Biomedicine & Pharmacotherapy 67 (2013) 777–789

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Original article

Potentiation of cytotoxicity of paclitaxel in combination with Cl-IB-MECA in human C32 metastatic melanoma cells: A new possible therapeutic strategy for melanoma

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ARTICLE INFO

Article history:
Received 3 July 2013
Accepted 10 August 2013

Keywords:
Paclitaxel
Cl-IB-MECA
Melanoma

Abbreviations:
Ac-DEVD-CHO, N-Ac-Asp-Glu-Val-Asp-CHO
AR, Adenosine receptor
Cl-IB-MECA, 2-chloro-*N*(6)-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide
DMSO, Dimethyl sulfoxide
DMEM-HG, Dulbecco's Modified Eagle's Medium–High Glucose
FBS, Fetal bovine serum
IAP, Inhibitors of apoptosis
LDH, Lactate dehydrogenase
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NR, Neutral red
NAC, *N*-acetyl-cysteine
PXT, Paclitaxel
ROS, Reactive oxygen species
TNF, Tumour necrosis factor

ABSTRACT

Metastatic melanoma monotherapies with drugs such as dacarbazine, cisplatin or paclitaxel (PXT) are associated with significant toxicity and low efficacy rates. These facts reinforce the need for development of novel agents or combinatory strategies. Cl-IB-MECA is a small molecule, orally bioavailable, well tolerated and currently under clinical trials as an anticancer agent. Our aim was to investigate a possible combinatory therapeutic strategy using PXT and Cl-IB-MECA on human C32 melanoma cells and its underlying mechanisms. Cytotoxicity was evaluated using MTT reduction, lactate dehydrogenase leakage and neutral red uptake assays, for different concentrations and combinations of both agents, at 24 and 48 h. Apoptosis was also assessed using fluorescence microscopy and through the evaluation of caspases 8, 9, and 3 activities. We demonstrated, for the first time, that combination of PXT and Cl-IB-MECA significantly increases cytotoxicity for clinically relevant concentrations. This combination seems to act synergistically in disrupting membrane integrity, but also causing lysosomal and mitochondrial dysfunction. When using the lowest PTX concentration (10 ng/mL), co-incubation with CI-IB-MECA (micromolar concentrations) potentiated overall cytotoxic effects and morphological signs of apoptosis. All combinations studied enhanced caspase 8, 9, and 3 activities, suggesting the involvement of both intrinsic and extrinsic apoptotic pathways. The possibility that cytotoxicity elicited by Cl-IB-MECA, alone or in combination with PXT, involves adenosine receptor activation was discarded and results confirmed that oxidative stress is only involved in cytotoxicity after treatment with PXT, alone. Being melanoma a very apoptosis-resistance cancer, this combination seems to hold promise as a new therapeutic strategy for melanoma.

1. Introduction

Malignant melanoma incidence is rising worldwide [1]. This deadliest form of skin cancer is highly aggressive and has a metastatic potential that is considerably greater than that of any other solid tumours [2]. Moreover, malignant melanoma presents high resistance to conventional chemotherapy and radiotherapy, especially once the metastatic process has begun [3]. Therefore, patients with metastatic melanoma have a poor prognosis, with an overall survival of 8 to 18 months [1]. Resistance to conventional chemotherapy is often related to overexpression of multidrug resistance proteins [3], efficient DNA repair mechanisms [4], overall resistance to endoplasmic reticulum stress [5] and pro-survival machinery that maintains melanoma cells alive by counteracting therapy- and microenvironment-induced apoptotic stimuli during tumour development and metastasis [6]. This last possibility is reinforced by studies, which reported melanoma cells to have low levels of spontaneous apoptosis, *in vivo*, comparatively to other tumour cells [6].

Apoptotic cell death is the major underlying mechanism of therapies targeting cancer cells [7]. The two major apoptotic

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http://dx.doi.org/10.1016/j.biopha.2013.08.003

pathways, the intrinsic or mitochondrial pathway, and the extrinsic or death receptor pathway [8], are subject to aberrant pro-survival signaling in cancer cells, being this mechanism implicated in the resistance of melanoma to standard anticancer therapies [9]. The intrinsic pathway involves outer mitochondrial membrane permeabilization through cooperation of proapoptotic Bcl genes family, including *BAD*, *BAX*, *BAK*, and *BNIP3L* [10]. Once mitochondrial membrane is disrupted, apoptogenic proteins are released into the cytosol, including cytochrome c, AIF, HtrA2/Omi, and Smac/DIABLO [11]. These proteins trigger caspases (cysteine aspartate proteases) cascade activation, beginning with initiator caspases like caspase 9. Upon activation of the executioner caspases, they cleave different cytoplasmic and nuclear substrates, resulting into DNA fragmentation [12]. On the other hand, extrinsic pathway involves cell membrane–bound death receptors activation, which belong to TNF receptor gene family, including CD95 and TRAIL-R1 [13]. Activation of CD95 or TRAIL-R1 by Fas or TRAIL, respectively, results in aggregation of these receptors and recruitment of FADD and caspase 8. Activation of caspase 8 can then initiate apoptosis by direct cleavage of downstream executioner caspases, including caspase 3 [14]. Moreover, TRAIL is able to induce both extrinsic and intrinsic apoptosis pathways in melanoma [15]. However, pre-existing TRAIL resistance, related with down regulation of caspase 8, was also seen in melanoma cells [16]. Thus, initiator caspases, like caspase 8, may control melanoma cell sensitivity to TRAIL, and strategies that result in their up regulation, may be useful for enhancement of TRAIL sensitivity in melanoma.

Caspase-dependent and/or independent processes result in lethal apoptosis, including features of cell shrinkage, nuclear fragmentation, and membrane blebbing [17]. Nevertheless, apoptotic events can be antagonized by pro-survival Bcl-2 proteins and inhibitors of apoptosis (IAP) [18]. Members of both families are widely overexpressed or over activated in melanoma [19]. Overexpression of Bcl-2, Mcl-1, Bcl-XL, NF-κB, TRAF-2, surviving, living, and chromosome x-linked IAP (XIAP) were found to occur in melanoma models [19,20]. This overexpression results in the blockage or hampering of apoptosis. Failure to activate apoptotic pathways, in response to drug treatment, may explain the observed resistance of melanoma to anticancer therapies [20]. Therefore, drugs that induce caspase activation, overcoming melanoma's apoptosis impairment and promoting cell death, have great potential for melanoma therapeutic intervention.

Drugs that target the mitotic spindle are among the most effective anticancer therapeutics currently in use. Taxanes, like paclitaxel (PXT), induce microtubules stabilization, leading to the arrest of cell proliferation and apoptosis [21]. PXT is widely used in many types of cancers, including melanoma [22]. Previous studies showed that PXT is an effective anti-melanoma agent in preclinical models [23,24] and it has been used in clinical trials since 1990 [25]. It is presently used in patients with metastatic melanoma, including patients whose disease has progressed after previous chemotherapy sessions [26]. PXT, as a single agent, showed comparable anticancer activity to common melanoma monotherapy [27]. Unfortunately, PXT is associated with high multidrug resistance [28] that limits the efficacy of this agent, especially when used alone [29]. Therefore, PXT is often given as part of a combination chemotherapy regimen [30]. PXT is used in combination with carboplatin in the treatment of patients with metastatic melanoma. However, this combination added significantly haematological toxicity without improving response or survival rates [31].

Targeting A_3 adenosine receptor (AR) by adenosine or synthetic agonists has been described to cause a differential effect on tumour or on normal cells, inhibiting cell growth of various tumour types such as melanoma [32]. Previously, our group demonstrated that micromolar concentrations of 2-chloro-*N*(6)-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (Cl-IB-MECA), a synthetic and selective A_3AR agonist, promotes cytotoxic effects on malignant melanoma cells [33]. Moreover, oral administration of Cl-IB-MECA to melanoma-bearing mice suppressed the development of melanoma lung metastases [34]. Additionally, Cl-IB-MECA is currently being evaluated in clinical trials (Phase1 and Phase 2) on patients with hepatocellular carcinoma, showing no serious drug-related adverse events or dose-limiting toxicities and good oral bioavailability [35]. Overall, Cl-IB-MECA is considered safe for non-cancer cells, therefore representing a promising therapeutic strategy for cancer treatment. In fact, the use of Cl-IB-MECA, alone or in combination, can improve the therapeutic index of a chemotherapeutic therapy, reducing the clinical dose while preserving efficacy against the cancer.

The aim of the present study was to investigate the potential synergistic cytotoxic effects of the combination of PXT with Cl-IB-MECA, on human C32 melanoma cells, using the lowest concentrations possible of both agents. Human C32 melanoma cells are a well-accepted model to evaluate cytotoxic compounds that can be of interest against melanoma [36]. In fact, PXT is a commonly used cytotoxic agent used on melanoma and Cl-IB-MECA is an oral drug with good potential anticancer properties. In this paper we provide evidence of a potentiation of C32 cells cytotoxicity by simultaneous treatment with PXT and Cl-IB-MECA, leading to apoptosis and necrosis. Based on the results we suggest a possible mechanism for this combination with the special focus on apoptotic pathways. The combination can, in fact, reveal a good potential on melanoma treatment by allowing to decrease PTX dosage and consequently decreasing its adverse effects and its multiresistance recurrence in melanoma.

2. Materials and methods

2.1. Chemicals

All reagents used were of analytical grade. Cl-IB-MECA, MRS 1220, MRE 3008F20, and CGS 15943 were obtained from Tocris Bioscience (Bristol, United Kingdom). Lactate dehydrogenase (LDH) assay kit was purchased from Promega Bioscience (VWR, Porto, Portugal). Caspase 3 and caspase 8 substrates and caspase inhibitor (*N*-Ac-Asp-Glu-Val-Asp-CHO, Ac-DEVD-CHO) were purchased from Calbiochem (Merck-Millipore, Interface, Amadora, Portugal). Bio-Rad RC DC protein assay kit was purchased to Bio-Rad (Amadora, Portugal). Fetal bovine serum (FBS), Glutamax, and Trypsin/EDTA were obtained from Gibco–Invitrogen, Alfagene (Carcavelos, Portugal). Dulbecco's Modified Eagle's Medium–High Glucose (DMEM-HG), penicillin/streptomycin (10,000 U/mL); PXT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red (NR), Hoechst 33258, caspase 9 substrate, *N*-acetyl-cysteine (NAC), dimethyl sulfoxide (DMSO) and all other chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich-Química SA, Sintra, Portugal) of the highest purity available.

2.2. Melanoma cell culture

Human C32 melanoma cells obtained from ECACC – SIGMA (Sigma-Aldrich-Química SA, Sintra, Portugal) were used in this study. Cells were seeded in DMEM-HG medium with 10% of FBS, 1% of a mixture of penicillin/streptomycin (10,000 U/mL; 10 mg/mL) and 1% of Glutamax, pH 7.4. Cells were incubated at 37 °C in a humidified atmosphere (95% air; 5% CO_2). For cell culture maintenance, cells were grown in monolayer and sub-cultivated twice a week. Cell passaging was done by trypsinization. All experiments were carried out with cells at 70%–80% confluence and from batches with passage number lower than 50.