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Association of *ABCC10* polymorphisms with nevirapine plasma concentrations in the German Competence Network for HIV/AIDS

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Abstract

Background—Nevirapine exhibits marked interpatient variability in pharmacokinetics. CYP2B6 activity and demographic factors are important, but there are a few data on drug transporters for nevirapine. ABCC10 (MRP7) is an efflux transporter highly expressed in liver, intestine, and peripheral blood cells. We investigated whether nevirapine is a substrate for ABCC10 and whether genetic variants contribute to variability in nevirapine plasma concentrations.

Methods—Accumulation of nevirapine was assessed in parental and ABCC10-transfected HEK293 cells (HEK293-ABCC10), CD4+ cells, and monocyte-derived macrophages from healthy volunteers (n =8). ABCC10 small interfering RNA studies were also conducted. DNA samples with paired plasma drug concentrations were available from 163 HIV-infected patients receiving nevirapine-containing regimens. Sequenom was used to screen 14 single nucleotide polymorphisms in *ABCC10*. Linear regression models were used to identify factors independently associated with nevirapine plasma concentration.

Results—Nevirapine accumulation was 37% lower in HEK293-ABCC10 cells compared with parental HEK293 cells (P =0.02), and this was reversed by cepharanthine (an ABCC10 inhibitor). After small interfering RNA knockdown of ABCC10, there was an increase in accumulation of

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nevirapine in CD4 cells (32%; $P = 0.03$) and monocyte-derived macrophages (38%; $P = 0.04$). Marked differences in the haplotype structure of *ABCC10* was observed between White and Black patients in the cohort. In Whites, an exonic single nucleotide polymorphism (rs2125739) was significantly associated with nevirapine plasma concentration ($P = 0.02$). Multivariate regression analysis identified carriage of a composite genotype of *ABCC10* rs2125739 and *CYP2B6* 516G > T ($P = 0.001$), time post dose ($P = 0.01$) and BMI ($P = 0.07$) to be independently associated with nevirapine plasma concentrations.

Conclusion—Nevirapine is a substrate for ABCC10 and genetic variants influence its plasma concentrations. ABCC10 in lymphocytes and macrophages may also contribute to variability in intracellular permeation of nevirapine. Further studies are required to determine the clinical implications of these findings.

Keywords

ABCC10; CD4+ lymphocyte; drug efflux transporter; HIV; monocyte-derived macrophage; nevirapine; single nucleotide polymorphism

Introduction

The non-nucleoside reverse transcriptase inhibitor nevirapine is widely prescribed in multidrug regimens to treat HIV-1 infection. Nevirapine has a long plasma half-life and a low barrier to viral genetic resistance with a single mutation in HIV-1 reverse transcriptase being sufficient to confer resistance [1]. The metabolism of nevirapine by CYP2B6, and to some extent CYP3A, has been well-described in the literature and genetic variability in the *CYP2B6* gene explains some of the variability in nevirapine pharmacokinetics [2–4]. However, the role of drug transporters in the disposition of nevirapine is less well understood. Previous work has suggested that nevirapine efflux from peripheral blood cells is independent of ABCB1 transport but other members of the ABCC family (ABCC1/2) may be involved [5].

The ABC transporter family is the largest family of drug efflux transporters and uses the energy generated from ATP hydrolysis to transport substrates across cellular membranes. Multidrug resistance-associated proteins (ABCC) are a subfamily of ABC transporters of which there are nine ABCC proteins. ABCC10 (MRP7) has been understudied until recently [6]. ABCC10 transports nucleoside analogues used in cancer chemotherapy and this can be reversed using the ABCC10 inhibitor, cepharanthine [7]. ABCC10 has been demonstrated to have wide tissue distribution at relatively high expression including in cells of the immune system [8].

To date, the only antiretrovirals that have been demonstrated to be ABCC10 substrates are ddC (zalcitabine) [9] and tenofovir [10]. Using cell lines expressing ABCC10, we sought to determine whether other antiretrovirals are substrates. We also investigated the expression of ABCC10 in specific immune cell subsets and used ABCC10-specific small interfering RNA (siRNA) to determine whether inhibition of ABCC10 increases intracellular accumulation of antiretroviral drugs. Finally, we also used high-throughput genotyping using Sequenom MALDI-TOF technology to investigate whether genetic variation in *ABCC10* contributes to variability in nevirapine plasma concentrations in HIV+ patients recruited as part of the German Competence Network for HIV/AIDS.

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Methods

Materials

Radiolabeled nevirapine, lopinavir, atazanavir, and efavirenz were purchased from Moravek Biochemicals (California, USA). Radiolabeled darunavir was a gift from Tibotec (Beerse, Belgium). HEK293 and HEK293-ABCC10 (ABCC10-expressing cells were designated C17 and C18) cells and the ABCC10 primary antibody were as described previously [11]. Healthy volunteer buffy coats were obtained from the National Blood Service (Liverpool, UK). CD4+ and CD14+ magnetic beads, macrophage colony stimulating factor, and transforming growth factor-β were purchased from Miltenyi Biotec (Surrey, UK). Cepharanthine (ABCC10 inhibitor) was purchased from Aktin Chemicals (Chengdu, Peoples Republic of China). ABCC10, GAPDH-positive control, and nontargeting negative-control siRNA were purchased from Dharmacon (Thermo Fisher, UK). Lipofectamine RNAiMAX was purchased from Invitrogen (Paisley, UK). A taqman gene expression assay for ABCC10 mRNA expression and Taqman Gene Expression master mix were purchased from Applied Biosystems (Warrington, UK). Sequence specific PCR primers and extend reaction oligonucleotides were obtained from Metabion GmbH (Martinsried, Germany).

Accumulation of radiolabeled antiretrovirals in ABCC10-expressing cell lines

Radiolabeled drugs were made up to 10 μmol/l with the corresponding nonradiolabeled drugs and were added to parental HEK293 and HEK293-ABCC10 cells and incubated for 30 min at 37°C. All incubations contained 0.3 μCi/ml radioactivity and were conducted in the presence and absence of cepharanthine (2 μmol/l; ABCC10 inhibitor). Samples were centrifuged at 9000 rpm for 1 min at 4–8°C and a supernatant sample (100 μl) representing the extracellular count taken and placed into a scintillation vial. Pellets were resuspended in 1 ml of ice-cold Hanks balanced salt solution (HBSS), centrifuged, resuspended in 100 μl of water to solubilize the cell pellet and transferred into a separate scintillation vial. Scintillation fluid (4 ml) was added to each vial, which were then placed in a scintillation counter. Data were expressed as the ratio of intracellular drug to extracellular drug assuming a cell volume of 1 pl for calculating the cellular accumulation ratio (CAR).

Separation of peripheral blood mononuclear cells from whole blood, isolation of subsets, and preparation of monocyte-derived macrophages

Healthy volunteer blood was layered over Ficoll-Paque (2 : 1 ratio) and centrifuged at 2000 rpm for 30 min (4°C). The interface layer containing peripheral blood mononuclear cells (PBMC) was then removed and washed with HBSS. PBMC samples were resuspended in Roswell Park Memorial Institute-1640 media (15% fetal calf serum) and placed in an incubator for 24 h (37°C, 5% CO_2) in order for monocytes to adhere. After incubation, media containing unadhered cells was removed to a universal tube and trypsin–EDTA was added to the culture flask to detach adhered monocytes. Cells were washed and resuspended in MACS buffer [phosphate-buffered saline (PBS), 2 mmol/l of EDTA, 0.5% bovine serum albumin]. Magnetic beads specific for cell lineage markers were added to lymphocyte samples and anti-CD14 beads were added to monocyte samples. Incubations and washes were conducted according to manufacturers' recommended guidelines. Cells were counted and resuspended to 2.5×10^6/ml in Roswell Park Memorial Institute-1640 media. Aliquots of CD14+ cells (2.5×10^6 cells) were resuspended in Iscove's Modified Dulbecco's Media (20% fetal calf serum) containing macrophage colony stimulating factor (10 ng/ml) and transforming growth factor-β (10 ng/ml) to promote differentiation into monocyte-derived macrophages (MDMs). Cells were cultured for 12 days, replenishing media every 3 days. Expressions of CD68, CD15, and CD89 were determined by flow cytometry to confirm lineage of the cells. In brief, cells (2×10^6) were trypsinized from culture flasks and washed twice in PBS before being resuspended in PBS containing antibodies for either CD68 (1 :

40), CD15 (1 : 40, FITC conjugated), or CD89 (1 : 20, phycoerythrin conjugated) and incubated at 4°C for 60 min. Samples were then washed three times in ice-cold PBS and if necessary incubated with PBS containing secondary antibody (1 : 400, phycoerythrin conjugated) for 60 min at 4°C. Cells were then washed before resuspension in CellFix for analysis on a Coulter Epics XL flow cytometer.

Expression of ABCC10 mRNA in immune cell subsets and human tissues

After magnetic bead isolation, total RNA was extracted from cells (2×10^6) using Tri reagent according to the manufacturer's protocols. RNA (2 μg) from the immune subset samples and human tissue panel were then reverse transcribed into cDNA and real-time quantitative PCR was performed on cDNA (40 ng). Thermal cycling conditions consisted of 15 min at 95°C followed by 50 cycles of 15 s at 95 and 60°C. Samples were then held at 4°C. Quantification of PCR products occurred in real time. Expression data were normalized to GAPDH using the comparative ΔΔCt method consisting of 2 raised to the power of the difference in the cycle threshold (Ct) between the reference gene (GAPDH) and the test gene.

RNAi-mediated knockdown of ABCC10 expression in primary CD4+ cells, CD14+ monocytes, and monocyte-derived macrophages

Pools (n=4) of siRNA duplexes specific for ABCC10 mRNA were purchased from Dharmacon along with relevant nontargeting (negative) and GAPDH-specific (positive) controls. siRNA was transfected into CD4+, CD14+, and MDMs using RNAimax (Invitrogen) under optimum conditions. The expression of ABCC10 mRNA was determined using real-time quantitative PCR as described above but relative to β-actin (Assay ID: 4352935E) expression as GAPDH was used as a positive siRNA control. Protein expression for ABCC10 was determined by flow cytometry essentially as described previously [12] but using ABCC10 primary antibody and isotype control (IgG) at 1 : 40 dilutions. A goat anti-mouse, FITC-conjugated secondary antibody was used at a 1 : 200 dilution.

Accumulation of nevirapine in immune cell subsets

CD4+, CD14+, and MDM cells (2×10^6) were incubated with nevirapine (10 μmol/l, 0.33 μCi/ml) for 30 min in complete media. Cells were then centrifuged (9000 rpm, 1 min) and an extracellular aliquot (100 μl) taken for liquid scintillation counting. Cells were then washed in ice-cold HBSS and pelleted (9000 rpm, 1 min) before being resuspended in water (100 μl). The CAR values were then calculated as described above.

Assessment of differential drug sensitivity to nevirapine in ABCC10-expressing cell lines

Cells were incubated in media containing a range of nevirapine concentrations (0.97–500 μmol/l) for periods of 3 and 7 days. Adefovir dipivoxil (0.97–500 μmol/l) was used as a positive control having been previously shown to be an ABCC10 substrate [9]. Drug sensitivity assays were carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described previously [9] and determination of cellular ATP content by the Cell Titer Glo Luminescent Assay. In brief, Cell Titer Glo substrate was added to cells in 96-well plates and incubated at room temperature for 30 min. Luminescence was then measured and IC_{50} values calculated from data obtained by MTT and ATP assaya using the Graphpad Prism software (version 5.0; GraphPad Software, Inc., California, USA).

Determination of the presence of nevirapine metabolism in ABCC10-expressing cell lines

NVP, standards of two known metabolites of NVP (3-OH NVP and 12-OH NVP) were gifts from Pfizer Global Research and Development (Sandwich, Kent, UK).

The liquid chromatography–mass spectrometry system used to assay NVP and its metabolism consisted of a pump and autosampler model Surveyor, and LCQ DecaXP ion trap detector (Thermo Fisher, UK). Chromatographic separation was performed at 37°C using a column oven, on Hypersil Gold (100 × 2.1 mm i.d., Thermo Fisher, UK), protected by a Column Saver with pre-column (Thermo Fisher, UK). The mobile phase consisted of solution A [high-performance liquid chromatography (HPLC) grade water 95%-acetonitrile 5% + 0.05% formic acid] and solution B (HPLC grade water 20%-acetonitrile 80% + 0.05% formic acid) and chromatographic run was performed at 0.4 ml/min using a gradient. At the start of the run the 100% of solution A was gradually increased to 80% in 3 min and then to 50% in 3.5 min. This condition was held for 1.5 min, after which the percentage of solution A was increased up to 100% and held for 4 min for a total run time of 6.5 min. Detector settings were electrospray ionization-positive polarity ionization, spray voltage (5 kV), capillary voltage (6 V), capillary temperature (360°C), shealth gas flow rate (65 l/h), and nitrogen cone flow (100 l/h). The main parent ion of NVP was isolated at 267 (m/z) with collision energy of 40 V and the main daughter ion was detected at 226 (m/z). NVP 3-OH was isolated at 283 (m/z) with collision energy of 35 V and the main daughter ion was detected at 242 (m/z). NVP 12-OH was isolated at 283 (m/z) with collision energy of 37 V and the main daughter ion was detected at 265 (m/z). Acetonitrile (150 ml) was added to 2×10^6 cells. After vortexing for 10 s, the mixture was centrifuged at 12 000 rpm for 10 min at 4°C. Supernatant was transferred into glass tube and treated by vortex–vacuum evaporation to dryness at 40°C. Each extract was reconstituted with 150 μl solution A and 20 μl were injected into the column.

Patients

The characteristics of the study population have been previously described ([3]). In brief, the study population was drawn from a cohort of HIV-infected White and Black patients from the German Competence Network for HIV/AIDS. Local research ethics committee approvals were obtained for the study. Written informed consent was obtained from all patients for collection of blood/DNA samples and subsequent genetic analysis. The study cohort consisted of a total of 163 HIV-infected patients (98 White; 65 Black) on nevirapine-containing HIV therapy for at least 3 months with an age range of 22–82 years (median: 41 years).

Quantification of plasma nevirapine concentrations

Plasma obtained from blood samples was heat inactivated, and nevirapine concentrations were determined (median time post dose was 10 h) using HPLC with UV-detection using previously validated assays as described elsewhere [13]. The Liverpool Laboratory participates in an external quality assurance scheme (KKGT, Nijmegen, The Netherlands).

Gene/single nucleotide polymorphism selection

A total of 14 single nucleotide polymorphisms (SNPs) in *ABCC10* were selected for screening which included seven haplotype tagging SNPs (htSNPs) from the HapMap dataset (Phase II, Release 23a, March 2008, Whites with Northern and Western European ancestry) using the Haploview software (http://www.broad.mit.edu/haploview/haploview). The criteria used for SNP selection included a minor allele frequency of more than 5% and pairwise linkage disequilibrium measure of $r^2 > 0.8$. Marker coverage for *ABCC10* was extended by 5000 base pairs upstream and downstream flanking regions to include any potential regulatory SNPs. Apart from the htSNPs, seven additional SNPs from the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP/), which were either exonic or mapped to untranslated or conserved regions, and hence potentially functionally important, were selected.

Genotyping

DNA was extracted from PBMCs using a QIAamp DNA Mini Kit (Qiagen, West Sussex, UK). SNP genotyping was performed using a Sequenom MassARRAY MALDI-TOF system (San Diego, California, USA). Sequence specific PCR primers and extend reaction oligonucleotides for each SNP was obtained from Metabion GmbH (Martinsried, Germany). A multiplex SNP assay (14 plex) was designed using software made available by Sequenom (https://mysequenom.com/tools/genotyping/default.aspx) for genotyping utilizing iPLEX chemistry with 10 ng of genomic DNA. For each assay, quality control procedures performed included use of replicate samples and negative template controls on the same 384-well plate. Each SNP genotype cluster plot was manually checked and scored. Any sample with a call rate of less than 80% and any assay with a call rate of less than 90% were removed from subsequent analysis.

Statistical analysis

Distribution of the data was assessed using the Shapiro–Wilk test. Differences in accumulation of nevirapine between the parental HEK293 and the ABCC10-expressing cells, differences in expression between siRNA-mediated knockdown samples and untreated samples, and differences in accumulation of nevirapine between immune cell subsets after siRNA-mediated knockdown of ABCC10 were assessed using the Mann–Whitney test. A P value of less than 0.05 was considered statistically significant. The Haploview software was used to investigate the haplotype structure of *ABCC10* in both Whites and Blacks who were represented in the German Competence Network for HIV/AIDS and to test the Hardy–Weinberg equilibrium. The parameters used for linkage disequilibrium analysis are D′ and r^2 [14]. Univariate statistical analysis was conducted by linear regression (continuous data). Log transformation of nevirapine plasma concentrations was performed to improve normality of the data. Association between each SNP and log transformed plasma nevirapine concentrations were analyzed using one-way analysis of variance (Parametric test). Multivariate analysis was conducted by linear regression using SPSS, v16.0 (SPSS Inc., Chicago, Illinois, USA) to identify independent predictors of nevirapine plasma concentration.

Ethics

Informed consent was obtained from the patients, and all clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki. The ethics committee of University of Bochum approved this study and issued an ethics approval statement.

Results

Accumulation of radiolabeled antiretrovirals in ABCC10-expressing cell lines

There was no significant difference between the accumulations of efavirenz, lopinavir, atazanavir, or darunavir in the parental HEK293 and the ABCC10-expressing cells (data not shown). There was a significant difference in the accumulation of nevirapine between the parental and ABCC10 cell lines C17 (21% lower than parental CAR; P=0.03; Fig. 1) and C18 (37% lower than parental CAR; P=0.02; Fig. 1). The efflux of nevirapine by ABCC10 was confirmed by the observed reversal by 2 μmol/l of cepharanthine (Fig. 1). To ensure that these observations were not compromised by differences in cellular metabolism of nevirapine, metabolism was assessed using mass spectrometry. The 3-OH and 12-OH metabolites of nevirapine were undetectable after 30-min incubation with ABCC10 cell lines (supplementary Fig. 3; Supplemental digital content 3, http://links.lww.com/FPC/A345).

Cytotoxicity of nevirapine and adefovir in ABCC10-expressing cell lines

In addition, drug sensitivity assays were conducted to assess whether ABCC10 influenced cytotoxicity of nevirapine. Adefovir dipivoxil was used as a positive control. ABCC10 was confirmed to influence the IC_{50} of adefovir being significantly higher in HEK293-C17 (7.62 μmol/l, $P = 0.04$) and HEK293-C18 (7.88 μmol/l, $P = -0.04$) compared with parental control cells (2.46 μmol/l) after 7 days incubation (supplementary Fig. 1a, c and e; Supplemental digital content 1, http://links.lww.com/FPC/A343) as determined by ATP assay. Similar results were obtained using MTT assay (supplementary Fig. 2a, c and e) showing HEK293-C17 and HEK293-C18 having a greater IC_{50} than HEK293 cell (6.36, 6.09, and 1.801 μmol/l, respectively). However, no significant toxicity was exhibited by nevirapine incubation with either ATP assay (supplementary Fig. 1b, d and f; Supplemental digital content 1, http://links.lww.com/FPC/A343) or MTT assay (supplementary Fig. 2b, d and f; Supplemental digital content 2, http://links.lww.com/FPC/A344).

Accumulation of nevirapine in CD4+ and monocyte-derived macrophages

The accumulation of nevirapine was assessed in MDM and CD4+ cells in the presence and absence of cepharanthine. Cepharanthine reduced the intracellular accumulation of nevirapine (Fig. 2) by 28% (P=0.03) and 30% (P=0.03) in CD4+ cells and MDMs, respectively (in contrast to the hypothesis). Nevirapine accumulation was similarly reduced by cepharanthine in CD14 cells but this was not significant. To address the potential lack of specificity of cepharanthine, siRNA mediated knockdown of ABCC10 was used to specifically 'inhibit' ABCC10 efflux as described previously [15]. After ABCC10 knockdown, nevirapine intracellular accumulation was increased by 32% (P=0.03) and 38% (P=0.04) in CD4+ cells and MDMs respectively when compared with untreated cells (Fig. 3b and d, respectively).

Haplotype structure of *ABCC10* gene in the German Competence Network for HIV/AIDS cohort

The study cohort was comprised of two different ethnicities (98 Whites and 65 Blacks). Therefore, the haplotype structure for *ABCC10* was investigated in these two ethnic groups using Haploview. *ABCC10* haplotype structure differed between Whites (Fig. 4a) and Blacks (Fig. 4b) in the study cohort. Minor allele frequencies for all *ABCC10* SNPs in Whites and Blacks in the study cohort are given in Supplementary Table 1 (Supplemental digital content 4, http://links.lww.com/FPC/A346).

Association of *ABCC10* single nucleotide polymorphisms with nevirapine plasma concentration

All SNP markers genotypes except rs2185631 were in Hardy–Weinberg equilibrium. Univariate regression analysis identified an exon 12 SNP marker, rs2125739, to be significantly associated with nevirapine plasma concentration in the whole cohort (R^2=0.05; P=0.04) and in White patients (R^2=0.07; P=0.02); however, none of the *ABCC10* SNPs were associated with nevirapine plasma concentration in Black patients. The analysis of log-transformed plasma nevirapine concentrations identified patients who were homozygous for the variant C allele of rs2125739 to show significantly lower nevirapine plasma concentrations in the whole cohort (4212 ng/ml; P=0.04) and in White patients (4176 ng/ml; P=0.004) than those that were heterozygous (whole cohort: 5931 ng/ml; Whites: 6185 ng/ml) or homozygous for the T allele (whole cohort: 5856 ng/ml; Whites: 5968 ng/ml; Fig. 5). Homozygosity for the variant allele also resulted in lower nevirapine plasma concentrations in Black patients (CC genotype: 4616 ng/ml; TC: 5270 ng/ml; TT: 5263 ng/ml), but was not statistically significant.

Predictors of nevirapine plasma concentration in HIV+ patients

Multivariate analysis by linear regression (step-wise model) was utilized to identify independent predictors of nevirapine plasma concentration in HIV+ patients represented in this cohort. We have previously reported a significant gene-dose effect between *CYP2B6* 516G > T and nevirapine plasma concentration in the same cohort [3]. Therefore, we explored the combined contribution of *CYP2B6* 516G > T SNP and *ABCC10* rs2125739 (composite genotype) on nevirapine plasma concentration as part of this multivariate analysis. Other covariates included in this model were sex, age, time post nevirapine dose, BMI, history of smoking, as well as alcohol use and ethnicity. Carriage of composite genotype of rs2125739 and G516T SNPs (R^2=0.09; P=0.001), time post dose (R^2=0.12; P=0.01), and age (R^2=0.18; P=0.03) were found to be independently associated with nevirapine plasma concentration.

Discussion

Plasma concentrations of nevirapine exhibit large interpatient variability [16,17]. Although lower nevirapine plasma concentrations are associated with reduced viral suppression [18,19], higher concentrations of the drug are implicated in toxicity and also the development of non-nucleoside reverse transcriptase inhibitor-resistant mutations in women who receive single-dose nevirapine for prevention of mother-to-child transmission [20]. Ethnicity, sex, body weight, underlying disease condition, and genetic factors are all associated with nevirapine plasma concentrations [21–23]. Association between polymorphisms in the *CYP2B6* gene and nevirapine plasma concentrations are well documented [3,4,24]. However, this association is not sufficient to explain all the pharmacokinetic variability seen in patients. Drug transporters expressed in key target tissues may have a role in drug intake and efflux, but little is known about the transporters that influence the disposition of nevirapine. Nevirapine differentially regulates the expression of ABCB1, ABCC1, ABCC2, and ABCC3 *in vitro* [25], but no study has definitively shown that nevirapine is a substrate for any transporter. With total nevirapine plasma concentrations related to both viral response and drug toxicity [18,19,26,27], investigation of factors having an impact on the intracellular and plasma concentrations of nevirapine is warranted. ABCC10 is a recently characterized transporter protein and its ubiquitous expression provided a rationale to explore its contribution to the transport of antiretroviral drugs including nevirapine.

The *ABCC10*-expressing embryonic kidney cell lines, C17 and C18, were used to establish that nevirapine is a substrate for ABCC10; the same in-vitro models have previously been used to characterize the transport of tenofovir and various other substrates of ABCC10 [9,11,28]. ABCC10 expressing C17 and C18 cells showed significantly lower nevirapine accumulation and the ABCC10 inhibitor, cepharanthine, reversed this. To confirm that these observations were not compromised by cellular metabolism of nevirapine, mass spectrometry was used. No metabolites of nevirapine were found to be present in these cells (supplementary Fig. 3; Supplemental digital content 3, http://links.lww.com/FPC/A345). ABCC10 showed high expression in immune cell subsets; hence the contribution of ABCC10 to nevirapine transport was assessed in CD4+ cells and MDMs using ABCC10-specific siRNA. In addition, the use of CD4+ cells and MDMs allowed easy assessment of the role of transporters in a HIV replication competent cell system. Cepharanthine resulted in a significant decrease in the accumulation of nevirapine in CD4+ and MDM cells (Fig. 2); and we hypothesize that although cepharanthine is a potent inhibitor of ABCC10, it may also inhibit the activity of influx transporters expressed in these cells. This phenomenon was also observed in our previous study of the interaction of cepharanthine with tenofovir [10], lending weight to the hypothesis that cepharanthine is able to inhibit as yet uncharacterized transporters in these cells. Intracellular accumulation of radiolabelled nevirapine was

significantly increased in siRNA-treated immune cells in comparison with untreated controls. The differences in the accumulation of nevirapine in the siRNA-treated primary cells are relatively small but are higher than differences observed with known pharmacological transport inhibitors [5]. Therefore, ABCC10 appears to be a contributing factor rather than a major determinant of cell-associated nevirapine. However, it should be noted that it was not possible to determine the intracellular unbound nevirapine concentration, which may be influenced to a larger extent. Also of interest, nevirapine has previously been shown to inhibit the activity of ABCC1, ABCC2 and ABCC3 [25], which have all been demonstrated to be expressed in peripheral immune cells [8,12,29]. Nonetheless, the experimental evidence obtained in this study confirms a role for ABCC10 in the efflux transport of NVP. Efavirenz, darunavir, lopinavir, and atazanavir were not found to be substrates of ABCC10.

There is a marked interindividual variability in the plasma concentrations of nevirapine in patients taking the same dose [21,30]. This is partially explained by genetic variability in the *CYP2B6* gene [3,4,24]. However, given the role of ABCC10 in nevirapine transport, we investigated whether SNPs in *ABCC10* contribute to variability in nevirapine plasma concentration. An exonic SNP in ABCC10, rs2125739, was significantly associated with nevirapine plasma concentration in Whites; the variant allele was significantly more prevalent in those with lower nevirapine plasma concentrations. The minor allele frequency for this SNP was similar in both Whites and Blacks represented in this cohort. Indeed, the same allele was also more prevalent in Blacks with lower nevirapine plasma concentration even though it did not reach statistical significance. This is possibly due to smaller sample numbers in the Black patient subset (n=65) compared with Whites (n=98). Bioinformatic analysis using the FastSNP software (http://fastsnp.ibms.sinica.edu.tw/) found rs2125739 to be located in a putative splice site. Splice site polymorphisms may affect pre-mRNA splicing and lead to an altered protein [31]. We previously identified tenofovir to be a substrate of ABCC10 and the same SNP was associated with tenofovir-related kidney tubular toxicity. Collectively with the data presented here, this suggests a direct functional effect of this polymorphism that now needs to be explored. However, it is important to stress that an effect on splicing has not been shown experimentally for this SNP and the association may be driven by linkage disequilibrium with other functional variants. Marked differences in ABCC10 haplotype structure and SNP allele frequencies were also identified between White and Black patients; this data may be of particular relevance when examining population pharmacokinetics of nevirapine in patient datasets stratified by ethnicity.

We previously reported the association between the *CYP2B6* 516G > T SNP and nevirapine plasma concentration in this same cohort [3]. A multivariate analysis identified the composite genotype of *CYP2B6* 516G > T and *ABCC10* rs2125739 (GGCC genotype) to be significantly associated with nevirapine plasma concentrations along with time post nevirapine dose and age. This provides evidence for the contribution of multiple alleles in multiple genes (along with other patient factors) to the ADME of nevirapine. Importantly, the carriage of these two SNPs were associated with nevirapine plasma concentrations independent of ethnicity, highlighting its importance in both White and Black patient populations. Furthermore, genetics is likely to be a better marker for exposure than ethnicity.

This study has certain limitations. First, the sample size is limited but is comparable with previous genetic association studies for nevirapine [3,4,24,32]. The stratification of the cohort for analysis in individual ethnic groups further reduced the sample size and these findings need to be replicated in larger, well-characterized cohorts. Second, we did not have any data on the intracellular nevirapine concentrations or treatment response. Intracellular concentrations could be more important than plasma concentrations with respect to efficacy and their association with *ABCC10* SNPs now needs to be explored. Finally, the impact of

polymorphisms in *ABCC10* on nevirapine plasma concentrations could contribute to associated adverse drug reactions. However, our cohort did not have any history on this and therefore conclusions on the role of ABCC10 in nevirapine-related toxicity could not be drawn.

Lower nevirapine plasma concentrations have been associated with reduced viral suppression [18,19]. Modulation of ABCC10 expression in the immune cell subsets may provide a therapeutic strategy to augment nevirapine efficacy. Indeed, this strategy has been successfully utilized *in vitro* and in animal models to overcome ABCC10-related multidrug resistant cancer phenotypes [33]. The combined delivery of docetaxel and cepharanthine resulted in higher intracellular accumulation of docetaxel and greater anti-tumour efficacy in ovarian cancer models. However, therapeutic intracellular 'boosting' of nevirapine with cepharanthine would not be possible due to the unexpected effect observed here (reduction of intracellular nevirapine concentrations). This unexpected observation is most likely to be mediated by inhibition of other, as yet uncharacterized, transporters of nevirapine. Nonspecific inhibition of any drug transporter has potential toxic implications and the contribution of ABCC10 to drug disposition in the liver and other tissues needs careful investigation.

In conclusion, our findings show a clear relationship between ABCC10 and nevirapine and add further to our understanding of the molecular pharmacology of nevirapine exposure. Using a limited sample size, we have also identified an association between *ABCC10* genetic variants and nevirapine plasma concentration. Validation of these pharmacogenetic findings in larger, ethnically diverse cohort of patients receiving nevirapine is now warranted to address the impact of *ABCC10* genetic variants on nevirapine disposition. Once validated, the impact of these variants on nevirapine-associated virological response and toxicity should be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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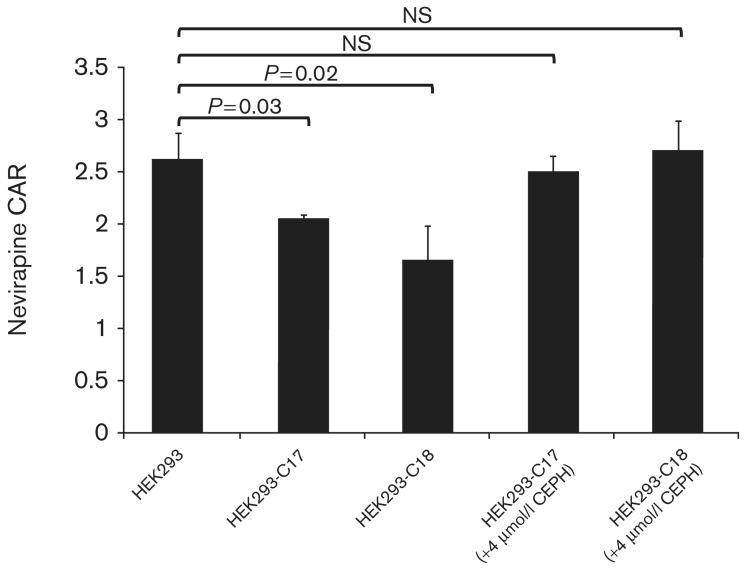

Fig. 1.

Accumulation of nevirapine in ABCC10-expressing cell lines. HEK293-C17 and HEK293-C18 cells, overexpressing ABCC10, showed significantly lower intracellular accumulation of nevirapine. Cepharanthine, a potent ABCC10 inhibitor, prevented this efflux. Data expressed as mean (n=6) ± SD. CAR, cellular accumulation ratio; NS, nonsignificant.

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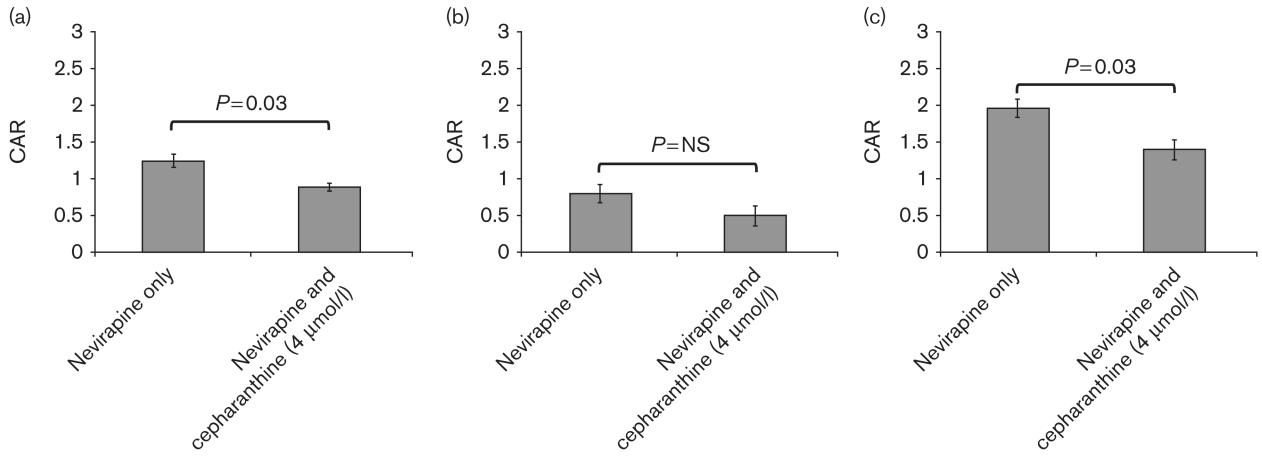

Fig. 2.

Impact of cepharanthine on the intracellular accumulation of nevirapine in primary immune cells. Nevirapine accumulation in (a) CD4, (b) CD14, and (c) MDM cells was lower when coincubated with cepharanthine, a potent ABCC10 inhibitor. Data expressed as mean (N=6)±SD.

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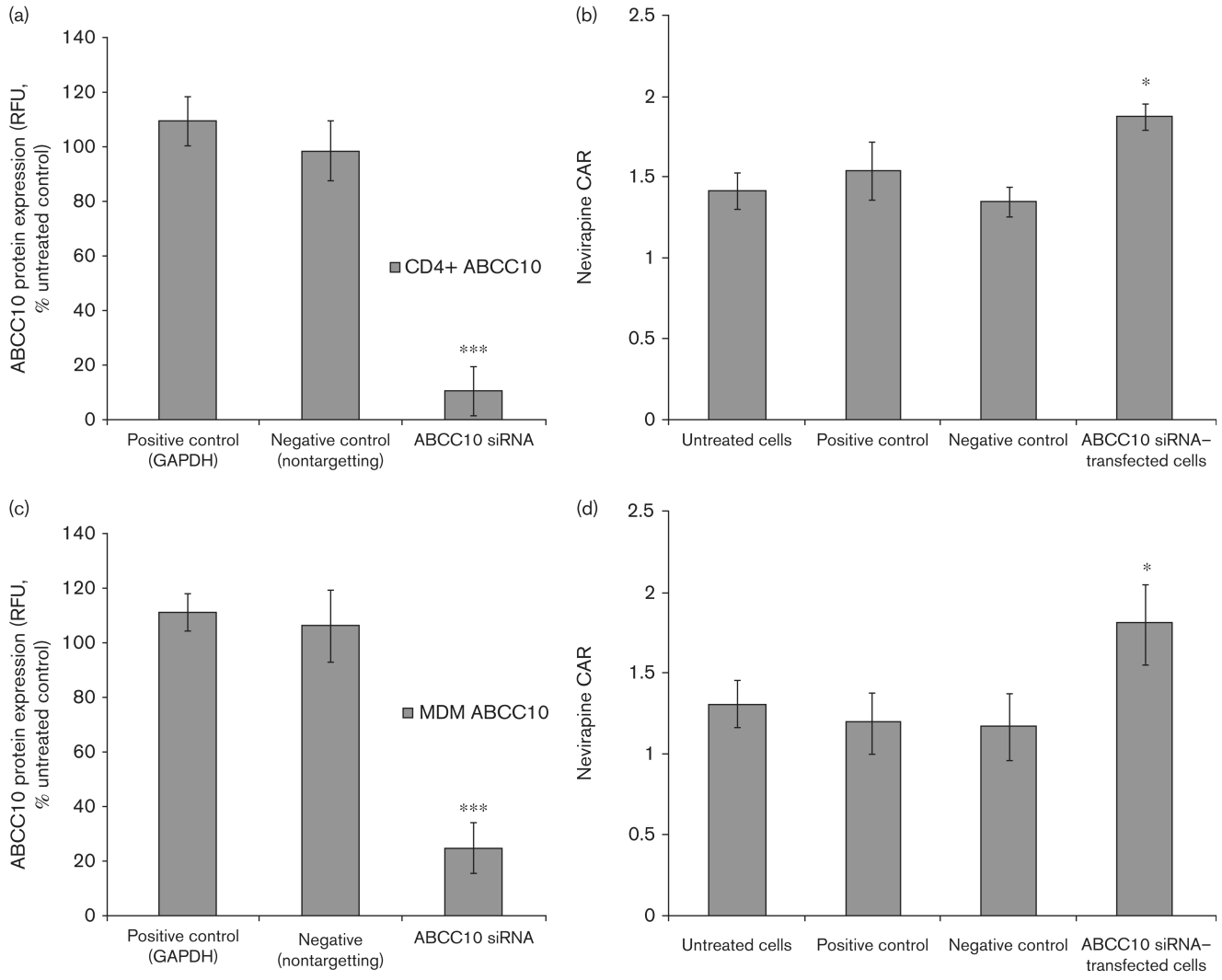

Fig. 3.

Nevirapine accumulation in primary immune cells after small interfering RNA (siRNA)-mediated knockdown of ABCC10. ABCC10 protein expression was determined by flow cytometry and was significantly lower in (a) CD4 and (c) monocyte-derived macrophages (MDM) cells 48 h post transfection. Lower ABCC10 expression was associated with a concomitant increase in nevirapine accumulation in (b) CD4 and (d) MDMs. Data expressed as the mean (n=6)±SD. *P<0.05, ***P<0.001. CAR, cellular accumulation ratio.

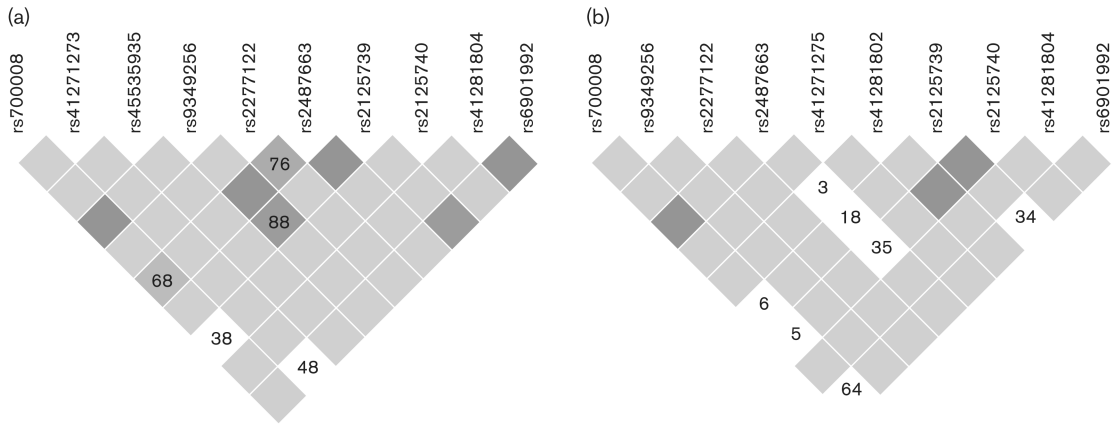

Fig. 4.

ABCC10 haplotype structure in ethnic groups in the German Competence Network for HIV/AIDS. Linkage disequilibrium (LD) pattern within the *ABCC10* gene in (a) Whites and (b) Blacks represented in the German Competence Network for HIV/AIDS. Dark grey boxes indicate strong LD, whereas light grey and white boxes indicate weak LD. The values in the box stand for pairwise LD measure as represented by D′.

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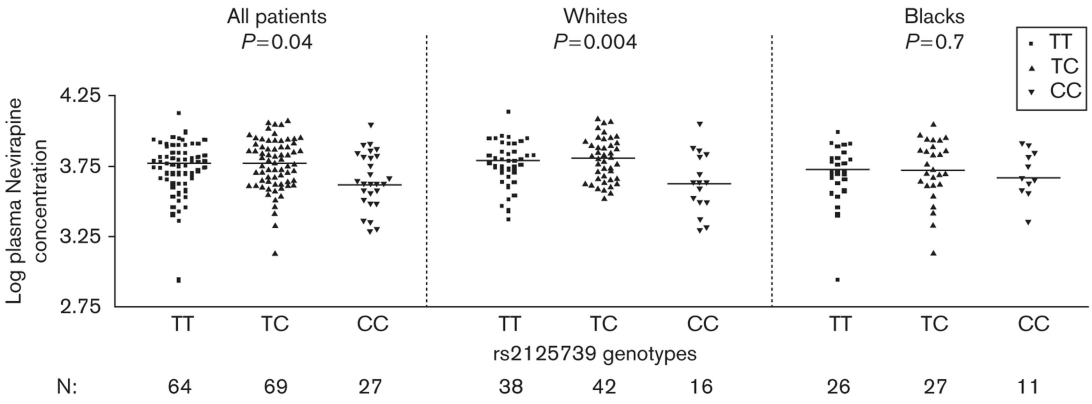

Fig. 5.

Association of ABCC10 single nucleotide polymorphism (SNP) rs2125739 with nevirapine plasma concentrations. Impact of *ABCC10* SNP rs2125739 on nevirapine plasma concentration in the whole cohort and within White and Black subset of patients within the German Competence Network for HIV/AIDS. For each genotype, log-transformed plasma nevirapine concentrations are represented here. *N*, number of patients in each genotype group.

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