Biochemical Pharmacology 87 (2014) 611–624

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

The novel pyrrolo-1,5-benzoxazepine, PBOX-6, synergistically enhances the apoptotic effects of carboplatin in drug sensitive and multidrug resistant neuroblastoma cells

Jennifer C. Lennon [a,b,*], Sandra A. Bright [a], Eilis Carroll [a], Stefania Butini [c], Giuseppe Campiani [c], Anne O'Meara [d], D. Clive Williams [a], Daniela M. Zisterer [a]

[a] School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, 152-160 Pearse Street, Dublin 2, Ireland
[b] The National Children's Research Centre, Crumlin, Dublin, Ireland
[c] European Research Centre for Drug Discovery & Development, University of Siena, Siena, Italy
[d] Our Lady's Childrens Hospital, Crumlin, Dublin, Ireland

ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form 19 December 2013
Accepted 20 December 2013
Available online 6 January 2014

Keywords:
PBOX-6
Carboplatin
Neuroblastoma
Apoptosis
Synergy

ABSTRACT

Neuroblastoma, a malignancy of neuroectoderrmal origin, accounts for 15% of childhood cancer deaths. Despite advances in understanding the biology, it remains one of the most difficult paediatric cancers to treat. A major obstacle in the effective treatment of neuroblastoma is the development of multidrug resistance (MDR). There is thus a compelling demand for new treatment strategies for this cancer that can bypass such resistance mechanisms. The pyrrolo-1,5-benzoxazepine (PBOX) compounds are a series of novel microtubule-targeting agents that potently induce apoptosis in various cancer cell lines, *ex vivo* patient samples and *in vivo* cancer models. In this study we examined the ability of two members, PBOX-6 and -15, to exhibit anti-cancer effects in a panel of drug sensitive and MDR neuroblastoma cell lines. The PBOX compounds potently reduced the viability of all neuroblastoma cells examined and exhibited a lower fold resistance in MDR cells when compared to standard chemotherapeutics. In addition, the PBOX compounds synergistically enhanced apoptosis induced by etoposide, carboplatin and doxorubicin. Exposure of drug sensitive and resistant cell lines to PBOX-6/carboplatin induced cleavage of Bcl-2, a downregulation of Mcl-1 and a concomitant increase in Bak. Furthermore, activation of caspase-3, -8 and -9 was demonstrated. Finally, gene silencing of Mcl-1 by siRNA was shown to sensitise both drug sensitive and multidrug resistant cells to carboplatin-induced apoptosis demonstrating the importance of Mcl-1 downregulation in the apoptotic pathway mediated by the PBOX compounds in neuroblastoma. In conclusion, our findings indicate the potential of the PBOX compounds in enhancing chemosensitivity in neuroblastoma.

1. Introduction

The paediatric malignancy neuroblastoma arises in cells of the neural crest [1]. It is both the most commonly diagnosed tumour in the first year of life and the most common extra cranial solid tumour in early childhood accounting for 15% of cancer deaths in young children [2]. Despite both advances in understanding the biology of the cancer and aggressive treatment strategies, including surgery, chemotherapy, radiation therapy, immunotherapy and differentiation therapy, it continues to be one of the most difficult paediatric cancers to successfully treat with more than 60% of patients presenting at diagnosis with adverse features with less than 40% of these whom achieve disease free long term survival [3]. A major obstacle in the effective treatment of neuroblastoma is the development of multidrug resistance (MDR) to a broad range of cytotoxic drugs [4–6] including vinca alkaloids, alkylating agents, platinum compounds and anthracyclines. This MDR phenotype often includes amplification of the proto oncogene MYCN, mutation/deletion of p53, deletion of loci at 1p or 11q and over expression of MDR proteins leading to altered drug transport and an increase in drug efflux [1]. There is therefore an urgent need for alternative approaches to treatment.

Abbreviations: Bcl-2, B cell lymphoma-2; BCRP, breast cancer resistance protein; CML, chronic myeloid leukaemia; DTT, Dithiothreitol; FAC, fluorescence associated cell sorter; FBS, foetal bovine serum; Mcl-1, Myeloid cell leukaemia 1; MDR, multidrug resistance; MRP1, multidrug resistance associated protein; MTA, microtubule targeting agent; PARP, Poly (ADP-ribose) polymerase; PBOX, pyrrolo-1,5-benzoxazepine.

* Corresponding author.
E-mail addresses: lennonje@tcd.ie (J.C. Lennon), brightsandi@gmail.com (S.A. Bright), e.carroll@dundee.ac.uk (E. Carroll), butini3@unisi.it (S. Butini), campiani@unisi.it (G. Campiani), amwom@eircom.net (A. O'Meara), clive.williams@tcd.ie (D.C. Williams), dzistrer@tcd.ie (D.M. Zisterer).

0006-2952/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2013.12.017

MDR proteins are members of a family of transmembrane ABC (ATP binding cassette) transporters that use ATP hydrolysis to mediate the export of hydrophobic drugs from the cell. Some of the best studied multidrug resistant proteins of the ABC family are ABCB1 (also called multidrug resistant protein (MDR-1) or *P*-glycoprotein (*p*-gp)), ABCC1 (also called multidrug resistance associated protein (MRP-1)) and breast cancer resistance protein (BCRP) [7]. The 170 kDa -gp is a transmembrane efflux pump with low substrate specificity and this MDR protein has been shown to be overexpressed in neuroblastoma patients where it predicts poor outcome [8] indicating that there is a need for alternative chemotherapeutic agents which are not substrates for these transporters.

Some PBOX compounds have been identified as novel microtubule-targeting agents (MTAs) [9] that possess the ability to potently induce cell cycle arrest and apoptosis in a number of cancer cell lines derived from both solid tumours and haematological malignancies [10–16]. Additionally these compounds have been shown to be efficacious in *in vivo* chronic myeloid leukaemia (CML) [14] and breast cancer animal models [17] and in *ex vivo* studies [11,14]. We previously demonstrated that PBOX-15 induces apoptosis both in *ex vivo* B-cell lymphocytic leukaemia (CLL) cells harbouring poor prognostic indicators and fludarabine resistance-associated p53 deletions [11] and in primary CML patient samples including those resistant to STI-571, the current frontline treatment for CML [13]. Unlike many chemotherapeutic agents, the PBOX compounds are not substrates for many MDR drug efflux pumps [12]. PBOX-6 and 15 were shown to induce comparable levels of apoptosis in both drug sensitive and MDR human promyelocytic leukaemia and ovarian carcinoma cell lines expressing either *p*-gp or BCRP, two of the main ABC transporters associated with MDR, indicating that they may possess advantages over standard chemotherapy which is currently in clinical use.

In this article, we extend our study of the PBOX compounds to neuroblastoma cells including those displaying resistance to chemotherapeutics currently used in the clinic. We show that two representative PBOX compounds reduce the viability of a panel of drug sensitive and MDR neuroblastoma cell lines with similar potency. We also delineate part of the mechanism of PBOX anti-cancer activity in these cells.

We demonstrate caspase-dependent apoptosis. Additionally we demonstrate an early downregulation of anti-apoptotic Mcl-1 and a concomitant upregulation in pro-apoptotic Bak. Importantly, we show that the PBOX compounds synergise with standard chemotherapy in both drug sensitive and MDR neuroblastoma cells and we elucidate part of the mechanism of PBOX potentiation of carboplatin-induced apoptosis in neuroblastoma cells. This study suggests that the PBOX compounds, either alone or in combination with standard chemotherapeutics, have potential as an effective therapy against neuroblastoma.

2. Materials and methods

2.1. Cell culture

SHSY5Y and SK-N-BE(1) cells were cultured in DMEM/F12 + GlutaMAX medium supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. Kelly and SK-N-BE(2)c cells were grown in RPMI 1640 + GlutaMAX medium supplemented with 10% (v/v) (FBS) and 1% (v/v) penicillin/streptomycin. SK-N-FI cells were grown in DMEM + Glutamax medium supplemented with 10% FBS and 1% penicillin/streptomycin. CHLA-90 cells were grown in IMDM medium supplemented with 1% (v/v) insulin/transferrin/selenium, 10% (v/v) (FBS) and 1% (v/v) penicillin/streptomycin. Cells were incubated in a humidified environment at 95% O_2 and 5% CO_2 and passaged twice a week.

2.2. Reagents

The pyrrolo-1,5-benzoxazepine compounds, 7-[(*N,N*-dimethylcarbamoyl)oxy]-6-(naphth-1-yl)pyrrolo[2,1-][1,5]benzoxazepine (PBOX-6) and 4-acetoxy-5-(1-(naphthyl)naphtho[2,3]pyrrolo[2,1-D][1,4]oxazepine (PBOX-15) were synthesised as described previously [18] and dissolved in ethanol. Vincristine (Tocris, Bristol, U.K), doxorubicin (Sigma Aldrich, St Louis, MO, USA) and carboplatin (Sigma Aldrich, St Louis, MO, USA) were dissolved in dH_20 and sterile filtered. Etoposide (Sigma Aldrich, St Louis, MO, USA) was reconstituted in DMSO (Sigma Aldrich, St Louis, MO, USA. All antibodies with the exception of cytochrome which was sourced from BD Bioscience (Oxford, UK) and Bax which was sourced from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained from Millipore (Cork, Ireland). Caspase inhibitors were purchased from Millipore (Cork, Ireland). All media and FBS were obtained from Invitrogen (Paisley, U.K.). The enhanced chemiluminescence reagents were supplied by Amersham Biosciences (Buckinghamshire, U.K.), The BCA reagents were from Pierce (Illinois, U.S.), the polyvinylidene difluoride membranes from Millipore (Cork, Ireland), while the protease inhibitors were obtained from Roche (Clare, Ireland). Unless otherwise stated all chemicals were obtained from Sigma-Aldrich (Sigma Aldrich, St Louis, MO, USA), cell culture materials were sourced from Greiner Bio-One GmbH (Stonehouse, U.K.).

2.3. AlamarBlue viability assay

Cells were seeded at densities varying from 25 000–80 000 cells/mL in a 96 well plate in a volume of 200 μL medium, left overnight to attach and treated with a range of concentrations of PBOX-6, vincristine, doxorubicin, etoposide and carboplatin for 72 h AlamarBlue (final concentration 10% (v/v)) was added to each well and left to incubate in the dark at 37 °C for 5 h. Fluorescence was measured using a SpectraMax Gemini plate reader (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 544 nm and 590 nm, respectively. The mean of each triplicate was calculated. Vehicle treated wells were set at 100% viability and treated wells were calculated as a percentage of the vehicle control. Dose response curves were plotted and IC_{50} values obtained using Prism GraphPad 4.

2.4. Flow cytometry

Following the required treatment, floating and adherent cells were harvested and fixed in 70% ethanol/PBS. Fixed samples were stored at −20 °C until required. Ethanol was removed by centrifugation and pellets were incubated in 400 μL FACS flow sheath fluid supplemented with 10 μg/mL RNase A (Sigma Aldrich, St Louis, MO, USA) and 100 μg/mL propidium iodide (Sigma Aldrich, St Louis, MO, USA). Cells were incubated in the dark at 37 °C for 30 min. Analysis was performed on a FACScalibur Fluorescence Associated Cell Sorter (FACS) (Becton–Dickinson, San Jose, CA, USA) using Cell Quest and Quanti-Quest software. Samples were first gated using a vehicle control to eliminate debris and cell aggregates from analysis. 10 000 cells from each sample were counted and results were visualised on histrograms.

Mitochondrial membrane potential was analysed using 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanineiodide (JC-1) (Invitrogen, Paisley, U.K.) an aggregate forming lipophilic dye. JC-1 was stored as a 200 μM solution in DMSO and added to cells at a final concentration of 5 μM for 20 min at 37 °C. Cells were collected by centrifugation at 300 × *g* for 5 min and the pellets were re-suspended in 400 μL PBS. Cells were treated with 50 mM Carbonylcyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial membrane depolariser, for 5 min as a positive control for