Identification of Drug Transporter Genomic Variants and Inhibitors That Protect Against Doxorubicin-Induced Cardiotoxicity

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BACKGROUND: Multiple pharmacogenomic studies have identified the synonymous genomic variant rs7853758 (G > A, L461L) and the intronic variant rs885004 in SLC28A3 (solute carrier family 28 member 3) as statistically associated with a lower incidence of anthracycline-induced cardiotoxicity. However, the true causal variant(s), the cardioprotective mechanism of this locus, the role of SLC28A3 and other solute carrier (SLC) transporters in anthracycline-induced cardiotoxicity, and the suitability of SLC transporters as targets for cardioprotective drugs has not been investigated.

METHODS: Six well-phenotyped, doxorubicin-treated pediatric patients from the original association study cohort were recruited again, and human induced pluripotent stem cell–derived cardiomyocytes were generated. Patient-specific doxorubicin-induced cardiotoxicity (DIC) was then characterized using assays of cell viability, activated caspase 3/7, and doxorubicin uptake. The role of SLC28A3 in DIC was then queried using overexpression and knockout of SLC28A3 in isogenic human-induced pluripotent stem cell–derived cardiomyocytes using a CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9). Fine-mapping of the SLC28A3 locus was then completed after SLC28A3 resequencing and an extended in silico haplotype and functional analysis. Genome editing of the potential causal variant was done using cytosine base editor. SLC28A3-AS1 overexpression was done using a lentiviral plasmid-based transduction and was validated using stranded RNA-sequencing after ribosomal RNA depletion. Drug screening was done using the Prestwick Chemical Library (n = 1200), followed by in vivo validation in mice. The effect of desipramine on doxorubicin cytotoxicity was also investigated in 8 cancer cell lines.

RESULTS: Here, using the most commonly used anthracycline, doxorubicin, we demonstrate that patient-derived cardiomyocytes recapitulate the cardioprotective effect of the SLC28A3 locus and that SLC28A3 expression influences the severity of DIC. Using Nanopore-based fine-mapping and base editing, we identify a novel cardioprotective single nucleotide polymorphism, rs11140490, in the SLC28A3 locus; its effect is exerted via regulation of an antisense long noncoding RNA (SLC28A3-AS1) that overlaps with SLC28A3. Using high-throughput drug screening in patient-derived cardiomyocytes and whole organism validation in mice, we identify the SLC competitive inhibitor desipramine as protective against DIC.

CONCLUSIONS: This work demonstrates the power of the human induced pluripotent stem cell model to take a single nucleotide polymorphism from a statistical association through to drug discovery, providing human cell-tested data for clinical trials to attenuate DIC.

Key Words: cardiotoxicity • CRISPR-Cas systems • doxorubicin • human induced pluripotent stem cells • myocytes, cardiac

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Clinical Perspective

What Is New?

• This study shows that patient-specific cardiomyocytes recapitulate the cardioprotective effect of the candidate gene association study–identified SLC28A3 (solute carrier family 28 member 3) locus and we functionally confirm for the first time the role of SLC28A3 in doxorubicin-induced cardiotoxicity.
• A novel genetic variant, rs11140490, is the potential causal variant in the SLC28A3 cardioprotective locus.
• The solute carrier transporter inhibitor desipramine protects against doxorubicin-induced cardiotoxicity through decreasing the intracellular uptake of doxorubicin into the heart.

What Are the Clinical Implications?

• We provide 2 potential therapeutic options to attenuate doxorubicin-induced cardiotoxicity: either repurposing US Food and Drug Administration–approved desipramine or therapy with SLC28A3-AS1 (SLC28A3 overlapping antisense long noncoding RNA).
• We propose that a simple clinical test to detect the presence of rs11140490 can be used to predict that a patient will be less likely to experience doxorubicin-induced cardiotoxicity and that, with future clinical trials, it may be possible for these patients to be treated with a longer duration (higher cumulative dose) of doxorubicin to enhance the efficacy of their chemotherapy.

Nonstandard Abbreviations and Acronyms

AIC  anthracycline-induced cardiotoxicity
Cas9  CRISPR-associated protein 9
CGAS  candidate gene association study
CRISPR  Clustered Regularly Interspaced Short Palindromic Repeats
DIC  doxorubicin-induced cardiotoxicity
GWAS  genome-wide association study
hiPSC  human induced pluripotent stem cells
hiPSC-CM  human induced pluripotent stem cell–derived cardiomyocyte
ISO  isogenic hiPSC line
SLC  solute carrier
SLC28A3  solute carrier family 28 member 3
SNP  single nucleotide polymorphism

Doxorubicin, a cytotoxic anthracycline antibiotic, is a common anticancer agent used to treat a wide variety of adult and childhood cancers. The cardiotoxicity of anthracyclines has been documented as occurring in 9% of treated adult patients,¹ and on average, it occurs within just 3.5 months after the final chemotherapy dose; 98% of patients experience cardiotoxicity within the first year.¹ Early cardiotoxicity leads to dose limitation or treatment discontinuation to the detriment of therapy. The cardiotoxicity of doxorubicin is also well understood to be dose dependent, with 65% and 85% of cancer patients experiencing a decline in left ventricular ejection fraction when treated with 550 and 700 mg/m² of doxorubicin, respectively.² However, despite attempts to severely limit cumulative dose, cardiotoxicity occurs in 14.5% of patients with breast cancer receiving the most common 240-mg/m² cumulative dose.³

Pharmacogenomic research has attempted to discover predictive DNA biomarkers for anthracycline-induced cardiotoxicity (AIC) and has so far identified about 75 AIC-associated loci.⁴ However, the true connection between these loci and cardiotoxicity is unknown, given that the majority of AIC pharmacogenomic studies lack functional validation of the identified associations. As a result, no US Food and Drug Administration–approved genetic biomarkers are currently being used in routine clinical practice that predict AIC,⁶ and only a single on-market drug, dexrazoxane, is approved to decrease the incidence of AIC.

SLC28A3 encodes solute carrier (SLC) transporter family 28 member 3 and is the most robustly replicated AIC-associated locus. This locus was initially discovered in a large, multicenter pediatric candidate gene association study (CGAS) that identified 2 single nucleotide polymorphisms (SNPs): rs7853758 (G > A, L461L), which is in a coding region but synonymous, and rs885004, which is located in intron 8. Both SNPs are in high linkage disequilibrium. rs7853758 is highly associated with a lower risk of developing doxorubicin-induced cardiotoxicity (DIC) in both discovery (n = 188; \( P_{\text{adj}} = 0.0071 \); odds ratio [OR] = 0.29) and replication (n = 156; \( P_{\text{adj}} = 0.0072 \); OR = 0.33) cohorts.⁷ It is important to note that this genetic association was replicated in a third additional multicenter independent cohort of 218 patients.⁶ The sensitivity and specificity (95% CI) of rs7853758 is 17.4 (7.8–31.4) and 64.6 (58.8–70.1), respectively.⁶ Despite identification of this SNP through 2 replication cohorts in CGAS, the mechanisms by which a synonymous variant can influence AIC is unclear. Thus, the validity of this locus in relation to AIC, the true causal variant and the cardioprotective mechanism of this locus, the role of SLC28A3 and other SLC transporters in AIC, and the suitability of SLC transporters as targets for cardioprotective drugs are critical uninvestigated topics.

Here, we show that patient-specific human induced pluripotent stem cell (hiPSC)–derived cardiomyocytes (hiPSC-CMs) recapitulate the cardioprotective effect of SNP rs7853758. SLC28A3 knockdown and overexpression using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) reduces and increases doxorubicin uptake.
into cardiomyocytes, respectively, altering their sensitivity to doxorubicin, thus confirming the role of this locus in DIC. Fine-mapping of the SLC28A3 locus uncovered that rather than the original CGAS-identified synonymous SNP (rs7853758), the linked SNP rs11140490 is the causal cardioprotective variant in that locus. Further mechanistic studies showed that rs11140490 exerts its action by regulating an SLC28A3-overlapping, antisense long noncoding RNA SLC28A3-AS1. Screening for other potential cardiac-specific SLC transporters in relation to DIC revealed that SLC22A4 and SLC22A17 are also implicated in DIC. Last, screening a drug library using hiPSC-CMs followed by in vivo validation in a mouse model of DIC discovered that the SLC competitive inhibitor, desipramine protects against DIC by without hindering doxorubicin chemotherapy efficacy. Together, these findings provide a novel genetic test for rs11140490 that can identify patients who are protected from DIC and 2 potential therapeutic options, either using the long noncoding RNA SLC28A3-AS1 or developing a derivative of desipramine to attenuate DIC.

METHODS

The data, analytic methods, and study materials are available to other researchers on reasonable request for purposes of reproducing the results. Detailed methods are provided in the Supplemental Material. RNA-sequencing data have been deposited in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) with accession code GSE165731.

Human Induced Pluripotent Cell Derivation and Cardiac Differentiation

Protocols and consents were approved by the institutional review boards of Northwestern University and University of British Columbia. Six well-phenotyped, doxorubicin-treated pediatric patients from the original association study cohort were recruited again with informed consent. Peripheral blood mononuclear cells were isolated from blood and reprogrammed to hiPSCs using CytoTune-iPS 2.0 Sendai Reprogramming Kit (Invitrogen).10 SNP karyotyping was performed using a whole-genome Infinium HumanCytoSNP–12 BeadChip (cardiac troponin T)–driven antibiotic selection cassette for cardiomyocyte purification.

CRISPR/Cas9–Mediated Gene Knockout and Overexpression

To generate SLC28A3 knockout gRNA expression vectors, gRNA targeting the start codon designed with minimal predicted off-target effect.13 Each gRNA was annealed and inserted into pSpCas9(5BB)-2A-Puro (PX459) V2.0 (48138; Addgene) plasmid that expresses puromycin resistance gene for downstream antibiotic selection, in addition to Cas9. One million cells were electroporated with 5 μg PX459 plasmid and positive clones were selected 24 hours after transfection using puromycin treatment for 48 hours. To generate SLC28A3 overexpressing cells, Human SLC28A3 Sequence-Verified cDNA was first amplified and cloned under the CAG promoter of a pAAVS1–Nst–CAG–DEST gateway cloning vector (80489; Addgene). AAVS1 (adenovirus-associated virus integration site 1) gRNA expression vector14 (pxAT2; 80494, Addgene), which expresses gRNA and Cas9, was used to target AAVS1 locus in the first intron of the PPP1R12C (protein phosphate 1 regulatory subunit 12C) gene.14 Cells were then electroporated with AAVS1 targeting plasmid and SLC28A3 overexpression donor plasmid. Positive clones were selected using neomycin treatment for 14 days.

Patient-Specific DIC Characterization

Patient-specific DIC was characterized by assays of cell viability using CellTiter–Glo 2.0 (Promega) and activated caspase 3/7 using Caspase 3/7–Glo (Promega) that were used per manufacturer’s instructions. Doxorubicin uptake was quantified using flow cytometry by measuring doxorubicin intrinsic phycoerythrin fluorescence at 1 and 3 hours after doxorubicin treatment and normalized to baseline fluorescence. All cells were stained with NucRed Live ReadyProbes Reagent (Invitrogen) to monitor cell viability.

SLC28A3 Locus Genetic Fine-Mapping

Fine-mapping of the SLC28A3 locus was then completed after SLC28A3 resequencing using MinION Nanopore sequencer and an extended in silico haplotype and functional analysis.

Genome Editing of Potential Causal Variant rs11140490

Locus-specific base-editor protein complex and gRNA were designed using Beditor,15 and the designed gRNA was cloned in the gRNA expressing plasmid (73797; Addgene). Then, 1 million cells were electroporated with 4 μg of the base editor–expressing plasmid (119861; PSI-Target-AID-NG; Addgene) and 4 μg of the gRNA-expressing plasmid (73797; lenti sgRNA[MS2]_puro; Addgene). Cells were then selected with puromycin 24 hours after transfection for 48 hours, clones were hand-picked, the target locus was polymerase chain reaction–amplified and Sanger sequenced to confirm the SNP editing in all clones.

SLC28A3-AS1 Overexpression in Isogenic hiPSCs

The SLC28A3-AS1 cDNA was cloned into pLenti-C-Myc-DDK-ires-Puro lentiviral vector ( OriGene), which was then cotransfected with packaging plasmids pSPAX2 (12260; Addgene) and pMD2.G (12259; Addgene) into Lenti-X 293T cells (Takara) to generate lentivirus. Virus-containing supernatant was collected at 48 and 72 hours posttransfection. Lentivirus was concentrated 1:100 from cleared supernatant using PEG-it (Systems Biosciences). Isogenic hiPSCs were then transduced and positive clones were selected with puromycin for 7 days to generate ISO[SLC28A3-AS1]. SLC28A3-AS1 overexpression was confirmed using stranded RNA-sequencing after ribosomal RNA depletion.

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Mouse Model of Doxorubicin-Induced Cardiomyopathy and Drug Administration

Procedures were followed in accordance with Stanford University’s institutional guidelines. In vivo validation was done using 10-week-old male C57BL/6J mice that were cotreated with doxorubicin (NovaPlus) and water as a control vehicle (n = 10), or with desipramine (Sigma) in the experimental groups (n = 8). At day 0, mice were treated with doxorubicin (3 mg/kg) intraperitoneally twice a week alone or with desipramine by Alzet pump infusion (20 mg·kg⁻¹·day⁻¹) for 3 weeks (days 0–21). For the control group, we treated mice with corn oil on the same schedule as desipramine administration. We recorded an echocardiogram once a week on days 0, 7, 14, and 21 and terminated the experiment at day 21.

Statistical Analysis

Data were analyzed in R version 4.0.3 and graphed in GraphPad Prism 6. Detailed statistical information is included in the corresponding figure legends. Data were presented as mean±SEM. Comparisons were conducted using a 1-way ANOVA test or an unpaired 2-tailed Student t test with significant differences defined as P<0.05 (*), P<0.01 (**), P<0.001 (***) and P<0.0001 (****). Our sample size (3 patients in each category) was based on the feasibility of handling this number of hiPSC lines. For dose–response curves, a log-logistic nonlinear regression model was used to estimate the value of the 4 parameters, and a t statistic was used to test for significant difference in LD₅₀ between groups using the drc package¹⁶ in R.

RESULTS

Investigation of the Protective Role of Variant rs7853758

Six well-phenotyped, doxorubicin-treated pediatric patients from the original CGAS cohort were specifically recruited again according to the original inclusion criteria¹⁷ (Table S1). These included 3 patients who were heterozygous for the rs7853758 variant and were protected from DIC (SLC₂₈ᵃʳ, SLC₂₈ᵃʳ, SLC₂₈ᵃʳ; collectively SLC₂₈ᵃʳ), and 3 control patients who did not protect this protective variant (they carry the reference allele) and developed DIC after the doxorubicin therapy (SLC₂₈ᵃʳ, SLC₂₈ᵃʳ, SLC₂₈ᵃʳ; collectively SLC₂₈ᵃʳ). Detailed patient data including age, sex, ethnicity, type of cancer, treatment regimen, and presence or absence of DIC are provided in Table S2. hiPSC lines were established from peripheral blood of patients using nonintegrating (Sendai virus–based) reprogramming and our well-established protocols.¹⁸,¹⁹ These lines showed normal hiPSC morphology (Figure S1A), expressed high levels of undifferentiated cell markers (Figure S1B and S1C), and were karyotypically normal (Figure S2). The genotypes of the rs7853758 SNP were validated using Nanopore-based sequencing (Figure 1A). Cardiomyocytes were generated using our established chemically-defined, small molecule–based monolayer differentiation system¹¹,²⁰ (Figure S3A), along with a TNNT2-based antibiotic selection cassette that consistently produces cardiomyocytes that are 80% to 98% TNNT2⁺ (Figure S3B and S3D). hiPSC-CMs express SLC₂₈ᵃʳ throughout the cell (Figure 1B).

Patient-Specific hiPSC-CMs Recapitulate the Cardioprotective Effect of rs7853758 Against DIC

We first aimed to model the cardioprotective effect of rs7853758 variant in patient–specific hiPSC-CMs. A cell viability assay completed at 72 hours after doxorubicin treatment demonstrated that the 3 SLC₂₈ᵃʳ lines recapitulated the protective effect of SNP ([rs7853758 G > A; L461L] LD₅₀ = 2.7 μmol/L; P<0.0001; n = 126) as compared with the SLC₂₈ᵃʳ lines (LD₅₀ = 1.38 μmol/L; n = 81) (Figure 1C; Figure S12A). Consistently, apoptosis as quantified by a caspase-3 and -7 activity assay completed 72 hours after doxorubicin treatment was significantly lower in SLC₂₈ᵃʳ lines (IC₅₀ = 1 μmol/L; P<0.001; n = 20) compared with the SLC₂₈ᵃʳ lines (IC₅₀ = 0.43 μmol/L; n = 20; Figure 1D). Because SLC₂₈ᵃʳ is an uptake transporter, we hypothesized that the lower level of DIC in the SLC₂₈ᵃʳ lines might be attributable to reduced doxorubicin uptake as a result of impaired SLC₂₈ᵃʳ function in these patients. To test this, we used a flow cytometry–based assay and found that intracellular doxorubicin uptake was >50% lower in SLC₂₈ᵃʳ as compared with SLC₂₈ᵃʳ (Figure 1E), which is consistent with the magnitude LD₅₀ (1.38 μmol/L versus 2.7 μmol/L) and IC₅₀ (0.43 μmol/L versus 1 μmol/L) changes on our in vitro DIC assays (Figure 1C). To investigate whether this cardioprotective effect seen in SLC₂₈ᵃʳ is attributable to altered SLC₂₈ᵃʳ protein expression, we then quantified SLC₂₈ᵃʳ in our patient-specific hiPSC-CMs using Western blot. SLC₂₈ᵃʳ expression in SLC₂₈ᵃʳ was significantly lower than SLC₂₈ᵃʳ (P = 0.03) (Figure S1F; Figure S12B).

SLC₂₈ᵃʳ Altered Expression Affects DIC in hiPSC-CMs

To further confirm the implication of SLC₂₈ᵃʳ in DIC regardless of patient–specific genetic, transcriptomic, and environmental background, and to isolate the effect of SLC₂₈ᵃʳ in relation to DIC, we next examined whether a gain or loss of function of SLC₂₈ᵃʳ altered DIC in an isogenic hiPSC line (ISO). The ISO line was derived from a healthy individual and its whole genome sequencing confirmed that it does not harbor any other DIC-associated loci identified by the original study. SLC₂₈ᵃʳ overexpression and knockout (KO) lines were generated through a CRISPR/Cas9–mediated approach. Disturbance at start codon region was confirmed by Sanger sequencing (Figure 2A and 2B) and altered SLC₂₈ᵃʳ expression in these lines was confirmed by quantitative reverse transcription poly-
merase chain reaction and Western blot showing 90% downregulation of SLC28A3 in ISO-KO (Figure 2C). The effect of SLC28A3 overexpression and KO on in vitro DIC was investigated using the above cell viability and caspase assays at 72 hours after doxorubicin treatment. The cell viability assay showed that the ISO...
Figure 2. SLC28A3 expression affects the severity of doxorubicin-induced cardiotoxicity by regulating doxorubicin uptake into cardiomyocytes.

A. Validation of CRISPR/Cas9–mediated SLC28A3 KO in an isogenic human induced pluripotent stem-cell line detected by Sanger sequencing, showing 8-bp deletion downstream of the TSS. B. Demonstration that 91% of the cell population acquire the introduced deletion. C. Validation of KO and AAVS1-based SLC28A3 OE by Western blot and reverse transcription polymerase chain reaction. D. Effect of doxorubicin (72 h) on viability in ISO (n = 45), ISO-OE (n = 14), and ISO-KO (n = 6) human induced pluripotent stem cell–derived cardiomyocytes. E. Effect of doxorubicin (72 h) on apoptosis measured by activated caspase 3/7 in ISO (n = 8), ISO-OE (n = 10), and ISO-KO (n = 6) human induced pluripotent stem-cell cardiomyocytes. F. Assessment of doxorubicin uptake through measurement of doxorubicin intrinsic fluorescence using flow cytometry–based assay (n = 6–9). D and E. A log-logistic nonlinear regression model was used to estimate the value of the 4 parameters, and a t-statistic was used to test for significant difference in LD$_{50}$ between groups. N = full independent experimental replicates. Error bars indicate SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by unpaired 2-tailed Student t-test (F). ISO indicates isogenic hiPSC line; KO, knockout; OE, overexpression; PAM, protospacer adjacent motif; SEM, standard error of the mean; SLC28A3, solute carrier family 28 member 3; and TSS, transcription start site.
overexpression hiPSC-CMs (LD₅₀ = 0.9 μmol/L) were ≈3.3-fold and 1.4-fold more sensitive to doxorubicin as compared with ISO-KO (LD₅₀ = 4 μmol/L; P=0.03) and ISO (LD₅₀ = 2.5 μmol/L; P<0.0001), respectively (Figure 2D). Likewise, caspase activity was ≈2.6-fold higher in ISO overexpression (LD₅₀ = 0.14 μmol/L) as compared with ISO (LD₅₀ = 0.53 μmol/L, P<0.0001) (Figure 2E). We next aimed to investigate the effect of SLC28A3 KO and overexpression on doxorubicin intracellular uptake. Doxorubicin uptake was significantly lower in ISO-KO as compared with doxorubicin treatment (Figure 2F). Similarly, doxorubicin uptake was significantly lower in ISO-KO compared with ISO (LD₅₀ = 0.53 μmol/L, P<0.0001) (Figure 3A–3B; Table S6).

SNPs are distributed as follows, 8 SNPs are located synonymous SNPs (Figures 3A–3B; Table S6). We then investigated the regulatory properties of all candidate SNPs constituting the HapSLC28A3 were in high linkage disequilibrium with an average D' and R² of 0.99 and 0.84, respectively (Figure S5A and S5B; Table S11). Regarding HapSLC28A3AS1, 7 structures were identified, Hap-I SLC28A3-AS1 to Hap-VII SLC28A3-AS1 (Figure S5C). In that, Hap-I SLC28A3-AS1 consists of the reference allelles for all 7 SNPs (ATCTAGA) and is inherited in 71.7% of the examined population, whereas Hap-I SLC28A3-AS1 comprises the variant alleles for all 7 SNPs (GCAGCGGAG) and is inherited in 17.7% of the examined population (Figure S5C). This finding confirms the linkage disequilibrium pattern identified by the Nanopore pipeline in all of the study patients.

To prioritize cardioprotective haplotype SNPs, we investigated the regulatory properties of all candidate SNPs (n = 24). Using ENCODE (Encyclopedia of DNA Elements) and Roadmap Epigenomics data, and DeepSEA algorithm, we examined the functional effect of each SNP on altering chromatin features (transcription factors, Dnase hypersensitive site, and histone marks) binding sites. Among all SNPs, rs11140490 and rs4877835 had the top chromatin regulatory effect as both SNPs predicted to alter the binding site of 206 and 204 chromatin features, respectively (Figure S4F; Tables S7–S8). Moreover, SNP rs11140490 has the most substantial regulatory effect as it is predicted to alter the binding sites of 43 features with log₂ fold change of ≥1, whereas rs4877835 is predicted to alter the binding sites of only 4 features with log₂ fold change of ≥1. It is unsurprising that the primary study significant association, rs7853758 does not show any significant chromatin regulatory effect (Figure 4G).

Fine-Mapping at the SLC28A3 Locus Prioritizes Potential Causal Variant

rs7853758 is a coding synonymous SNP located in exon 14 and thus does not affect the amino acid sequence. Because of the tag SNP and linkage disequilibrium issues associated with GWAS, we expected that rs7853758 is linked (coinherited) to additional SNPs including the causal one(s). To elucidate this, we next fine-mapped the SLC28A3 locus to identify the potential causal variant. We sequenced the SLC28A3 gene in all 6 SLC6a and SLC6d patients using a Nanopore MiniIon sequencer and SNPs were called using Nanopolish (see Supplemental Methods). For all patients, Nanopore genotypes of the original association study hit, rs7853758 were in concordance with the GWAS association hit, rs7853758 were in concordance with the GWAS association study patients. These findings show that SLC28A3 is implicated in DIC regulation through affecting doxorubicin uptake into cardiomyocytes.
Figure 3. Fine-mapping at the SLC28A3-SLC28A3-AS1 locus identifies rs11140490 as the potential causal cardioprotective variant. 

A, Location of the Hap^{SLC28A3} comprising 24 SNPs that are coinherited only in SLC^{var}-protected patients. SNP rs11140490 marked by red rectangle is located at the splice site of the first exon of an overlapping long noncoding RNA, SLC28A3-AS1 (adapted from Magdy et al^{29}).

B, Consequence of coinherited Hap^{SLC28A3} SNPs (n = 24). C, Overall prioritization of candidate causal SNPs based on functional annotation analyses including, expression quantitative trait loci annotation, chromatin regulatory analyses, and overlapping with RR in cardiac tissues. D, Editing of rs11140490 (CT > TT) in 2 patient-specific human induced pluripotent stem cell lines using a cytosine base editor (Target-AID-NG). E, Effect of doxorubicin (72 h) on viability in SLC^{var-rs11140490 (CT)} (2 lines; n = 31) and SLC^{var-rs11140490(CT > TT edited)} (n = 75) human induced pluripotent stem cell–derived cardiomyocytes. F, Assessment of doxorubicin uptake through measurement percentage of cells with doxorubicin intrinsic fluorescence using flow cytometry–based assay in SLC^{var-rs11140490 (CT)} and SLC^{var-rs11140490(CT > TT edited)} (n = 4).

Log-transport nonlinear regression model was used to estimate the value of the 4 parameters, and t-statistic was used to test for significant difference in LD_{50} between groups. N = full independent experimental replicates. Error bars indicate SEM, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by unpaired 2-tailed Student t test (F). eQTL indicates expression quantitative trait loci; FA, functional annotation (adapted from Magdy et al^{29}); RR, regulatory regions; SEM, standard error of the mean; SLC28A3, solute carrier family 28 member 3; SLC28A3-AS1, SLC28A3 overlapping antisense long noncoding RNA; and SNP, single nucleotide polymorphisms.
Because DIC affects cardiomyocytes, we performed an additional regulatory analysis exclusively focusing on human cardiac tissue, and for that we used ensemble regulatory build that includes transcription factors, histone mark, and Dnase hypersensitive regions. Six SNPs, rs11140490, rs4877835, rs4877831, rs7047898, rs885004, and rs10868137 were found to be located in at least 1 regulatory region in human cardiac tissue (Table S9). Last, to investigate further regulatory consequences of these candidate SNPs, we used the Genotype-Tissue Expression project database (https://www.gtexportal.org/home/) to investigate which of the identified candidate SNPs have been shown to be an expression quantitative trait loci. All candidate SNPs except rs7858075 have been previously identified as expression quantitative trait loci in cultured fibroblasts, thyroid, and brain tissues (Table S10). Although the expression quantitative trait loci identified by the Genotype-Tissue Expression database are located in noncardiac tissues, these associations emphasize the regulatory function of these SNPs. These findings, when taken together, suggest that SNP rs11140490, located at the splice site of the first exon of SLC28A3-AS1, is the SNP with the highest likelihood to be the causal cardioprotective SNP (Figure 3C).

Editing hiPSC-CM rs11140490 Confirms Causality in Relation to Protection Against DIC

After we prioritized rs11140490 to be the top candidate causal variant within the DIC-associated SLC28A3–SLC28A3-AS1 locus, we confirmed the causality of this variant. Using a base editor–mediated approach, we edited the SNP rs11140490 in hiPSCs from 2 SLC28A3 patients that harbor the heterozygous genotype CT back to the reference genotype TT. The cytosine base editor that we have used is composed of a catalytically inactive “dead” Cas9 fused to cytosine base editor cytidine deaminase that converts a cytosine base to a thymine base. It is important to note that the cytosine base editor does not induce a DNA double-strand break. We cloned the cytidine deaminase into the backbone of a catalytically inactive Cas9 fused to a single guide RNA targeting the SNP rs11140490 to create a specificity under the control of a doxorubicin promoter. We then performed a dose–response analysis of doxorubicin uptake in these hiPSCs and compared the uptake of doxorubicin and a doxorubicin metabolite and/or SLC transporters, 12 of which have been shown to either affect SLC transporters or to be located in noncardiac tissues, including liver and kidney. To test this hypothesis, we investigated the effect of altered expression of SLC28A3-AS1 on DIC phenotype. We overexpressed SLC28A3-AS1 in an isogenic cell line (ISO SLC28A3-AS1) by transducing isogenic hiPSCs with the SLC28A3-AS1 cDNA cloned into pLenti-MYC-DDK-IRES-Puro lentivirus expression vector (PS100069; OriGene). SLC28A3-AS1 overexpression was confirmed by stranded RNA sequencing after ribosomal RNA depletion (Figure 4A). We then assessed the effect SLC28A3-AS1 overexpression on SLC28A3 expression in cardiomyocytes and showed that SLC28A3 is significantly downregulated in ISO SLC28A3-AS1 cardiomyocytes after doxorubicin treatment (Figure 4B). We then investigated the effect of SLC28A3-AS1 overexpression on DIC and doxorubicin uptake as before. ISO SLC28A3-AS1 hiPSC-CMs were significantly more resistant to doxorubicin (LD50 = 8.4 μmol/L; P < 0.0001) as compared with ISO (LD50 = 1.8 μmol/L; Figure 4C). Moreover, doxorubicin uptake was significantly impaired in ISO SLC28A3-AS1 cardiomyocytes when compared with ISO at both 1 hour (P = 0.001) and 3 hours (P < 0.001) after doxorubicin treatment (Figure 4D). These results show that the regulation of SLC28A3-AS1 is a potential mechanism by which SNP rs11140490 exerts its cardioprotective effect.

Implication of Other SLC Transporters on DIC Regulation

After we provided proof of principle for the importance of SLC transporters in DIC by showing that SLC28A3 expression and genomic variants affect the severity of DIC, we examined other potential SLC transporters that might affect DIC. To date, there are more than 450 identified SLC transporters, 12 of which have been shown to either transport doxorubicin or a doxorubicin metabolite and/or their genes harbor SNPs that are significantly associated with doxorubicin clinical outcomes. We examined which of these 12 SLC transporters are expressed in adult heart tissue, fetal heart tissue, and hiPSC-CMs. Only 3 additional SLC transporters met these criteria; SLC22A4, SLC22A3, and SLC22A17 were selected for further investigation (Figure 4E). For each of these transporters, we generated CRISPR/Cas9–mediated KO hiPSC...
Figure 4. The cardioprotective role of SLC28A3-AS1.

A. Relative expression of SLC28A3-AS1 in ISO hiPSC compared with ISO transduced by viral plasmid encoding SLC28A3-AS1 cDNA to overexpress SLC28A3-AS1 (ISO SLC28A3-AS1) assessed by RNA-Seq (n = 2). B. SLC28A3 relative expression in ISO and ISO SLC28A3-AS1 hiPSC-CMs (n = 5–11) assessed by real-time PCR. C. Effect of SLC28A3-AS1 overexpression on cell viability after doxorubicin (72 h) treatment; ISO (n = 17), ISO SLC28A3-AS1 (n = 12). D. Effect of SLC28A3-AS1 overexpression on doxorubicin uptake 1 h and 3 h after doxorubicin treatment (n = 6–14).

E. Relative human cardiomyocyte expression of SLC transporters (n = 12) previously identified as transporting doxorubicin or a doxorubicin metabolite and/or by genetic associations with doxorubicin clinical outcomes. Red dashed line denoted for the expression cutoff for SLC transporter selection. F. Effect of knocking out doxorubicin-relevant SLC transporters on doxorubicin uptake into patient-derived cardiomyocytes [SLC28A3KO, SLC22A4KO, SLC22A3KO, and SLC22A17KO (n = 5–13)]. G. Effect of knocking out potential cardiac-specific SLC transporters on cell viability after doxorubicin treatment [SLC28A3KO (n = 14), SLC22A4KO (n = 58), SLC22A3KO (n = 17), and SLC22A17KO, (n = 10), ISO (n = 128)].

C and G, Log-logistic nonlinear regression model was used to estimate the value of the 4 parameters and a t-statistic was used to test for significant difference in LD50 between groups. N = full independent experimental replicates. Error bars indicate SEM; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 by unpaired 2-tailed Student t test (B, D, and F). DOX indicates doxorubicin; hiPSC-CMs, human induced pluripotent stem cell–derived cardiomyocytes; ISO, isotype; SEM, standard error of the mean; SLC, solute carrier; SLC28A3, solute carrier family 28 member 3; and SLC28A3-AS1, SLC28A3 overlapping antisense long noncoding RNA.
(SLC22A4\textsuperscript{KO}, SLC22A3\textsuperscript{KO}, and SLC22A17\textsuperscript{KO}) and differentiated them to cardiomyocytes (Figure S7). We examined the effect of altered expression of each candidate transporter on doxorubicin intracellular uptake and cell viability after doxorubicin treatment. Expectedly, SLC22A3, SLC22A4, SLC28A3, and SLC22A17 KO resulted in a significant decrease in doxorubicin uptake in isogenic hiPSC-CMs (Figure 4F). DIC quantification revealed that SLC22A4\textsuperscript{KO} cardiomyocytes (LD\textsubscript{50} = 3.8 μmol/L, n = 58; \(P<0.0001\)), SLC28A3\textsuperscript{KO} cardiomyocytes (LD\textsubscript{50} = 3.3 μmol/L, n = 17; \(P<0.0001\)), and SLC22A17\textsuperscript{KO} cardiomyocytes (LD\textsubscript{50} = 3 μmol/L, n = 10; \(P=0.005\)) were less sensitive to doxorubicin and more protected against DIC as compared with ISO (LD\textsubscript{50} = 1.9 μmol/L, n = 128; \(P < 0.0001\)). However, knocking out SLC22A3 had a detectable but not significant effect on DIC (LD\textsubscript{50} = 2.1 μmol/L, n = 17; \(P = 0.5\); Figure 4G).

**High-Throughput Drug Screening Reveals SLC Inhibitor Desipramine as Novel Cardioprotective Drug Against DIC in hiPSC-CMs and Murine**

Because SLC transporters affect DIC and represent well-founded druggable targets, we examined whether SLC substrates can alter doxorubicin uptake and subsequently regulate DIC in hiPSC-CMs. We tested this by first screening 17 drugs with well-established roles in affecting SLC transporter efficacy in relation to doxorubicin uptake (Table S12). To eliminate potential toxicity from high doses of the SLC substrate drugs, LD\textsubscript{50} was determined in our ISO cardiomyocytes for the 17 drugs to determine relevant maximum tolerable dose (Figure S8). This target-focused screening revealed that desipramine was the only substrate that altered doxorubicin uptake at both 1 hour and 3 hours after doxorubicin treatment. Doxorubicin uptake was significantly lower in cells that were cotreated with desipramine (3 μmol/L) plus doxorubicin (3 μmol/L), as compared with cells treated with doxorubicin (3 μmol/L) only, after 1 hour (\(P=0.008\)) and 3 hours (\(P=0.04\)) after treatment (Figure S9A and S9B). Because desipramine significantly decreased doxorubicin transport into cardiomyocytes, we next inspected whether lower doxorubicin intracellular concentration in desipramine pretreated cells affect the magnitude of DIC.

Next, we aimed to expand our screening for novel cardioprotectors by testing the cardioprotective role of the Prestwick Chemical Library that includes 1219 drugs. Cells were pretreated with 3 μmol/L of relevant drugs for 24 hours, then cotreated with relevant drug (3 μmol/L) and doxorubicin (10 μmol/L) for 72 hours, after which DIC was quantified. For each plate, untreated and doxorubicin (10 μmol/L) only–treated cells were included to serve as negative and positive controls, respectively. Drug library screening revealed 34 cardioprotective molecules that attenuated cell death after doxorubicin treatment (Figure 5A). Drug repurposing is significantly faster and more cost-effective than de novo drug discovery approaches, and thus we focused on the top US Food and Drug Administration–approved drugs identified from our screening. It is interesting that the top 10 significant cardioprotectants included 6 US Food and Drug Administration–approved drugs (fluoxetine, butoconazole, perospirone, tetracaine, propofol, and desipramine [Figure 5B]); of these drugs, desipramine again showed the most significant protection against doxorubicin-induced cardiomyocyte death (\(P=0.007\); 2.5-fold; Figure 5A–5B).

To further validate these results, we then investigated the cardioprotective effect of these US Food and Drug Administration–approved cardioprotective drugs against 10 log-doses of doxorubicin. This analysis showed that desipramine has the strongest cardioprotective effect when compared with cells treated only with doxorubicin (LD\textsubscript{50} = 10.66 μmol/L; \(P<0.0001\)), followed by propofol (LD\textsubscript{50} = 4.77 μmol/L; \(P=0.003\)), then tetracaine (LD\textsubscript{50} = 4.65 μmol/L; \(P<0.001\)), then butoconazole (LD\textsubscript{50} = 4.4 μmol/L; \(P=0.01\); Figure 5C and 5D; Figure S9C).

On the basis of these findings, we selected desipramine as our lead cardioprotective drug, and thus, we then investigated whether desipramine could attenuate DIC in vivo. For that, we treated mice with doxorubicin (intraperitoneal 3 mg/kg 2× per week for 3 weeks) plus desipramine (20 mg/kg day\textsuperscript{-1} infusion for 3 weeks) or water as a vehicle control. Doxorubicin treatment results in a steady decline in cardiac function, as assessed by fractional shortening. Importantly, cardiac function was significantly less attenuated by doxorubicin at three weeks with desipramine (\(P<0.05\)), compared with vehicle treatment (Figure 5E and 5F; Figure S12B and S12C, Table S13). To rule out the possibility that desipramine could diminish the chemotherapeutic effect of doxorubicin, we studied 8 cancer cell lines—representing breast, liver, colorectal, prostate, uterus, cervix, and bone cancers—and found that cotreatment with desipramine did not impede the anticancer efficacy of doxorubicin in any of these lines (Figure 5F; Figure S10).

**DISCUSSION**

The identification of reliable predictive genomic biomarkers for DIC and the discovery of efficient cardioprotectants are indispensable to enhance the clinical utility of doxorubicin in cancer treatment. The identification of gene variants predictive of altering DIC through GWAS has provided impetus for developing platforms to confirm these GWAS hits, moving them from “association” to “confirmed mechanism.” In this work, we demonstrated that the patient-specific hiPSC-CM model is ideal for studying the implication of transporter inhibitors and genetic variants in DIC. We show that patient-specific cardiomyocytes recapitulate the cardioprotective effect of the CGAS-identified SLC28A3 locus; confirm for the first time the role of SLC28A3 in DIC independent of patient-specific genetic...
Figure 5. Multimodality drug screening identifies DESP as a novel effective cardioprotectant against DOX-induced cardiotoxicity. 

A. Prestwick Chemical Library screening (n = 1219) in relation to DOX-induced cardiotoxicity (n = 5). All drugs were used at 3 μmol/L. Red dashed line indicates cell viability 72 h after DOX treatment (10 μmol/L); the top 10 significant cardioprotective drugs based on cell viability are labeled. B. Bar plot showing top 10 significant cardioprotective drugs (on the basis of P value) compared with DOX alone (72 h; 10 μmol/L)–treated cells. Non–FDA-approved drugs are indicated with teal bars. C. Further validation of top FDA-approved drugs (identified from the Prestwick Library screening) against 10 log doses of DOX. D. Effect of cotreatment of DESP (3 μmol/L) and DOX (72 h) on human induced pluripotent stem cell–derived cardiomyocyte viability (DOX [n = 42]; DOX + DESP [n = 35]). E. Percent change in ventricular fraction shortening normalized to baseline, after 3 weeks of DOX treatment (3 mg/kg IP; n = 10) compared cotreatment (n = 8) of DESP (20 mg·kg⁻¹·d⁻¹; Alzet pump) and DOX (3 mg/kg IP) in mice. F. Ventricular fractional shortening at baseline, 1, 2, and 3 weeks after treatment. G. Assessment of cell viability of MCF-7 (Michigan Cancer Foundation 7), a breast cancer cell line, after 72 h of DOX and DESP cotreatment (n = 12–20). A log-logistic nonlinear regression model was used to estimate the value of the 4 parameters, and t-statistic was used to test for significant difference in LD₅₀ between different groups. F = full independent experimental replicates. Error bars indicate SEM; *P < 0.05, **P ≤ 0.01, ***P < 0.001, ****P < 0.0001 by unpaired 2-tailed Student t test (A–C and E) and by ANOVA with post hoc testing (F). DESP indicates desipramine; DOX, doxorubicin; FDA, US Food and Drug Administration; and SEM, standard error of the mean.
background; and critically reveal that another SNP within this cardioprotective locus, rs11140490 has the highest likelihood to be causal. Furthermore, this platform allowed us to discover that the SLC competitive inhibitor, desipramine protects against DIC without hampering doxorubicin chemotherapy efficacy.

The SLC28A3 genetic variant rs7853758 is the most robustly replicated AIC strongly associated cardioprotective loci. We show that rs7853758 is in perfect linkage disequilibrium with 23 other SNPs of which 22 are noncoding variants, forming the cardioprotective Hap. Pinpointing causative SNP within this locus is crucial for clinical translation because testing for the causal variant guarantees the detection of the best possible clinical correlation with AIC. Almost 93% of phenotype-associated genetic variants are noncoding. Using the ENCODE (Encyclopedia of DNA Elements) project dataset, it has been shown that ≈80% of GWAS-identified noncoding SNPs are not the causal SNP. Using our recently developed cost-effective fine-mapping pipeline, we found that rs11140490, but not the CGAS-identified hit rs7853758, is the variant with the highest likelihood to be causal in DIC.

Editing only the rs11140490 cardioprotective genotype (CT) back to the reference genotype (TT) in patient-specific hiPSC-CMs renders these cardiomyocytes more susceptible to DIC confirming the causality of this novel variants. It is interesting that editing rs11140490 increase the sensitivity of hiPSC-CMs by ≈28% which is modest and thus denotes that there might be more SNPs within the Hap that interact in an additive manner with rs11140490 to predispose to protection against DIC.

Going forward, we propose that a simple clinical test to detect the presence of rs11140490 can be used to predict that a patient will be less likely to experience DIC and that, with future clinical trials, it may be possible for these patients to be treated with a longer duration (higher cumulative dose) of doxorubicin to enhance the efficacy of their chemotherapy. Similarly, the rs11140490 genetic testing could be used as a part of a polygenic cardiotoxicity risk stratification score for doxorubicin-containing chemotherapy regimens such that the protective effect of rs11140490 could balance out for a risk factor that would have otherwise prevented the administration of a relevant doxorubicin-containing chemotherapy regimen to a particular patient.

SLC28A3encodes a cardiac-specific uptake transporter that has no or marginal expression in several cancer cells and hence, represents a highly druggable target to screen for cardioprotective agents. A large number of drugs have been identified as being trafficked by SLC transporters, which explains the substantial role of these transporters in both drug pharmacokinetics and pharmacodynamics and emphasizes the importance of this class of transporters in drug response disposition. Our high-throughput drug screening discovered that treating patient-specific cardiomyocytes with the SLC inhibitor desipramine protects against DIC through decreasing the intracellular uptake of doxorubicin into human heart. Our results suggest that a single dose of 3 μmol/L desipramine 24 hours before the administration of doxorubicin in addition to another dose of 3 μmol/L desipramine coadministered with doxorubicin is sufficient for protecting against DIC.

Desipramine is a tricyclic antidepressant sold under the brand name Norpramin and was first patented in 1962. The typical adult dose of desipramine is 100 mg to 200 mg/day. In more severely ill patients, the dosage may be gradually increased to 300 mg/day if necessary. The typical therapeutic concentration is 100 to 300 ng/mL with daily dosing. Our 3-μmol/L in vitro dose would be 798 ng/mL; it is important to note that our preliminary data suggest that just 2 doses, or potentially 1 dose per cycle, of doxorubicin would be required to attenuate DIC. We would therefore suggest that these desipramine doses are potentially within the acceptable clinical range, although further animal model work to confirming dosing strategy is required before clinical trial.

Desipramine is far from an ideal drug, being the most potent sodium channel blocker among its group and causing cardiotoxicity when used chronically. Desipramine-treated patients have been shown to have significantly lower rates of sinus pauses and junctional rhythm, but significantly higher rates of single or paired premature atrial contractions and runs of supraventricular tachycardia as well as lengthening of the QT interval. The solution to this is to develop a desipramine derivative without the sodium channel blockade effect, although approach eliminates the primary advantage of repurposing a drug like desipramine to attenuate DIC. Still, the advantage of using our hiPSC-CM platform for subsequent testing will be a major advantage in such an effort.

The majority (56%) of candidate drugs have failed in clinical trial because of lack of efficacy, most likely because the preclinical models used to test the drug does not recapitulate what happens in humans or in the specific target cell type. Additionally, studies staring with genetic correlations in genes encoding targets increases the success rate in clinical development by 2-fold. Patient-derived hiPSC-CMs provides a unique platform that first permits a thorough validation of GWAS-identified AIC-associated loci, and second recapitulate alteration in DIC phenotype in a human-relevant manner. Hence the utilization of hiPSC-CMs in the development of cardioprotectants substantially improves the potential of developing novel derivatives of desipramine that have the same SLC28A3 competitive inhibition effect as desipramine without the well-known side effects common to the tricyclic antidepressant drug family.

It is clear that hiPSC-CMs do not fully mimic the human whole-body model and do not recapitulate all the steps of drug pharmacokinetics and pharmacodynamics. However, hiPSC-CM generation methodologies of the present have enhanced the robustness, purity, maturation, and scalability.
to a point where these cells are suitable for a wide range of disease modeling and drug response assays. Here we demonstrated that patient-specific cardiomyocytes recapitulate intra-individual variability in genomic-dependent DIC susceptibility. We show that hiPSC-CMs are appropriate to study drug response-associated loci especially for genes with a known mechanism of action such as transporter-encoding genes. The integration of CRISPR/Cas9-based genetic editing to our patient-specific hiPSC model proves to be a powerful tool in identify causal genetic variations in relation to a specific drug response. The expansion of the utility of patient-specific hiPSC-CMs to study additional anthracycline-relevant loci, as well as those of other anticancer agents, will help to identify patient-/population-specific chemotherapeutic-induced cardiotoxicity genetic biomarkers and new cardioprotective agents. Ultimately, the information derived from this platform may allow physicians to tailor chemotherapeutics doses on the basis of patient genotype, bringing the promise of personalized medicine to the field of cardio-oncology.

ARTICLE INFORMATION
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PWB and TM designed the research for this work. TM, M.J., H.-H.K., C.J.W., D.L.-L., H.F., M.R-T., M.G., H.J., and P.W.B. performed the experiments; G.F. and D.B. designed and the performed mouse experiments; C.J.D.R. and B.C.C determined the anthracycline-induced cardioxopotency phenotype to be used for patient recruitment and their differentiated derivatives. T.M. and P.W.B. supervised the project. T.M. and P.W.B. wrote the final article with input from all other authors.

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Disclosures
None.

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