Biochemical Pharmacology 83 (2012) 858–865

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

DNA demethylation increases sensitivity of neuroblastoma cells to chemotherapeutic drugs

Jessica Charlet [a], Michael Schnekenburger [b], Keith W. Brown [a,*], Marc Diederich [b]

[a] University of Bristol, School of Cellular and Molecular Medicine, Medical Sciences Building, University Walk, Bristol, BS8 1TD, UK
[b] Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, L-2540, City of Luxembourg, Luxembourg

ARTICLE INFO

Article history:
Received 18 November 2011
Accepted 10 January 2012
Available online 18 January 2012

Keywords:
Neuroblastoma
Chemotherapeutic drugs
MYCN
DNA methylation
5-Aza-2′-deoxycytidine

ABSTRACT

Neuroblastoma is a common embryonal malignancy in which high-stage cases have a poor prognosis, often associated with resistance to chemotherapeutic drugs. DNA methylation alterations are frequent in neuroblastoma and can modulate sensitivity to chemotherapeutic drugs in other cancers, suggesting that manipulation of epigenetic modifications could provide novel treatment strategies for neuroblastoma. We evaluated neuroblastoma cell lines for DNA demethylation induced by 5-Aza-2′-deoxycytidine, using genome-wide and gene-specific assays. Cytotoxic effects of chemotherapeutic agents (cisplatin, doxorubicin and etoposide), with and without 5-Aza-2′-deoxycytidine, were determined by morphological and biochemical apoptosis assays. We observed that the extent of genome-wide DNA demethylation induced by 5-Aza-2′-deoxycytidine varied between cell lines and was associated with expression differences of genes involved in the uptake and metabolism of 5-Aza-2′-deoxycytidine. Treatment of neuroblastoma cells with a combination of chemotherapeutic drugs and 5-Aza-2′-deoxycytidine significantly increased the levels of apoptosis induced by cisplatin, doxorubicin and etoposide, compared to treatment with chemotherapeutic drugs alone. The variable demethylation of cell lines in response to 5-Aza-2′-deoxycytidine suggests that epigenetic modifiers need to be targeted to suitably susceptible tumours for maximum therapeutic benefit. Epigenetic modifiers, such as 5-Aza-2′-deoxycytidine, could be used in combination with chemotherapeutic drugs to enhance their cytotoxicity, providing more effective treatment options for chemoresistant neuroblastomas.

1. Introduction

Neuroblastoma (NBL) is one of the commonest solid childhood cancers, which arises from neural crest cells of the sympathetic nervous system and causes about 15% of all paediatric oncology deaths [1]. NBLs diagnosed antenatally or in the newborn period have a good prognosis, unlike in older children, who have a poor outcome [2].

High-risk tumours with disseminated disease (stages 3–4) often bear *MYCN* amplification [3] and are mostly fatal [4]. Other genetic alterations in NBL include loss of chromosome 1p, loss of 11q and gain of 17q, which are independent of *MYCN* status [1,2]. Genes found mutated in NBL include the tumour suppressors *PHOX2B*, mutated in a few cases of inherited NBL [5], *NF1*, mutated in 6% of primary NBLs [6] and p53, which is mutated in 2% of NBLs, however other functional defects in the p53 pathway such as *MDM2* amplification are found in NBL [7]. The proto-oncogene *ALK* is frequently mutated in familial NBL [8] and in about 10% of sporadic cases, where it is associated with poor prognosis [9].

In addition to genetic abnormalities, epigenetic deregulation plays an important role in NBL pathogenesis, including aberrant promoter DNA hypermethylation of tumour suppressor genes such as *RASSF1A*, *CASP8* and *DCR2* [10–13]. DNA hypermethylation of individual genes e.g. *CASP8* may be associated with poor outcome in NBL [11,14,15] but methylation at multiple CpG islands may be more closely associated with poor prognosis [16]. A recent genome-wide analysis of DNA methylation in NBL has identified large-scale genomic alterations [17], as we have described in Wilms' tumour [18], suggesting that both large-scale and gene-specific epigenetic changes contribute to the pathogenesis of embryonal tumours.

One important phenotypic consequence of DNA hypermethylation events may be resistance to chemotherapeutic drugs [19], leading to treatment failure as observed in relapsed NBLs [20,21], in which apoptosis genes may be epigenetically silenced [22]. Inhibitors of DNA methyltransferases, such as 5-azacytidine and 5-Aza-2′-deoxycytidine (5-Aza-dC), have been used to successfully re-sensitise cancer cells to chemotherapeutic drugs [23–27],

* Corresponding author. Tel.: +44 0 117 33 12071; fax: +44 0 117 33 12091.
E-mail addresses: J.Charlet.07@bristol.ac.uk (J. Charlet), michael.schnekenburger@lbmcc.lu (M. Schnekenburger), keith.brown@bristol.ac.uk (K.W. Brown), marc.diederich@lbmcc.lu (M. Diederich).

0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2012.01.009

suggesting that this could be a beneficial strategy for therapy-resistant NBL. Clinical chemotherapy resistance in NBL has previously been shown to be reflected in neuroblastoma cell cultures [21], demonstrating that neuroblastoma cell lines provide relevant in vitro models for studying mechanisms of drug resistance and the re-establishment of drug sensitivity. We therefore asked whether inhibition of DNA methylation by 5-Aza-dC in human NBL cell lines could increase their sensitivity to clinically relevant cytotoxic drugs.

Here we show, for the first time, that pre-treatment of NBL cell lines with 5-Aza-dC significantly increases their sensitivity to cisplatin, etoposide and doxorubicin. Interestingly, NBL cell lines vary in their extent of DNA demethylation in response to 5-Aza-dC, associated with altered expression of transporters and enzymes involved in the metabolism of 5-Aza-dC. These results reveal a possible novel epigenetic strategy to fight high-stage, aggressive and chemoresistant NBLs, whilst highlighting the necessity to target treatment to those tumours that are most responsive to demethylating agents.

2. Materials and methods

2.1. Cell culture and treatments

The NBL cell lines BE(2)-M17, SK-N-AS and SHSY-5Y were from our collaborator Dr C. McConville, University of Birmingham, UK, who obtained them directly from ECACC (Porton Down, Salisbury, UK). All cell lines originated from patients that had undergone chemotherapy [28]. Simple tandem repeat (STR) fingerprints (D13S317, D16S539 and D5S818) were initially tested in the McConville lab and further verified at 2 loci (D16S539 and TH01) in the Brown lab. All STR results matched those in the Cell Line Integrated Molecular Authentication database (bioinformatics.ist-ge.it/clima/). Additionally, all lines were karyotyped in the McConville lab and assayed by qPCR for *MYCN* amplification in the Brown lab and results were completely consistent with published results.

NBL cell lines were cultured in DMEM/F12-HAM medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids (Sigma) at 37 °C in a humidified 5% CO_2 incubator. Prior to treatment, cells were seeded for 24 h at 10^5 cells per well in 6-well dishes. Cisplatin was obtained from Teva pharmaceuticals (Platosin®, Bucharest, Romania) at a stock concentration of 1 mg/ml. Doxorubicin, etoposide and 5-Aza-dC (5-Aza-2′-deoxycytidine) were purchased from Sigma and were at stock concentrations of 1 mM in H_2O, 50 mM in DMSO and 10 mM in DMSO respectively. Single drug treatments were for 24 h; combinatorial treatments were performed by pre-treatment with 5-Aza-dC at 2 μM for 3 days and then the chemotherapeutic drug was added on the fourth day for 24 h.

2.2. Morphological determination of apoptosis

1 μg/ml Hoechst 33342 (Sigma) was directly added to the cells and cultures were incubated for 10–15 min at 37 °C, then 1 μg/ml propidium iodide (Sigma) was added and cells were analysed on a fluorescence microscope for viable, apoptotic, necrotic and secondary necrotic cells by observing cell colour and nuclear morphology. Each field of view contained approximately a hundred cells that were counted by eye.

2.3. Statistical analysis

The Chi^2 test was used to determine whether there was a significant increase in apoptosis for the combination treatments (5-Aza-dC plus chemotherapeutic drugs) compared to separate treatments.

2.4. Whole genome DNA methylation analysis

Genomic DNA was extracted from cells with the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's protocol. Methylation sensitive restriction analysis (MSRA) was used to investigate genomic DNA methylation in the cell lines. Briefly, 1 μg DNA was digested with either HpaII or MspI (New England Biolabs) and digests were run on an agarose gel [27]. Percentage methylation loss was determined using ImageJ software (rsbweb.nih.gov/ij/) by comparing the intensity of the high molecular weight band in the HpaII lane to the undigested control lane.

2.5. Methylation-specific PCR (MSP)

Bisulphite conversion of genomic DNA was performed with the MethylDetector kit (Active Motif) according to manufacturer's protocol. Bisulphite converted DNA was amplified with gene specific primers (Table 1) for M (methylated) and UM (unmethylated) DNA by end-point PCR, using HotStarTaq DNA Polymerase (Qiagen) according to manufacturer's protocol. PCR amplicons were then run on a non-denaturing polyacrylamide gel.

2.6. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted with the RNeasy elution kit (Qiagen) according to the manufacturer's protocol. RNA was DNase treated with the TURBO DNA-free kit (Applied Biosystems) and cDNA was synthesised using the Thermoscript RT-PCR system (Invitrogen). Gene-specific primers (Table 1) were used to measure mRNA levels with the SYBRGreen kit (Invitrogen) on an MX3000P real-time PCR machine (Stratagene). The amount of target gene was normalised to the endogenous level of *TBP*.

Table 1
Primers used in qRT-PCR and MSP. Sequences of primers used in PCR; M = MSP primers specific for methylated DNA, UM = MSP primers specific for unmethylated DNA.

Method	Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
qRT-PCR	*RASSF1A*	TGCGACCTCTGTGGCGACTTCAT	TAGTGGCAGGTGAACTTGCAATGCG
	DNMT1	TCAGCAAGATTGTGGTGGAG	CAAGTTGAGGCCAGAAGGAG
	DNMT3A	TGCCAAAACTGCAAGAACTG	CAGCAGATGGTGCAGTAGGA
	DNMT3B	TTTGGCCACCTTCAATAAGC	GGTCCTCCAATGAGTCTCCA
	TBP	GCCCGAAACGCCGAATAT	CCGTGGTTCGTGGCTCTCT
	CDA	TGCCCCTACAGTCACTTTCC	CGGGTAGCAGGCATTTTCTA
	DCK	TCTCCATCGAAGGGAACATC	TCAGGAACCACTTCCCAATC
	ENT1	TCTTCTTCATGGCTGCCTTT	CCTCAGCTGGCTTCACTTTC
	ENT2	TCCTCATGTCCATCGTGTGT	AGCTCAGCTTTGGTCTCCAG
MSP	*RASSF1A* (M)	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA
	RASSF1A (UM)	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA