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Anticancer activity and radiosensitization effect of methyleneisoxazolidin-5-ones in hepatocellular carcinoma HepG2 cells

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ABSTRACT

Parthenolide (PTL), a well-known sesquiterpene lactone of natural origin with α,β-unsaturated carbonyl structure, has proven to show promising anti-cancer properties. In this report, anti-proliferative potential of two synthetic methyleneisoxazolidin-5-ones, MZ-6 and MZ-14, with the same structural motif, has been investigated in human hepatoma HepG2 cells. The effects on apoptosis induction and DNA damage were evaluated. All compounds decreased the number of live cells and increased the number of late apoptotic cells. However, only MZ-14 was able to induce DNA damage. Both synthetic compounds increased intracellular reactive oxygen species (ROS) generation and mitochondrial membrane potential changes at the same level as PTL. Additionally, cell survival was analyzed after a combined treatment, in which HepG2 cells were preincubated for 24 h with MZ-6, MZ-14 or PTL and irradiated with different doses of X-rays. The inhibition of cell survival was assessed by the clonogenic assay. We have shown that the clone formation was strongly inhibited by the combined treatment. The synergistic effect was observed for all three compounds but MZ-6 was significantly more effective. It is interesting to note that in HepG2 cells MZ-6 was the least cytotoxic of the tested compounds, did not induce DNA damage and was less active than the others in the clonogenic cell survival assay. It seems advantages from the point of view of the further *in vivo* studies that the compound with the lowest cytotoxic activity showed the strongest sensitizing effect.

1. Introduction

A limited success of currently used antitumor therapies is a driving force for seeking new agents with anticancer potential and development of new strategies for cancer treatment. Natural products play an important role in the anticancer drug discovery process. A vast number of important anticancer agents has been obtained from plants, either directly, by extracting an active component or, more often, by structural modifications of natural compounds or the synthesis of their analogs with improved pharmacological properties. Many plant-derived compounds with anticancer potential are characterized by the α-methylene-γ-lactone structural motif [1,2]. Such compounds can react as the Michael-type acceptors with mercapto (-SH) groups in cysteine residues of enzymes, other functional proteins and free intracellular glutathione, leading to the formation of covalent adducts [3,4] and consequently disrupting key biological processes in the cells [5]. The cytotoxic action of α-methylene-γ-lactones was shown to result in inhibition of proliferation, induction of apoptosis, inhibition of metastasis or sensitization of tumor cells to antineoplastic agents [2,5].

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Parthenolide (PTL, Fig. 1), isolated from feverfew (*Tanacetum parthenium*), is the most extensively studied representative of α-methylene-γ-lactones. Strong cytotoxic activity of this compound was demonstrated *in vitro* in different cancer cell lines, including collateral, hepatoma, pancreas and breast cancer [6–8]. Moreover, PTL was shown to synergistically enhance the action of various known anticancer drugs that act through different mechanisms, improving their profiles *in vitro* and in animal models [9]. Very important new direction in PTL-based studies is sensitization of tumor cells to antineoplastic agents and radiation [9–11].

In our search for new compounds with anti-cancer activity, we have concentrated on the synthesis of simple heterocycles with the same α-methylene-γ-lactone motif as in PTL. In our previous reports, we have shown the synthesis and cytotoxic activity of a series of 4-methyleneisoxazolidin-5-ones, containing an additional nitrogen atom in the γ-lactone ring [12]. Several of these compounds showed strong cytotoxicity against leukemia (HL-60 and NALM-6) and breast cancer (MCF-7 and MDA-MB-231) cells [12,13]. The anticancer activity of the most promising compound, 3-*iso*propyl-2-methyl-4 methyleneisoxazolidin-5-one, designated MZ-6 was explored on breast cancer (MCF-7 and MDA-MB-231) cell lines. MZ-6 inhibited proliferation, induced apoptosis and inhibited migration of MCF-7 cells [8,14].

The aim of this study was to investigate the cytotoxicity and possible anticancer activity of MZ-6 and its analog, 2-methyl-4-methylene-3-(4-methylphenyl)*iso*xazolidin-5-one, designated MZ-14 (Fig. 1) in human hepatocellular liver carcinoma cell line HepG2. The effects of MZ-6 and MZ-14 on apoptosis induction, DNA damage, reactive oxygen species (ROS) generation and mitochondrial membrane potential changes were evaluated. Additionally, MZ-6 and MZ-14 were tested for their ability to sensitize HepG2 cells to X-radiation. For comparison, PTL was included in the study.

2. Materials and methods

2.1. Materials and general procedures

PTL was purchased from Tocris Bioscience (Bristol, UK). 3-*iso*propyl-2-methyl-4-methyleneisoxazolidin-5-one (MZ-6) and 2-methyl-4-methylene -3-(4-methylphenyl)*iso*xazolidin-5-one (MZ-14) were synthesized in a two-step reaction sequence, published earlier [12–14]. For all experiments PTL and the tested compounds were dissolved in DMSO and further diluted in culture medium to obtain less than 0.1% DMSO concentration. In each experiment controls without and with 0.1% DMSO were performed. DMSO in this concentration had no effects on the observed parameters.

2.2. Cell culture

The HepG2 human hepatocellular liver carcinoma cell line (ATCC) was cultured in DMEM with 4.5 g/L D-glucose, supplemented with 10% FBS (Biological Industries, Beit-Haemek, Israel), glutamine (2 mM) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37 °C in a 5% CO_2 atmosphere and were grown until 80% confluent.

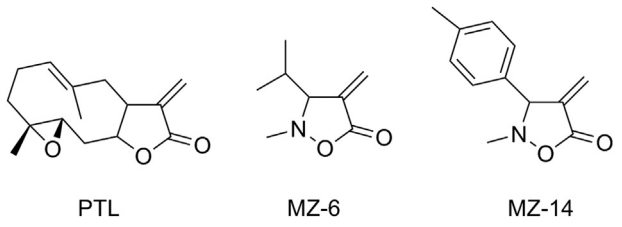

Fig. 1. Structure of parthenolide (PTL) and the synthetic compounds, 3-*iso*propyl-2-methyl-4-methyleneisoxazolidin-5-one (MZ-6), 2-methyl-4-methylene -3-(4-methylphenyl)*iso*xazolidin-5-one (MZ-14), with the same as in PTL α-methylene-γ-lactone motif, additionally modified by introduction of a nitrogen atom.

2.3. Metabolic activity—MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay, which measures activity of cellular dehydrogenases, was based on the method of Mosmann [15]. Briefly, cells were seeded into 96-well plates (about 1.5×10^4 cells per well, in 100 μL) and then left to adhere and grow for 24 h. Subsequently, 100 μL of the tested compounds in the medium was added to a final concentration of 0–250 μM, for 24 h, followed by the addition of 100 μl MTT, 3 mg/mL in PBS, for the next 3 h. After the incubation, the medium was removed. Remaining insoluble formazan crystals were dissolved in 100 μL DMSO. The absorbance of the blue formazan product was measured at 570 nm in the plate reader spectrophotometer Infinite M200 (Tecan, Austria) and compared with control (untreated cells). All experiments were performed three times in triplicate. The concentration of tested compounds required to inhibit cell viability by 50% (IC_{50}) was calculated using Microsoft Excel software for semi-log curve fitting with linear regression analysis.

2.4. Analysis of apoptosis by flow cytometry

Apoptotic cell death was determined using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen). The cells were seeded on 6-well plates (6.0×10^5 cells per plate) and left to adhere for 18–24 h. Subsequently, the growth medium was replaced by a fresh one supplemented with the tested compounds in the desired concentrations. The cells incubated without tested compounds were used as a control. After 24 h of treatment all the cells from each plate were harvested by trypsinization and stained with annexin V and propidium iodide (PI) according to the manufacturer guidelines. Apoptosis of cells was analyzed by flow cytometry with the use of BD LSR Fortessa apparatus. Early and late apoptosis was visualized and quantified by constructing a dot-plot using BD FACSDiva software.

2.5. Alkaline comet assay

The cells were seeded on wells in 24-well plates (1.5×10^5 cells per well) and left to adhere for 18–24 h. Subsequently, the medium was changed to the fresh one, containing (or not) different concentrations of tested compounds, followed by incubation at 37 °C for 2 h. At the end of the incubation time, all the cells from each well were harvested, spun down and resuspended in 0.5 mL of the cold medium. The comet (single cell gel electrophoresis) assay was performed as previously described [16,17]. Briefly, the cell suspensions were mixed with an equal volume of 2% low melting agarose (Type VII, Sigma), poured on microscope slides pre-coated with 0.5% regular agarose (Type I-A, Sigma) and put on ice for agarose solidification. For the cell lysis, the slides were immersed for 1 h in a cold lysis buffer comprising 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH = 10. After lysis, the cells were subjected to electrophoresis (direct DNA damage estimation, i.e. single- and double DNA breaks). Electrophoresis was preceded by 40-min incubation in 1 mM EDTA and 300 mM NaOH (DNA unwinding) and then performed in the same solution at 1.2 V/cm for 30 min at 10 °C. After electrophoresis, the slides were washed with 0.4 M Tris, pH = 7.5 and stained with 4,6-diamidine-3-phenylindole dihydrochloride (DAPI, 1 μg/mL). The pictures of a 100 randomly