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Identification of genes associated to
2',2'-difluorodeoxycytidine resistance in HeLa cells
with a lentiviral short-hairpin RNA library

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ABSTRACT

Resistance to the cytotoxic nucleoside analog 2',2'-difluorodeoxycytidine (dFdC) used in cancer chemotherapy is a frequent cause of treatment failure. Although several molecular mechanisms that cause resistance to dFdC have been identified, many cells acquire dFdC resistance by unknown mechanisms. We have used a short-hairpin RNA (shRNA) library in a lentiviral vector that contains ≈ 5000 shRNAs designed against genes encoding kinases, phosphatases, tumor suppressors and DNA binding proteins to perform a loss-of-function screen to identify genes causing dFdC resistance in HeLa cells when their expression is decreased. 155 cell lines with shRNA expression were isolated from the screen and several of these cell lines were in repeated experiments verified to show resistance to dFdC compared to wild-type cells. DNA sequencing of the shRNA vector integrated in the cellular genome was used to determine the shRNA expressed in the cells and the putative target genes were identified by sequence analysis. 16 cell lines with putative target genes previously not associated to dFdC resistance were identified. Chemically synthesized short-interfering RNAs (siRNAs) directed against the target genes were used to verify that the decreased expression of the identified genes caused dFdC resistance. Using these techniques we identified two splicing factor proteins, serine/arginine-rich splicing factor 3 (SRSF3) and splicing factor proline/glutamine-rich (SFPQ), that induced resistance to dFdC as well as other pyrimidine nucleoside analogs when their expression was decreased in HeLa cells.

Keywords: nucleoside analog, short-hairpin RNA, drug resistance, chemotherapy, small-interfering RNA, RNA interference

1. INTRODUCTION

2',2'-difluorodeoxycytidine (dFdC)¹ is a cytosine nucleoside analog that is used in chemotherapy for a broad range of different malignant tumours [1]. It is currently used in treatment of pancreatic adenocarcinoma, breast cancer, non-small cell lung cancer, ovarian cancer, bladder cancer as well as head-neck cancer [1]. The cytotoxic effects of dFdC depend on import of the compound into cancer cells and its intracellular phosphorylation since the unphosphorylated form of the nucleoside analog is pharmacologically inactive. DFdC is imported into cells by nucleoside transporter proteins in the cell membrane and phosphorylated in the cytosol to its mono-, di- and triphosphate forms [2, 3]. The phosphorylation of dFdC to its triphosphate form is sequentially catalysed by deoxycytidine kinase (dCK), UMP-CMP kinase and nucleoside diphosphate kinases. The phosphorylated forms of dFdC have multiple modes of action and affect several intracellular targets. DFdC-TP is a substrate for DNA polymerases during DNA replication and inhibits this process by inducing chain termination during DNA strand elongation [4]. DFdC-DP binds ribonucleotide reductase and inhibits *de novo* dNTP synthesis catalysed by this enzyme [5]. Other possible modes of action for phosphorylated dFdC include inhibition of DNA topoisomerases [6].

Many patients treated with dFdC chemotherapy for malignant tumours fail to respond to the drug or develop resistance during the treatment. Several predictive markers for the response to dFdC chemotherapy have been identified [1]. These include the expression levels of dCK and nucleoside transporter proteins. However, these markers only partially explain the

¹ **Abbreviations:** araC, 1- β -arabinofuranocylcytosine; dCK, deoxycytidine kinase; dFdC, 2',2'-difluorodeoxycytidine; 5-FdUrd, 5-fluorodeoxyuridine; RNAi, RNA interference; shRNA, short-hairpin RNA; siRNA, short-interfering RNA; SFPQ, splicing factor proline/glutamine-rich; SRSF3, serine/arginine-rich splicing factor 3.

response to dFdC and the molecular mechanisms that cause resistance to dFdC are not known for many of the patients treated with this drug.

Short-hairpin RNA (shRNA) mediated gene silencing has in the last few years emerged as an efficient technique to selectively silence the expression of specific genes [7]. The shRNA technique is based on the expression of short double-stranded RNA molecules that in a sequence specific manner cause degradation of the target mRNA through endogenous RNA interference (RNAi) machinery and subsequent decreased production of the protein. This technique has successfully been used to study the loss-of-function effects of specific genes [8-10]. Libraries with large number of shRNAs that target multiple genes have also been used in functional screens to study cellular signalling pathways and to identify genes or novel gene functions [11-15].

We decided to use a human shRNA library in an attempt to identify genes that when the expression is decreased cause resistance to dFdC in a cancer cell line. A shRNA library in lentiviral vectors expressing ≈ 5000 shRNAs was used to induce suppression of gene expression in HeLa cells and the cells were treated with dFdC. Surviving cells that exhibited resistance to dFdC were isolated and cultured. The dFdC resistance of the cells were verified and the putative target genes were identified based on DNA sequence analysis of the integrated shRNA vector. In summary, we identified two novel genes that induced resistance to dFdC when their expression was decreased.

2. MATERIALS AND METHODS

2.1 Cell culture, lentiviral transduction and clonogenic assay

HeLa cells (American Type Culture Collection, VA, USA) were cultured in Dulbecco's modified eagle medium with GlutaMAX (Invitrogen, CA, USA) supplemented with 10 % fetal calf serum (Invitrogen, CA, USA), 10 U/ml penicillin G and 10 mg/ml streptomycin sulphate at 37 °C with 5 % CO₂. The RNAi consortium Human shRNA Library [16] was a kind gift of Dr. Jonas Larsson (Lund University, Sweden). The HeLa cells were seeded at 4×10^3 cells/cm² and incubated overnight. The media was then replaced with fresh cell culture media supplemented with 8 µg/ml polybrene (Sigma-Aldrich, Germany) and the lentiviral shRNA library with a calculated multiplicity of infection of one viral particle per cell. At 24 h after viral transduction the cell culture media containing 3 µM dFdC (Sigma-Aldrich, Germany) were added and the cells were incubated for 24 h in cell culture flasks. A clonogenic assay was performed to isolate cells resistant to dFdC [17-18]. In summary, the cells were trypsinized and seeded in 10 cm cell culture Petri dishes. After 24 h incubation the cell culture media were changed and after two weeks of culture cell colonies that had >50 cells were isolated with Cloning Cylinders (Millipore Corporate, MA, USA) and cultured independently. Control cell cultures that were not transduced with the lentiviral shRNA library were cultured in parallel and treated with dFdC as described above. Colony fixation-staining solution with glutaraldehyde 6 % (v/v) (Sigma-Aldrich, Germany), crystal violet 0.5 % (wt/v) (Sigma-Aldrich, Germany) in H₂O was added for 30 minutes to fixate and stain the cells. Colonies with > 50 cells were counted under a microscope.

2.2 DNA sequencing of shRNA encoding DNA inserts and identification of putative target genes

The dFdC resistant cell lines isolated from the screening were cultured and the DNA sequences of the proviral shRNA insert were determined. Genomic DNA from the cell lines were purified with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The proviral shRNA insert were PCR amplified from the genomic DNA with Crimson Taq DNA polymerase (New England Biolabs, MA, USA) and flanking oligonucleotide primers (5'-GAGGGCCTATTTCCCATGAT and 5'-GACGTGAAGAATGTGCGAGA). The PCR products were purified and cloned into the pGEM-T Vector (Promega, WI, USA) and their DNA sequences were determined with a cycle sequencing method using the BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0 cycle sequencing kits (Applied Biosystems, CA, USA) and analysed on a 3130xl capillary sequencer (Applied Biosystems, CA, USA). The 21-nucleotide DNA sequences encoding shRNA stems were determined and searching the NCBI database with the BLAST algorithm identified the putative target genes.

2.3 Verification of cellular dFdC resistance

The individual cell lines isolated in the screening were seeded in 96-well plates with 1×10^3 cell/well. After 4 h incubation, the cell culture medium was replaced with medium containing different concentrations of dFdC. The medium was changed after 48 h incubation. After another 48 h, the plates were washed and the Cell Proliferation Kit II (XTT) (Roche, IN, USA) was used to quantify the cell. The plates were analyzed with a Wallac 1420 Victor Plate Reader (Perkin Elmer, MA, USA).

2.4 Verification of target genes causing dFdC resistance

We used transient transfection of HeLa cells with siRNAs to verify the putative target genes associated with dFdC resistance. Ambion Silencer Select Predesigned or verified siRNAs (Applied Biosystems, CA USA) designed against SRSF3 (5'-AGAGCUAGAUGGAAGAACATT), SFPQ (5'-GCACGUUUGAGUACGAAUATT) and dCK (5'-CAACUUCGAUUAUCUUCAATT) were used for these experiments as described in the manufacture's instructions. 0.2 µl siPORT™ NeoFX™ (Applied Biosystems, CA USA) agent was used for transfection in each well of 96-well plate, and 600 cells in 80 µl medium was added afterward by the reverse transfection method. 48 hours post-transfection fresh cell culture medium containing dFdC, 1-β-arabinofuranosylcytosine (araC) (Sigma-Aldrich, Germany) or 5-fluorodeoxyuridine (5-FdUrd) (Sigma-Aldrich, Germany) was added. The XTT assays were performed as described above using a Labsystems Multiskan MCC/340 ELISA reader (Labsystems, Helsinki, Finland).

2.5 Cell proliferation assay with DNA incorporation of ³H-dThd

HeLa cells were cultured in 6-well plates to log phase. Two days after transfection with siRNAs, the cell media were replaced with fresh media containing 1 µM ³H-dThd (Sigma-Aldrich, Germany). After 2 hours incubation, the cells were washed three times to remove non-incorporated ³H-dThd, trypsinized, centrifuged and resuspended in 1 ml 10 % trichloric acid (Sigma-Aldrich, Germany) and then incubated 30 minutes at 4°C. DNA was precipitated by centrifugation and resuspended in 400 µl 0.5 M NaOH and 0.5 % SDS (Sigma-Aldrich, Germany). After neutralization with 100 µl 2 M HCl, the samples were mixed with 3 ml scintillation fluid and radioactivity of DNA-incorporated ³H-dThd was determined with a scintillation counter.

3. RESULTS

3.1 Isolation of cell lines expressing shRNAs associated to dFdC resistance

We decided to use a human shRNA library to suppress the expression of multiple genes and screen for shRNAs that induced resistance to dFdC in HeLa cells. A lentiviral shRNA library that contains ≈ 5000 individual 21-mer nucleotide shRNA constructs directed against kinases, phosphatases, tumor suppressors, DNA binding and modification enzymes was used for the screening [16]. We used a clonogenic assay to identify cell clones resistant to dFdC. First, we tested multiple dFdC concentrations to determine the lowest concentration of the nucleoside analog that reproducibly killed all cells and resulted in no formation of cell colonies in the experimental design. We found that $3 \mu\text{M}$ dFdC was the lowest concentration that in three independent experiments showed no formation of cell clones and this concentration was used for the screening (Figure 1). HeLa cells (3×10^6) were transduced with equivalent number of lentiviral particles and cultured for 24 h before addition of dFdC to allow the shRNAs to exert effects and suppress gene expression. $3 \mu\text{M}$ dFdC was added to the cells which were subsequently plated on Petri dishes (5×10^3 or 5×10^4 cells / plate) and cultured with dFdC in the cell culture medium. The cells were thereafter cultured in medium without dFdC and cell colonies formed in the plates were isolated and harvested from the plates. A total of 216 cell colonies were isolated from the screening and 155 of these could be expanded and cultured separately. There were no surviving cell colonies in the negative control cell cultures that consisted of HeLa cells that had not been transduced with the lentiviral shRNA library.

3.2 Sequence determination of shRNAs and identification of target genes

We determined the DNA sequences of the shRNA vector inserts to identify putative genes targeted by the shRNAs. Genomic DNA was isolated from the cell lines derived from

the clones and the regions of the integrated lentiviral DNA vector sequence that encoded the shRNA with 21-nucleotide stems were amplified by PCR, cloned into a plasmid vector and sequenced. The shRNAs contain by design hairpin structures that interfere with PCR amplification and DNA sequencing. We tested multiple PCR conditions for the individual cell clones. We were able to PCR amplify and determine the DNA sequence of the integrated lentiviral shRNA vectors from 85 cell lines. Several of the generated cell lines contained identical shRNA sequences and 33 cell lines that contained unique shRNAs were used for further studies.

3.3 Verification of cellular dFdC resistance

The HeLa cell lines that contained unique shRNA sequences were repeatedly tested for the sensitivity to 50 and 100 nM dFdC to verify that they retained resistance. This assay showed that 16 of the 33 cell lines exhibited relative resistance to dFdC compared to wild-type cells in the assay conditions ($p < 0.05$) (Figure 2). These cell lines expressed shRNA sequences that corresponded to 13 different putative gene targets (Table 1). All the gene targets were identified in several of the original 85 cell lines characterized with either two identical shRNA or two different shRNA directed against the same putative target gene.

3.4 Verification of target genes

To verify that the putative gene targets identified caused resistance to dFdC we designed and used chemically synthesized siRNAs to transiently transfect HeLa cells and test their sensitivity to dFdC. The siRNAs were designed from unrelated DNA sequence of the same gene target compared to the shRNA in the lentiviral vector. Only two of the 13 tested siRNAs showed consistently significant resistance to dFdC ($p < 0.05$) and these were the siRNAs directed against two splicing factors: serine/arginine-rich splicing factor 3 (SRSF3)

and splicing factor proline/glutamine-rich (SFPQ). The siRNAs directed against these two genes resulted in resistance to dFdC similar to a siRNA directed against dCK (Figure 3A). SiRNAs against the splicing factors also resulted in resistance to the pyrimidine nucleoside analogs araC and 5-FdUrd (Figure 3B and 3C).

3.5 Effects on cell proliferation

We assayed DNA incorporation of ^3H -dThd to determine the cell proliferation rate in cells transfected with the siRNAs designed against the two splicing factors SRSF3 and SFPQ. Cells transfected with siRNA against SRSF3 showed decreased DNA incorporation of ^3H -dThd suggesting that these cells had a lower proliferation rate compared to control cells transfected with non-targeting siRNA (Figure 4). There was no difference in ^3H -dThd incorporation between control cells and cells transfected with siRNA directed against SFPQ or dCK.

4. DISCUSSION

It is important to understand the molecular mechanisms of dFdC resistance to develop and improve its application in cancer chemotherapy. Several genes that cause dFdC resistance have been identified and the majority of these genes encode proteins that are known to be involved in the transport or metabolism of the compound [1]. However, since these identified genes cannot explain all patients with dFdC resistant tumour it is still important to identify novel genes that induce resistance also to understand the molecular mechanism how dFdC exerts its effects. We have used an shRNA library in a functional screen to identify genes that when their expression is decreased induce dFdC resistance. Using this method we identified 16 cell lines that encoded unique shRNA sequences and exhibited resistance to dFdC. Sequence analysis showed that the shRNA expressed in these cells targeted 13 putative genes with diverse functions (Table 1). However, of these 13 genes we were only able to verify two genes that cause dFdC resistance when their expression was decreased in unrelated experiments with siRNAs directed at other gene sequences. These experiments suggest that either the suppression of the gene expression by the transiently transfected siRNAs used for verification was less potent than that of the consistently expressed shRNA, or that other gene targets might be affected by the continuous expression of shRNA in cell lines. However, the identification of identical shRNAs in several of the independently isolated cell lines as well as the identification of several shRNAs with similar putative gene targets strongly suggests that the identified shRNAs are associated to dFdC resistance.

The two genes identified that both exhibited dFdC resistance when their expression was decreased by the shRNA in the screening and with independently designed siRNAs encode two splicing factors SRSF3 and SFPQ. The splicing factor family contains a heterogeneous group of proteins that constitute part of the spliceosome [19]. These proteins contain an RNA recognition motif for binding RNA and a domain for binding other proteins.

SFPQ has been most carefully studied of these two proteins and it is involved in spliceosome formation and catalysis of pre-mRNA splicing [20-22]. However, SFPQ also has other cellular functions and in association with other protein since it has been shown to be involved in non-homologous DNA recombination and DNA damage response [23, 24]. Similarly, SRSF3 has been shown to have other functions unrelated to its role in pre-mRNA splicing. A recent study has shown that the expression of this protein is up-regulated in epithelial ovarian cancer cells and that it is required for tumor growth and cell survival [25]. We do presently not know the molecular mechanisms by which the decreased expression of two splicing factor proteins causes dFdC resistance. We used ^3H -dThd DNA incorporation as a measurement of cell proliferation and showed that cells that were transfected with siRNA against SRSF3 exhibited decreased proliferation rate compared to control cells transfected with non-targeting siRNAs. However, we did not observe significant difference in proliferation rate for cells transfected with siRNAs against SFPQ compared to control cells. Accordingly, it is not likely that cell cycle effect alone, and the resulting decreased incorporation of dFdC into DNA, can account for the decreased sensitivity to dFdC in the cells with siRNA targeting the two splicing factors. Decreased expression of both SFPQ and SRSF3 also induced resistance to other nucleoside analogs such as araC and 5-FdUrd. These findings suggest that decreased expression of the splicing factors induce drug resistance by a molecular mechanism that is shared by several different nucleoside analogs.

Li and co-workers have performed a genome-wide association study with multiple cell lines to analyse single nucleotide polymorphisms and mRNA expression levels to identify genes that affected dFdC sensitivity [26, 27]. Using these techniques they have identified genes and genetic variations associated to nucleoside analog resistance. In the present study we have shown that the shRNA library-mediated gene silencing is also a useful tool for identification of novel genes that are associated to nucleoside analog resistance, and we

believe that this technique will be important in studies to identify novel drug targets as well as elucidate the pharmacological mechanism of chemotherapeutic drugs.

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6. ACKNOWLEDGEMENTS

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7. LEGENDS FOR FIGURES

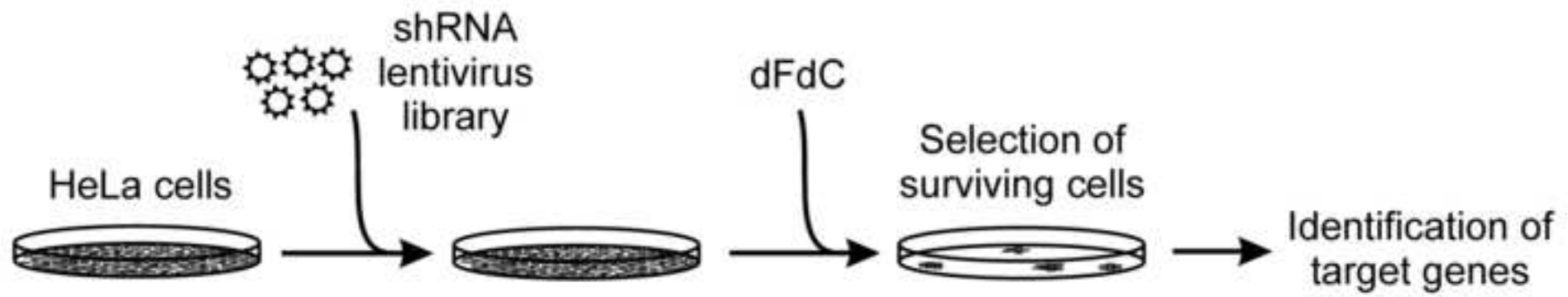
Figure 1. Sensitivity of HeLa cells to dFdC in a clonogenic assay with 5×10^3 (o) or 10×10^3 (♦) cells per 100 mm cell culture plate.

Figure 2. Sensitivity of 33 HeLa cell lines expressing unique shRNAs derived from the screening to 50 or 100 nM dFdC. *, cell lines exhibiting significant resistance to either dFdC concentration compared to control cells ($p < 0.05$). wt, wild-type HeLa cells.

Figure 3. Sensitivity of HeLa cells transfected with siRNAs against the splicing factors SRSF3 and SFPQ to 0.1 μ M dFdC (A), 5 μ M araC (B) or 1 μ M 5-FdUrd (C). SiRNAs against dCK were used as positive control for dFdC and araC resistance. Negative control cells (control) were transfected with non-targeting siRNA. **, $p < 0.01$.

Figure 4. ^3H -dThd incorporation into DNA of HeLa cells transfected with siRNAs against the splicing factors SRSF3 and SFPQ or the nucleoside kinase dCK. Negative control cells (control) were transfected with non-targeting siRNA. **, $p < 0.01$

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DNA sequence of shRNA stem	Putative gene target	Abbreviation	Clones
ACAATGGCAACAAGACGGAAT	serine/arginine-rich splicing factor 3	SRSF3	4
TGGAAGTGTGCAATGGTGAAA	serine/arginine-rich splicing factor 3	SRSF3	2
CCCAGAATCCAACAATAGCGT	protein kinase, AMP-activated, gamma 1 non-catalytic subunit	PRKAG1	1
GCTTGTCTGCATTTCTCCTAA	protein kinase, AMP-activated, gamma 1 non-catalytic subunit	PRKAG1	2
CCACAGTTTCCTAAGCGCATA	PDLIM1 interacting kinase 1 like	PDIK1L	1
CTAAGCAGTATCAAGAGCCAA	PDLIM1 interacting kinase 1 like	PDIK1L	1
GAGAGTTCTTGCATTGGAACT	telomeric repeat binding factor 2, interacting protein	TERF2IP	6
GACCAACAAATCTCAAGCCCT	splicing factor proline/glutamine-rich	SFPQ	4
CCAAGCAGCACCATCATCCAT	EPH receptor A4	EPHA4	4
CACAGGTTATACGAGGGACAA	pyridoxal (pyridoxine, vitamin B6) kinase	PDXK	3
CAGCCGAAGTCTCATCTCAGT	sedoheptulokinase	SHPK	3
TACATTGTGAGCTCTGGTTAT	mitogen-activated protein kinase kinase 4	MAP2K	2
GAAGTTGATTCAGATCCAAGA	mutL homolog 1, colon cancer, nonpolyposis type 2	MLH1	2
GAATCACAGACTTTGGACAAA	caspase 8, apoptosis-related cysteine peptidase	CASP8	2
GTTCAGTTCCTTATCTACCAA	mitogen-activated protein kinase 14	MAPK14	2
GCAGCAGACTCCACTCCACTT	NFk light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	2
CAGACCCTAATGATCCAAATA	SFRS protein kinase 1	SRPK1	2
CTGGCGGAATACTGCATCAAA	ubiquitin-conjugating enzyme E2R 2	UBE2R2	2
TAACTATTGTGGACCAGACAA	POT1 protection of telomeres 1 homolog	POT1	2

Table 1 – DNA sequences of the shRNA stems associated to dFdC resistance and the putative gene targets. The number of cell clones expressing identical shRNA of the original 85 characterized cell lines is indicated.

Figure 1
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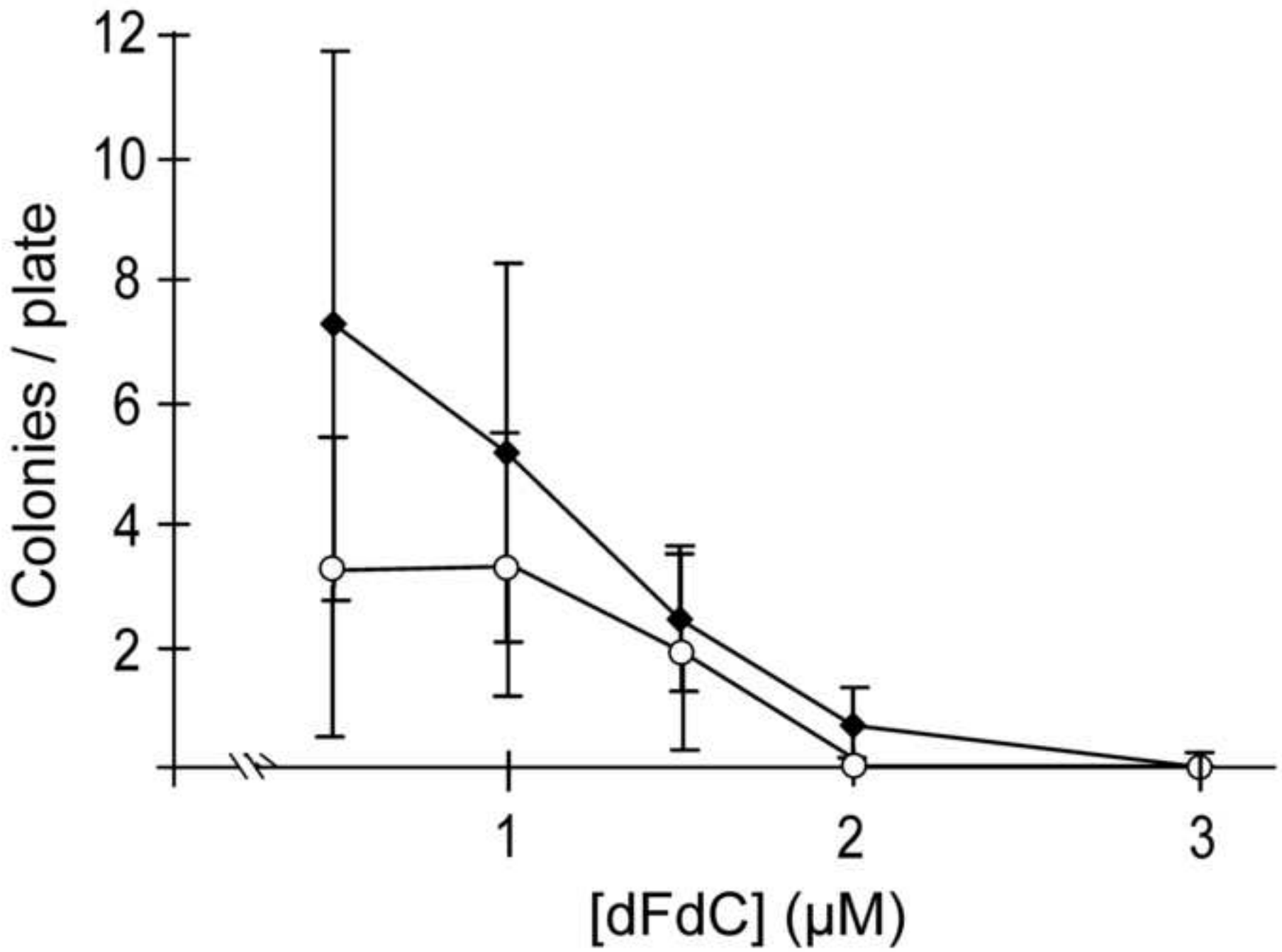


Figure 2
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