC22A2) and OCT3 (SLC22A3) was detected in either the lymphoma cell lines or the primary cell samples (data not shown).

To prove the expression of OCT1 on the protein level, we performed a Western blot analysis. To evaluate the specificity of the OCT1 antibody purchased from Aviva Systems Biology, we prepared plasma membranes from the CHO cells stably transfected with OCT1 and from nontransfected cells. The Western blot results showed no differentially labeled bands in the OCT1-expressing cells in comparison to mock CHO cells, suggesting that the antibody was not specific enough (Supplemental Fig. 1). A more specific antibody was not available.

Expression of ABC Transporter mRNAs in Lymphoma Cell Lines. As detected by qualitative RT-PCR, ABC transporters involved in drug efflux were expressed in lymphoma cell lines (Fig. 2), with the highest levels of ABCB1 (multidrug resistant 1 [MDR1]) in the control lymphocytes and in L-428 and HUT-78 cells. Multidrug-resistance protein 1 (MRP1) mRNA was expressed consistently in the lymphoma cell lines. SUDHL-4 demonstrated lower mRNA expression of MRP1 than the other cell lines tested. MRP2 mRNA was expressed differentially, with high expression in L-428 cells. Karpas-422 and Jurkat cells had comparable expression, whereas HUT-78 cells demonstrated relatively lower expression. Raji and SUDHL-4 cells and the control lymphocytes did not show MRP2 expression. MRP4 mRNA was expressed at very low levels in all of the cell lines.

Interaction of OCT1 with Antineoplastics. The potential interaction of different classes of antineoplastics with OCT1 was determined by the inhibition of the uptake of a radiolabeled model organic cation, [3H]MPP. The basic [3H]MPP transport rate for CHO cells stably transfected with OCT1 was 2070 ± 20 fmol/5 min, n = 10, and in nontransfected mock cells was 220 ± 2 fmol/5min, n = 10. OCT1-mediated [3H]MPP uptake was strongly reduced to 25.4 ± 3.7% (P < 0.001), 16.7 ± 3.0% (P < 0.001), and 28.5 ± 6.4% (P < 0.001) by the topoisomerase inhibitors mitoxantrone and irinotecan (Fig. 3B) and by the microtubule inhibitor paclitaxel (Fig. 3C), respectively. Ifosfamide, trofosfamide, bendamustine (Fig. 3A), etoposide (Fig. 3B), and vinblastine (Fig. 3C) inhibited MPP uptake by CHO-OCT1 cells less strongly to 55.7 ± 6.6%, (P < 0.001), 71.4 ± 6.3% (P < 0.01), 75.9 ± 6.3% (P < 0.05), 73.4 ± 4.5% (P < 0.001), and 55.4 ± 5.2% (P < 0.001), respectively. The other antineoplastics tested did not inhibit OCT1-mediated uptake significantly (Fig. 3).

The affinity of OCT1 for the three antineoplastics with the strongest inhibition of MPP uptake, irinotecan, mitoxantrone, and paclitaxel, was determined by Dixon plot analysis (Fig. 4). OCT1 showed a high affinity for irinotecan, with a Ki...
value of 1.7 μM, whereas the affinities for mitoxantrone and paclitaxel amounted to calculated \( K_i \) values of 85 and 50 μM, respectively, as indicated by the intersection of the straight lines.

Although these studies show the interactions of the selected antineoplastic drugs with OCT1, they do not prove that the antineoplastics are taken up. Therefore, we attempted to measure the uptake of \([3H]paclitaxel\) into OCT1-expressing CHO cells. After being washed three times with ice-cold PBS, a very high radioactivity background was found. We then compared the background activity of \([3H]paclitaxel\) with that of \([3H]MPP\) (background dpm) after washing. The background activity of mock-transfected CHO cells, which do not transport MPP, was only 9% for 20 nM \([3H]MPP\) contained in 5 μl of uptake buffer (Supplemental Fig. 2). In contrast, the background activities of \([3H]paclitaxel\) were 105 and 164% for 20 and 100 nM \([3H]paclitaxel\), respectively, contained in 5 μl of uptake buffer (Supplemental Fig. 2, A and B). Because paclitaxel obviously adhered to the surfaces of the cells and plastic plates, we were not able to evaluate OCT1-mediated paclitaxel transport.

**OCT1-Mediated Chemosensitivity.** In OCT1-expressing CHO cells (Fig. 5A, white bars) but not in control CHO cells (Fig. 5A, hatched bars), exposure for 15 min to 100 μM irinotecan decreased cell viability measured 24 h later by the MTT assay to 65.5 ± 4.8% (***, \( P < 0.001 \)). Simultaneous incubation of cells with irinotecan and the OCT1 inhibitor TEA significantly restored cell viability compared with treatment with irinotecan alone to 93.5 ± 6.2% (§, \( P < 0.05 \)). Similar results were obtained by simultaneous treatment of OCT1 cells with irinotecan and corticosterone, leading to viability levels of 90.7 ± 7.4%. However, the difference was not significantly different compared with treatment with irinotecan alone (Fig. 5A, N.S.). TEA and corticosterone without irinotecan had no effect on the cell viability of OCT1-expressing CHO cells. CHO cells without OCT1 were unaffected by any treatment (Fig. 5A, hatched bars).

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**Fig. 2.** RT-PCR screening of ABC transporters in lymphoma cell lines. A qualitative expression profiling of efflux transporters was performed by RT-PCR and visualized with ethidium bromide on agarose gels. K, Karpas-422; R, Raji; S, SUDHL-4; J, Jurkat; H, HUT-78; L, L-428; HP-1, healthy person sample-1; HP-2, healthy person sample-2; +, positive control; −, negative control.

**Fig. 3.** Inhibition of OCT1-mediated MPP uptake by several antineoplastics. A–D, \([3H]MPP\) (20 nM \([3H]MPP\) plus 980 nM unlabeled MPP) uptake by human OCT1 stably expressed in CHO cells was measured in the presence of 100 μM of different antineoplastics: alkylating drugs (A), topoisomerase inhibitors (B), microtubule inhibitors (C), and antimetabolites (D). Data are means ± S.E.M. of three independent experiments with four repeats each. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).
L-428 cells expressing a high level of OCT1 mRNA (Fig. 1A) demonstrated a decrease of cell viability of 14.3 ± 2.5% \((P < 0.001)\) with irinotecan treatment (Fig. 5B, black bars, **). On the simultaneous exposure of cells with irinotecan and TEA, L-428 cells showed a viability decrease of 13.4 ± 2.3% \((P < 0.001)\) compared with that of untreated cells. When irinotecan and corticosterone were applied together, L-428 cells demonstrated a decrease of cell viability of 12.9 ± 2.3% \((P < 0.01)\). Thus, in contrast to OCT1-expressing CHO model cells, TEA and corticosterone did not restore the irinotecan-induced cytotoxicity in L-428 cells. SUDHL-4 cells, which did not express OCT1, were not affected by treatment with irinotecan under any condition tested (Fig. 5B, hatched bars).

Treatment of OCT1-expressing CHO model cells with 100 \(\mu M\) paclitaxel decreased cell viability (white bars) to 57.3 ± 4.2% \((P < 0.001)\). Nonexpressing CHO cells were not affected by paclitaxel. This decrease was significantly reversed on simultaneous treatment with paclitaxel and TEA or paclitaxel and corticosterone to 87.9 ± 3.9 and 86.3 ± 3.8%, respectively. In comparison with CHO-OCT1 cells treated with paclitaxel alone, the observed reversal with TEA as well as corticosterone was 30% and statistically significant (Fig. 6A; §§§, \(P < 0.001\)). The cell viability of OCT1-expressing CHO cells treated with paclitaxel plus TEA or corticoste-
Successful cancer therapy depends on the effective uptake of chemotherapeutic agents into tumor cells. Early in the 1960s, murine leukemia L5178 cells were described as methotrexate-resistant cells. The resistance was due to the low capability of L5178Y cells to transport methotrexate (Fischer, 1962). Meanwhile, it is clear that the uptake of methotrexate into the cells is mediated by reduced folate carrier (RFC) (for a review, see Matherly et al., 2007). Low expression of RFC results in decreased chemosensitivity. Transcriptional and posttranslational down-regulation of transporters could lead to the resistance of tumor cells to methotrexate, as demonstrated by the methylation and polymorphism of RFC in osteosarcoma cells (Yang et al., 2008). Activation of protein kinase Cα stimulated by antiepileptic drugs such as phenobarbital induced the internalization and subsequent proteasomal degradation of RFC. Consequently, the uptake of methotrexate in tumor cells decreased as demonstrated by drug resistance in acute lymphatic leukemia patients (Halwachs et al., 2009, 2011).

Because uptake transporters play a role in chemosensitivity as described above, OCTs may serve such a purpose, because they have a broad substrate specificity (Koepsell et al., 2007). These transporters are expressed in many tissues, including kidney, liver, and blood-brain barrier. Transporting positively charged endogenous (e.g., epinephrine and noradrenaline) and exogenous substrates (e.g., MPP, TEA, and metformin) (Koepsell et al., 2007), OCTs are involved in drug distribution (Shang et al., 2003). Cisplatin, a platinum-based antineoplastic, is specifically transported by OCT2 (Ciarimboli et al., 2005), whereas OCT6 transports the antineoplastic doxorubicin (Okabe et al., 2005). We recently demonstrated the involvement of OCT3 in the uptake of the antineoplastics irinotecan, melphalan, and vincristine as well as the OCT3-mediated cytotoxicity of these drugs in renal carcinoma cell lines (Shnitsar et al., 2009).

In the present study, we found that OCT1 mRNA is expressed in lymphoma cell lines at higher levels than in lymphocytes from healthy donors. In CLL patient samples, OCT1 mRNA was expressed at higher levels than in control lymphocytes, making this transporter a prime candidate for transporter-specific tumor therapy with antineoplastic substances. However, the expression of OCT1 at the protein level could not be proven, because of a lack of a specific antibody for human OCT1.

Because little was known about the interaction of OCT1 with antineoplastic agents, we tested their inhibitory potentials on OCT1-mediated [3H]MPP uptake. We selected 20 substances covering the major groups of antineoplastics used in chemotherapy. At 100 μM each, irinotecan, mitoxantrone, and paclitaxel inhibited OCT1-mediated [3H]MPP uptake by >50%. This is the first time that the interaction of irinotecan, mitoxantrone, and paclitaxel has been shown for OCT1. Thereby, OCT1 showed a high affinity for irinotecan ($K_i = 1.7$ μM) and an intermediate affinity for paclitaxel and mitoxantrone ($K_i = 50–85$ μM). Kinetically, the observed inhibition was noncompetitive, leaving open whether these antineoplastics are actually transported by OCT1 or only block its action. To examine whether one of the antineoplastics is transported by OCT1 into CHO cells, we performed uptake studies with radioactively labeled paclitaxel. Because paclitaxel heavily bound to the cells and the plastic plates, it was not possible to calculate OCT1-mediated uptake. A very high binding capacity of radioactively labeled paclitaxel on platelets (672 pmol/10⁹ platelets) was reported by Wild et al.
We calculated the binding capacities of CHO-mock cells for 20 and 100 nM [3H]paclitaxel to be 62.5 and 510.9 pmol/10⁹ cells, respectively. In contrast, the binding capacity of 20 nM [3H]MPP was only 4.4 pmol/10⁹ CHO-mock cells. Heavy binding did not allow us to prove the transport of [3H]paclitaxel.

MTT assays were carried out to test the impact of OCT1 expression on the sensitivity of CHO model cells and lymphatic tumor cells to irinotecan and paclitaxel. In these experiments, cells were exposed to the antineoplastics for only 15 min to minimize uptake by diffusion and to focus on OCT1-mediated drug uptake. Exposure to antineoplastics for 30 to 120 min led to the massive destruction of OCT1-expressing cells and control cells after subsequent overnight incubation at 37°C. Therefore, we could not generate reproducible cell viability results by the MTT assay at incubation periods exceeding 15 to 30 min.

The topoisomerase I inhibitor irinotecan is used as a first-line therapy in metastatic colorectal cancer in combination with fluorouracil and leucovorin (Saltz et al., 2000). As opposed to control cells not expressing OCT1, cell proliferation was significantly inhibited in OCT1-expressing CHO cells treated with irinotecan. When the activity of OCT1 was blocked by an excess of the substrate TEA or the addition of the nontransported inhibitor corticosterone (Wessler et al., 2001), irinotecan failed to reduce cell viability in OCT1-expressing CHO cells. These results are compatible with the assumption that irinotecan is taken up by OCT1, leading to reduced cell viability in OCT1-expressing cells.

To test whether irinotecan could be used in the treatment of lymphomas expressing OCT1, we performed similar experiments using the lymphoma cells L-428 with high OCT1 mRNA expression and SUDHL-4 cells showing no expression of OCT1 mRNA. MTT tests demonstrated lowered cell viability of irinotecan-treated L-428 cells but not of SUDHL4 cells. Compared with OCT1-expressing CHO model cells, L-428 cells were less affected by irinotecan. The reduced antineoplastic effect in these cells could be due to the expression of efflux ABC transporters such as MDR1 and MRPs (Tofolli et al., 2003). Indeed, by RT-PCR, we found high expression of MDR1, MRP1, and MRP2 in L-428 cells. As opposed to OCT1-expressing CHO cells, TEA and corticosterone did not reduce the small antineoplastic effect of irinotecan on L-428 cells. Thus, the role of OCT1 in conferring sensitivity of L-428 cells to irinotecan is not clear at present.

OCT1-mediated [3H]MPP uptake also was inhibited in CHO cells by the addition of paclitaxel to the transport buffer. Paclitaxel is active against a broad range of cancers, such as lung cancer and head and neck cancer. It is used in palliative therapy of ovarian and breast cancers resistant to chemotherapy (Rowinsky and Donehower, 1995).

OCT1-expressing CHO cells incubated with paclitaxel demonstrated significantly reduced cell viability. To confirm the OCT1 activity-dependent action of paclitaxel, we incubated cells with paclitaxel in the presence of TEA and corticosterone. Cell viability was not reduced by TEA or corticosterone alone, but both OCT1 inhibitors completely reversed the cytotoxic effect of paclitaxel in OCT1-expressing CHO cells.

The lymphoma cell lines L-428 and SUDHL-4 also were tested for OCT1-mediated cytotoxicity of paclitaxel. OCT1-expressing L-428 cells demonstrated reduced cell viability, whereas SUDHL-4 cells, which do not express OCT1, were not affected. The reduced cell viability was significantly reversed upon the application of the OCT1 substrate TEA together with paclitaxel. The OCT1 inhibitor corticosterone also reversed the lowered cell viability, suggesting that OCT1 mediates the uptake and cytotoxic activity of paclitaxel not only in OCT1-expressing model cells but also in the lymphoma cell line L-428.

Because OCT1-expressing cells are more sensitive to irinotecan and paclitaxel than nonexpressing cells, we hypothesize that the use of antineoplastics transported by OCT1 could help to improve targeting of these drugs. The efficiency of OCT1 will depend on its affinity for antineoplastics and the maximal transport rate as well as on the role of simple diffusion and the activities of efflux ABC transporters. Furthermore as described previously for RFC, transcriptional, posttranscriptional, and posttranslational regulation may well determine the efficiency of OCT1 in the chemosensitivity.

The affinity of OCT1 for irinotecan (Kᵣ = 1.7 μM) is in the same range as the maximal plasma concentration (2.9 μM at a dosage of 350 mg/m²) (Chabot et al., 1995). For paclitaxel, the free plasma concentration (0.85 μM at a dosage of 175 mg/m²) (Sonnichsen and Relling, 1994) is much lower than the Kᵣ (50 μM). If, however, the maximal uptake rate (V_max) for paclitaxel were high enough, then OCT1 could still improve the sensitivity of the cells to this antineoplastic. A high membrane permeability of the antineoplastics would counteract OCT1-mediated uptake by quick equilibration of intracellular and extracellular drug concentrations, which is one possible reason for the relatively small effects of OCT1 expression on the sensitivity toward antineoplastics. Another reason is the expression of efflux ABC transporters in most of our cells. Inhibiting the efflux ABC transporters could potentiate the susceptibility of cancer cells to antineoplastics that are transported by OCT1.

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Authorship Contributions

Participated in research design: Gupta, Bureckhardt, and Hagos.
Conducted experiments: Gupta and Henjakovic.
Contributed new reagents or analytic tools: Wulf, Koepsell, and Bureckhardt.
Performed data analysis: Gupta and Hagos.
Wrote or contributed to the writing of the manuscript: Gupta, Wulf, Koepsell, Bureckhardt, and Hagos.

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Figure 1

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