Biochemical Pharmacology 103 (2016) 29–39

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Proteasomal inhibition potentiates drugs targeting DNA topoisomerase II

Ka C. Lee [a], Rebecca L. Bramley [a], Ian G. Cowell [a], Graham H. Jackson [b], Caroline A. Austin [a,*]

[a] Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom
[b] Institute for Cellular Medicine, Newcastle University, Newcastle upon Tyne NE2 4HH, United Kingdom

ARTICLE INFO

Article history:
Received 12 November 2015
Accepted 22 December 2015
Available online 12 January 2016

Keywords:
DNA topoisomerase II
Proteasome
Mitoxantrone
Velcade
MG132

ABSTRACT

The reaction mechanism of DNA topoisomerase II (TOP2) involves a covalent double-strand break intermediate in which the enzyme is coupled to DNA via a 5′-phosphotyrosyl bond. This normally transient enzyme-bridged break is stabilised by drugs such as mitoxantrone, mAMSA, etoposide, doxorubicin, epirubicin and idarubicin, which are referred to as TOP2 poisons. Removal of topoisomerase II by the proteasome is involved in the repair of these lesions. In K562 cells, inhibiting the proteasome with MG132 significantly potentiated the growth inhibition by these six drugs that target topoisomerase II, and the highest level of potentiation was observed with mitoxantrone. Mitoxantrone also showed the greatest potentiation by MG132 in three Nalm 6 cell lines with differing levels of TOP2A or TOP2B. Mitoxantrone was also potentiated by the clinically used proteasome inhibitor PS341 (Velcade). We have also shown that proteasome inhibition with MG132 in K562 cells reduces the rate of removal of mitoxantrone or etoposide stabilised topoisomerase complexes from DNA, suggesting a possible mechanism for the potentiation of topoisomerase II drugs by proteasomal inhibition.

1. Introduction

Type II DNA topoisomerases (TOP2s) play a role in several cellular processes including replication, transcription, chromosome condensation and segregation and permit the alteration of DNA topology by allowing one double-stranded DNA segment to pass through another. They achieve this by introducing an enzyme-bridged double-strand break into the first DNA segment, where each monomer of the dimeric enzyme remains covalently attached to the ends of the DSB via a 5′-phosphotyrosyl linkage. The second DNA segment is then "passed" through the enzyme-bridged DNA gate, and the break is re-ligated. The enzyme-bridged gate is normally a short lived intermediate, but a group of drugs, known as "TOP2 poisons" inhibit the religation step resulting in the formation of an unusual type of DSB in which the TOP2 protein remains covalently linked to the DNA. These breaks are cytotoxic, hence the utility of TOP2 poisons such as etoposide, epirubicin and mitoxantrone in cancer therapy. Cells deficient in KU or LIG4 are extremely sensitive to TOP2 poisons, as are cells treated with the DNA-PK_{cs} inhibitor NU7026, implicating NHEJ in the repair of TOP2-induced DSBs [1–6]. However, TOP2-linked DSBs do not activate DNA-PK in vitro [7] and various lines of evidence suggest cellular processing is required before TOP2-induced breaks elicit a DNA damage response [8,9]. A number of potential mechanisms exist to remove TOP2 adducts from DNA to allow repair by NHEJ, including cleavage by an AP lyase activity such as KU [2,10]; removal of the DNA end bearing the TOP2 by a nuclease such as MRE11 [11–13] or proteolysis of the TOP2 protein followed by the action of a 5′-tyrosyl DNA phosphodiesterase, TTRAP/TDP2 [14–17].

A number of studies have implicated the proteasome pathway in regulating TOP2 protein levels, although the precise situation is complex and probably depends on cell type and the nature of the stresses that cells are exposed to in different experimental situations. Two topoisomerase orthologues exist in mammalian cells, *TOP2A* and *TOP2B* [18–20] and both are targeted by topoisomerase poisons such as etoposide [21]. TOP2A but not TOP2B is degraded in a cell cycle dependent manner in a way that appears to involve the ubiquitin–proteasome pathway [22] and TOP2A degradation has also been observed in a number of conditions that generate cellular stress, including adenovirus infection, glucose starvation and oxidative stress [23–27]. The degradation of TOP2A is ubiquitin and ATP dependent, and a number of ubiquitin ligases including BRCA1, FBW7, MDM2, BMI1/RING1A and ECV have been reported to associate with TOP2A [26–31]. TOP2 poisons trigger proteasome dependent decreases in TOP2 protein levels. It has been reported that etoposide and teniposide trigger reduction of TOP2A and TOP2B protein [9,28,30,32,33], whilst ICRF-193 (a catalytic inhibitor) triggers proteasome dependent degradation of only TOP2B

* Corresponding author.
E-mail address: caroline.austin@ncl.ac.uk (C.A. Austin).

http://dx.doi.org/10.1016/j.bcp.2015.12.015
0006-2952/

[34,35]. Stabilisation of TOP2 by proteasomal inhibition has been reported to overcome resistance to TOP2 poisons [36,37]. Here we show that inhibition of the proteasome with MG132 or PS341 potentiates the growth inhibition by some drugs targeting topoisomerase II. In addition, we show that in K562 cells proteasomal inhibition by MG132 reduces the rate of removal of etoposide or mitoxantrone stabilised covalent topoisomerase II complexes from the genomic DNA following drug removal from the media.

2. Material and methods

2.1. Cell lines

The CML cell line K562, the human pre-B cell line Nalm-6 and the *TOP2A*$^{+/-}$ and *TOP2B*$^{-/-}$ derivatives of Nalm-6 [38] were cultured in RPMI 1640 medium (Life Technologies, UK) containing 10% foetal bovine serum (Life Technologies, UK) and antibiotics (Life Technologies, UK). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Nalm-6 and its derivatives have a normal p53 status [39], whilst K562 is null for p53 [40,41].

2.2. Growth inhibition assays

Cells were seeded in 96 well plates (Greiner-Bio-ONE, UK) and incubated at 37 °C, 5% CO_2 for 24 h prior to drug treatment (2000 cells per well for K562 or 10,000 cells per well for Nalm 6 cell lines). Cells were then treated with varying concentrations of anti-topoisomerase II drug alone (Sigma Aldrich, UK) or in combination with the proteasome inhibitor MG132 (Sigma) or PS341 (Cambridge Bioscience, UK) and incubated for 120 h. 50 μL XTT reagent (50:1 XTT reagent to electron coupling reagent, XTT Cell Proliferation kit, Roche, UK) was added per well and cells were incubated for a further 4 h. Absorbance values were obtained using the Bio-Rad 550 Microplate Reader (Bio-Rad, USA) and analysed using GraphPad Prism software (GraphPad Software, USA), version 4.03. Growth inhibition values were determined by setting the values obtained with no drug as 100% for the etoposide-alone data and with MG132/PS341 alone as 100% for the etoposide plus MG132/PS341 data.

The IC_{50} values (concentration at 50% growth inhibition) of anti-topoisomerase II drug alone versus IC_{50} of drug in combination with proteasome inhibitor were used to calculate potentiation factors (Pf_{50}). The inhibitory concentration of TOP2 poison in the presence of proteasome inhibitor was divided by the concentration of TOP2 poison alone for each separate experiment. The mean Pf_{50} values in the tables represent the mean of at least 3 individual Pf_{50} values.

2.3. In vitro trapped in agarose DNA immunostaining (TARDIS)

TOP2 adducts on genomic DNA were generated by treating K562 cells with 100 μM etoposide or 5 μM mitoxantrone for 2 h prior to embedding cells in agarose on microscope slides (Lonza, USA). To inhibit the proteasome, cells were treated with 50 μM MG132. Cells were collected at the times shown after drug removal and TOP2A and TOP2B complexes were quantified by TARDIS analysis as previously described [42–44]. Briefly, cells were mixed with molten LMP agarose (Lonza, USA) and spread thinly on slides. Agarose embedded cells were then extracted with 0.1% SDS and 1 M NaCl leaving "nuclear ghosts" consisting of genomic DNA coupled to any TOP2 protein-DNA complexes. TOP2 complexes were then detected by quantitative immunofluorescence from several fields of cells per slide. Microscopy was carried out using an Olympus IX81 motorised microscope fitted with an Orca-AG camera (Hamamatsu) and suitable narrow-band filter sets. Images were analysed using Volocity software (Perkin-Elmer). Experiments were carried out at least in triplicate and data are presented as mean of means obtained for each replicate for each treatment ± SEM. For the data in Fig. 9A, rabbit polyclonal antibodies 18511α and 18513β were employed [45], and for the data in Figs. 9B and 10, antibodies 4566-TOP2A and 4555-TOP2B were used. 18511α was raised in-house to recombinant human TOP2A generated in yeast, whilst 18513β, 4566-TOP2A and 4555-TOP2B were raised to GST-TOP2 C-terminal domain fusion proteins generated in bacteria.

2.4. Standard immunofluorescence

Cells were plated in PBS (Life Technologies, UK) onto poly lysine-coated slides (VWR, UK) and after allowing 10 min for cells to adhere, they were fixed in PBS containing 4% paraformaldehyde (Sigma, UK). Immunofluorescence was carried out using rabbit anti-TOP2A (4566) and mouse anti-TOP2B (MAB6348, R&D Systems) and Alexa-488 and Alexa-594 coupled anti-rabbit and anti-mouse secondary antibodies respectively (Life Technologies, UK) as described [46].

2.5. Western blotting

Cells were washed in ice cold PBS and pelleted in 2 ml microfuge tubes. Whole-cell extracts were prepared and Western blotting was performed as described previously [47]. Poly K48-linked ubiquitin was detected with the rabbit monoclonal APU2 (Merck-Millipore, UK) [48]. Autorads were quantified using a GelDoc EX imager (Bio-Rad). Blots were stripped and re-probed for actin, and APU2 signals were normalised to actin.

3. Results

3.1. The proteasome inhibitor MG132 potentiates the growth inhibitory effects of anti-topoisomerase II drugs in K562 cells

K562 cells were incubated with a combination of MG132 and one of six drugs that target DNA topoisomerase II. These included mitoxantrone, mAMSA and etoposide, (an anthracenedione, an acridine and an epipodophyllotoxin, respectively), and three anthracyclines; doxorubicin, its epimer epirubicin, and idarubicin. In order to investigate whether proteasome inhibition affected the growth inhibitory effect of these DNA topoisomerase II-targeting agents, K562 cells were treated with a range of concentrations of anti-topoisomerase II drug with or without MG132 and growth inhibition was measured by XTT staining. The concentration of

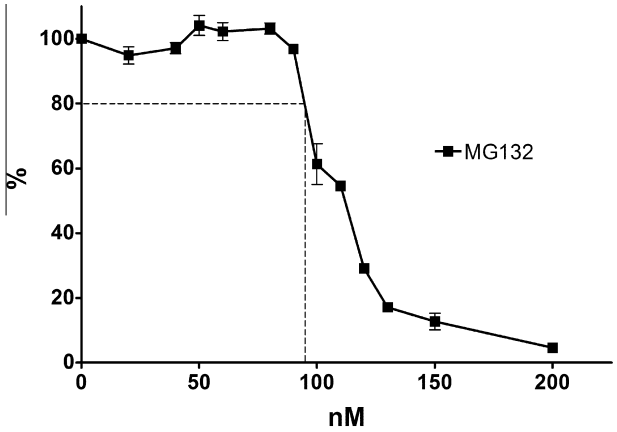

Fig. 1. MG132 dose response in K562 cells. The IC_{20} of MG132 in K562 cells was determined by growth inhibition assays. Cells were treated with increasing concentrations of MG132 and stained with XTT after 5 days of incubation. Error bars represent the mean ± SEM of 4 separate experiments.