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A harmine-derived beta-carboline displays anti-cancer effects *in vitro* by targeting protein synthesis

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ABSTRACT

Growing evidence indicates that protein synthesis is deregulated in cancer onset and progression and targeting this process might be a selective way to combat cancers. While harmine is known to inhibit DYRK1A and intercalate into the DNA, tri-substitution was shown previously to modify its activity profile in favor of protein synthesis inhibition. In this study, we thus evaluated the optimized derivative CM16 *in vitro* anti-cancer effects unfolding its protein synthesis inhibition activity. Indeed, the growth inhibitory profile of CM16 in the NCI 60-cancer-cell-line-panel correlated with those of other compounds described as protein synthesis inhibitors. Accordingly, CM16 decreased in a time- and concentration-dependent manner the translation of neosynthesized proteins *in vitro* while it did not affect mRNA transcription. CM16 rapidly penetrated into the cell in the perinuclear region of the endoplasmic reticulum where it appears to target translation initiation as highlighted by ribosomal disorganization. More precisely, we found that the mRNA expression levels of the initiation factors EIF1AX, EIF3E and EIF3H differ when comparing resistant or sensitive cell models to CM16. Additionally, CM16 induced eIF2α phosphorylation. Those effects could explain, at least partly, the CM16 cytostatic anti-cancer effects observed *in vitro* while neither cell cycle arrest nor DNA intercalation could be demonstrated. Therefore, targeting protein synthesis initiation with CM16 could represent a new promising alternative to current cancer therapies due to the specific alterations of the translation machinery in cancer cells as recently evidenced with respect to EIF1AX and eIF3 complex, the potential targets identified in this present study.

1. Introduction

Among various processes that enable the continuous growth, multiplication and dissemination of malignant cells (Hanahan and Weinberg, 2011), protein synthesis plays an important role in the onset and progression of cancer. Growing evidence indicates that targeting mRNA translation as a cancer therapy has the potential of selectively eradicating cancerous cells (Bhat et al., 2015; Nasr and Pelletier, 2012; Spilka et al., 2013). In eukaryotic cells, mRNA translation occurs in four stages: initiation, elongation, termination and ribosome recycling. Among these, initiation is believed to be pivotal in the regulation of translation (Sonenberg and Hinnebusch, 2009), and is often altered in cancer through dysregulation of expression and/or phosphorylation status of translation initiation proteins, including eIF2α, eukaryotic translation initiation factor 3 (eIF3) and members of the eukaryotic translation initiation factor 4F complex (eIF4F) (Bhat et al., 2015; Blagden and Willis, 2011; Silvera et al., 2010). Translational control contributes to maintain several oncogenic programs (Silvera et al., 2010) and is reciprocally affected by oncogenic signaling pathways, which include MAPK and PI3K-AKT-mTOR (Bader et al., 2005; Topisirovic and Sonenberg, 2011). In response to energy and nutrient demand, mTOR is activated by the PI3K signaling cascade and

Abbreviations: DYRK1A, dual specificity tyrosine phosphorylation regulated kinase 1A; EIF1AX, eukaryotic translation initiation factor 1A, X-linked; EIF3E, eukaryotic translation initiation factor 3 subunit E; EIF3H, eukaryotic translation initiation factor 3 subunit H; eIF2α, eukaryotic translation initiation factor 2 subunit 1

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promotes the assembly of the eIF4F complex resulting in the cap-dependent translation (Hay and Sonenberg, 2004; Ma and Blenis, 2009). Thus, the elevated activity of the translation machinery components and regulators makes them potential selective therapeutic targets to combat cancer cells (Bhat et al., 2015; Blagden and Willis, 2011; Malina et al., 2012).

Harmine, a natural β-carboline, is known to exert anticancer properties through i) the inhibition of DYRK1A (Göckler et al., 2009; Seifert et al., 2008), a protein kinase linked to tumorigenesis (Abbassi et al., 2015; Laguna et al., 2008; Pozo et al., 2013) and ii) DNA intercalating properties (Nafisi et al., 2010; Sarkar et al., 2014). Although displaying more potent growth inhibition than harmine itself, 2,7,9-*tri*-substituted β-carbolines exhibit no effect on DYRK1A, but instead were found to be possible protein synthesis inhibitors (Frédérick et al., 2012). Optimization of their pharmacological and physico-chemical properties led to the identification of CM16 as the lead compound, which to the best of our knowledge is the first harmine-derived beta-carboline (Meinguet et al., 2015) to be studied for its potential as protein synthesis inhibitor of cancer cells. The present study examines the anti-cancer properties of CM16 at the cellular level and on protein synthesis in three cancer cell models of different histological origins, i.e. the melanoma SKMEL-28, the glioma Hs683 and the breast cancer MDA-MB-231 models. We discovered EIF1AX and eIF3 complex members, recently identified as potential cancer targets, as possible regulators of cancer cell sensitivity to CM16.

2. Materials and methods

2.1. Cell lines and compounds

The human cancer cell lines, oligodendroglioma Hs683 (ATCC code HTB-138), melanoma SKMEL-28 (ATCC code HTB-72) and breast adenocarcinoma MDA-MB-231 (ATCC HTB26), as well as the normal human cell lines, skin fibroblasts NHDF (Lonza CC-2509), and lung fibroblasts NHLF (Lonza CC-2512) were selected for the current investigation. Cells were cultivated at 37 °C with 5% CO_2 in RPMI culture medium supplemented with 10% FBS, 200U penicillin-streptomycin, 0.1 mg/ml gentamicin and 4 mM L-glutamine, or fibroblast medium FBM supplemented with 2% fetal bovine serum, 0.1% insulin, rhFGF-B and gentamicin. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine at 37 °C and 5% CO_2. CM16 was synthetized as previously described (Meinguet et al., 2015).

2.2. MTT colorimetric assay

Cells were first grown in 96 well plates for 24 h and then treated with CM16 at different concentrations up to 100 μM or left untreated for 72 h. Viability was estimated by means of the MTT - 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma, Bornem, Belgium) mitochondrial reduction into formazan as previously described (Mosmann, 1983). Two experiments were performed in non-cancerous cell lines (NHDF and NHLF) and three in cancerous cell lines (Hs683, SKMEL-28 and MDA-MB-231), each in sextuplicate.

2.3. Quantitative videomicroscopy

Computer-assisted phase contrast microscopy was performed as previously described (Debeir et al., 2008). Briefly, Hs683, SKMEL-28 or MDA-MB-231 cells were seeded in 25 cm^2 culture flasks and left untreated or treated with CM16 at their GI_{50} concentrations determined with the MTT colorimetric assay or at the concentration 10 times higher. Pictures of one field were taken every four min during a 72 h period and further compiled into a short movie (Debeir et al., 2008). Quantitatively, global growth ratio was determined based on cell counting of pictures corresponding to 24 h, 48 h and 72 h in comparison to 0 h. Experiments were performed once in triplicates.

2.4. Fluorescence assays

2.4.1. CM16 cell penetration and distribution analysis

The fluorescence properties of CM16 were first determined in a phosphate buffer at pH 7.4 as ex/em: 330/439 nm. To qualitatively analyze the CM16 cell penetration and distribution, tumor cells (Hs683, SKMEL-28 and MDA-MB-231) were seeded on glass coverslips in cell culture plates and, after attachment, treated with 5 μM CM16 or left untreated. For imaging of the living cells, medium was removed and PBS was added. Coverslips were rapidly transferred to a slide and images were captured with the Imager M2 fluorescence microscope (Carl Zeiss, Zaventem, Belgium) coupled with the AxioCam ICm1 and AxioImager software (Carl Zeiss, Zaventem, Belgium). The experiment was conducted twice in duplicate and three images per condition were taken.

2.4.2. Ribosome fluorescent staining

Endoplasmic reticulum (ER) staining of cells was performed with glibenclamide ER-tracker red dye (Molecular Probes - Life Technologies, Merelbeke, Belgium) by fluorescence microscopy. Cells were seeded on coverslips in cell culture plates and left untreated or treated with CM16 after attachment (at least 24 h). Following treatment, the dye solution (1 μM in PBS) was incubated with the samples for 30 min at 37 °C. The staining solution was replaced with cell culture medium and sample-containing coverslips were transferred to microscope slides. The imaging of living cells was performed similarly to description above (item a) with fluorescence microscope (Carl Zeiss, Zaventem, Belgium). Experiments were conducted once or twice, depending on the cell line, in duplicate and five images per condition were taken.

2.5. Protein neosynthesis evaluation

2.5.1. Fluorescence method

To evaluate the effects of CM16 on neosynthesized proteins the Click-iT AHA alexa fluor 488 kit (Invitrogen, Life Technologies, Merelbeke, Belgium) was used. A methionine analog L-azidohomoalanine is incorporated in newly synthesized proteins and reacts with an alkyne coupled to alexa 488 fluorescent dye allowing measurement (ex/em: 495/520 nm). Hs683, SKMEL-28 and NHDF cells were seeded in 96 wells plates and left untreated or treated with CM16 or positive control, i.e. cycloheximide (Santa Cruz Biotech., Heidelberg, Germany). The treatment was followed by the addition of L-azidohomoalanine (1/1000) for four h. After fixation with formaldehyde, the neosynthesized proteins were stained according to the manufacturer's recommendations. Normalization according to cell number was carried out as described in the user manual with Hoescht counterstaining. The fluorescent signal was measured in a microplate reader (SynergyMX Biotek, Winooski, USA: ex/em: 350/460 nm). Experiments were performed each in sextuplicate.

2.5.2. ^{35}S Methionine incorporation

MDA-MB-231 or NHDF cells were seeded (50,000 cells per well in a 12 well plate format) one day prior to the labeling experiment. On the day of the experiment, cells were incubated with CM16 at the indicated concentrations for a total of 1 h and 20 min. During the last 20 min, [^{35}S]-methionine/cysteine (150–200 μCi/ml) (Perkin Elmer, Waltham, MA) was added to the cells. At the end of the incubation, cells were washed twice with ice-cold PBS and labeling reactions were terminated through the addition of RIPA buffer. Newly synthesized radiolabeled proteins were precipitated on 3 MM Whatman paper (pre-blocked with 0.1% L-methionine) using trichloroacetic acid (TCA) and washed twice with 5% TCA, followed by two washes of ethanol. Samples were then dried and quantitated using scintillation counting. CPMs were normal-