Chemico-Biological Interactions 231 (2015) 27–34

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Boldine, a natural aporphine alkaloid, inhibits telomerase at non-toxic concentrations

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ARTICLE INFO

Article history:
Received 27 September 2014
Received in revised form 31 January 2015
Accepted 23 February 2015
Available online 3 March 2015

Keywords:
Cancer
Boldine
Telomerase
hTERT
Splicing variants

ABSTRACT

In a preliminary screening study of natural alkaloids, boldine, an aporphine alkaloid, showed an interesting dose and time dependent anti-proliferative effect in several cancer cell lines. Cytotoxicity of boldine in human fibroblasts was considerably lower than the telomerase positive embryonic kidney HEK293 and breast cancer MCF-7 and MDA-MB-231 cells. Whether boldine can inhibit telomerase was investigated here using a modified quantitative real-time telomere repeat amplification protocol (q-TRAP). This test showed that boldine inhibits telomerase in cells treated with sub-cytotoxic concentrations. Telomerase inhibition occurs via down-regulation of hTERT, the catalytic subunit of the enzyme. Boldine changed the splicing variants of hTERT towards shorter non-functional transcripts as well. A direct interaction of boldine with the enzyme may also be involved, though thermal FRET method did not detect any substantial interaction between boldine and synthetic telomere sequences. This study advocates boldine as a valuable candidate for telomerase-targeted cancer care. This study suggests that derivatives of boldine could be potent anti-cancer drugs.

1. Introduction

Telomeres, the terminal DNA at chromosome ends, progressively shorten during each cell division and limit the replicative life span of dividing cells [1]. It is believed that continuous proliferation and avoidance of replicative senescence are caused by the action of telomerase, the cellular ribonucleoprotein reverse transcriptase that is responsible for telomere elongation [2,3]. Telomerase is active at extremely low levels in most human normal tissues, whereas it is strongly reactivated in more than 85% of human tumors [4–6]. Therefore, telomerase has been proposed as a critical anticancer target [7], the inhibition of which has the potential to serve as an anti-cancer strategy [8].

Natural compounds with extensive structural diversity are valuable resources for drug discovery. Several natural small molecules have been discovered so far that arrest proliferation of cancer cells by inhibiting telomerase [9–12]. This study has focused on evaluation of cytotoxicity and telomerase inhibitory effects of boldine (1,10-dimethoxy-2,9-dihydroxyaporphine), a natural aporphine alkaloid found abundantly in *Peumus boldus* [13], on cancer cells. This strong antioxidant has shown to have several pharmacological activities, such as anti-inflammatory, antipyretic, antiatherogenic, antiplatelet, antitumor promoting, cytoprotective and tyrosinase inhibitory properties [14,15]. In addition, boldine has been suggested as a therapeutic agent in the treatment of diabetes mellitus [16]. It has also shown to attenuate catecholamine oxidation-induced brain mitochondrial dysfunction [17]. Aporphinoids in general exhibit a wide range of biological properties [18,19], for example isolated aporphines of plant *Cassytha filiformis* (Lauraceae) possess *in vitro* antiproliferative properties in a number of cancer and non-cancer cell lines [20,21]. Several of the aporphine alkaloids extracted from natural herbs and related derivatives of boldine are known as inhibitors of topoisomerase I or II [22].

In this study boldine showed strong suppression of proliferation in embryonic kidney cells HEK293 and two telomerase-positive breast cancer cells; consequently its ability to inhibit telomerase activity and probable involved mechanisms were investigated. By our knowledge, no other report exists on the ability of boldine to inhibit telomerase.

2. Materials and methods

2.1. Cell culture

Each of the breast cancer cell lines (MCF-7 and MDA-MB-231), human embryonic kidney 293 cells (HEK293) from DSMZ

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http://dx.doi.org/10.1016/j.cbi.2015.02.020
0009-2797/

(German Collection of Microorganisms and Cell Cultures) and normal human foreskin fibroblast cells (HFF) (Royan Institute, Iran) were cultivated in Dulbecco's modified Eagle's medium supplemented (DMEM High Glucose with stable Glutamine) with 10% fetal bovine serum (FBS gold), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. All the materials were purchased from PAA, Austria. All the cell types were sub-cultured routinely when reached to almost 80% confluence and the cell viability was briefly estimated using trypan blue exclusion method [23]. All the following experiments were performed with cells in the logarithmic growth phase. Berberine and boldine were purchased from Sigma–Aldrich and dissolved in absolute ethanol (Merck) at a concentration of 50 mM (stock solution) and stored at −20 °C until use. Each stock solution was serially diluted in medium before use and the maximum final concentration of ethanol in cell cultures did not exceed 0.1%. Berberine was used as a known compound with telomerase inhibitory effect [24] and the data collected for boldine was compared with that of MCF-7 treated with berberine as a control.

2.2. Cytotoxicity assay

Cell viability was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (Sigma–Aldrich) assay [25]. Briefly, cells were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with each compound at serially diluted concentrations. After various incubation times, MTT at 0.5 mg/ml final concentration was added and incubated 4 h to be reduced to blue formazan by viable cells. Absorbance of the dye, after dissolving in DMSO containing 10% SDS and 1% acetic acid, was measured at 570 nm using a plate reader (BioTek, USA) and cell viability was analyzed using Gen5 software version 1.06. The assay was carried out at least in three independent logical repeats each of which includes samples in triplicates. The concentration of boldine that caused cell growth decrease to 50% of untreated controls, IC_{50}, was determined from the dose–response curves. The results are presented as means ± SD.

2.3. Telomerase assay (SYBR Green q-TRAP assay)

Telomerase activity was measured using a SYBR-Green quantitative-telomere repeat amplification protocol (q-TRAP) based on the method of Hou et al. [26] with some small modifications [27]. Briefly the MCF-7 cells after 48 h incubating with various concentrations of boldine and/or berberine were washed with PBS, lysed in a buffer containing 10 mM Tris–HCl pH = 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM Phenylmethylsulfonylfluoride (PMSF), 5 mM beta-mercaptoethanol, 0.5% CHAPS and 10% glycerol and incubated for 30 min at 4 °C. Protein concentration of the cell lysates was measured using microBradford assay with plate reader (BioTek, USA) and analyzed with Gene5 software version 1.06. Using SYBR Green PCR Master Mix (GenetBio, South Korea), q-TRAP assay was performed to compare telomerase activity in equal amounts of protein extracts from samples in a real-time thermal cycler Rotor-Gene 3000 (Corbett Research). The reaction mixtures including 1X SYBR Green master mix, 1 μg protein of cell extract, 10 pmol TS (5′-AATCCGTCGAGCAGATT-3′) and 5 pmol ACX (5′-GCGCGGCTTACCCTTACCCTTACCCTAACC-3′) primers were incubated 30 min at 25 °C. Then the amplification of telomerase products was started at 94 °C for 10 min to activate the hot-start Taq polymerase and the 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C with signal acquisition. The threshold cycle value (Ct) determined for each sample by using Rotor-Gene 6.01 software was compared with those of the standards generated from serially diluted cell lysate of untreated MCF-7 control. The experiment has been repeated at least four times independently each including triplicates for each concentration of boldine.

2.4. q-TRAP-ligand assay

In a q-TRAP assay two enzymes are involved; first telomerase elongates the synthetic substrate, TS, and then the products are amplified by a hot-start Taq polymerase. In q-TRAP-ligand experiments (also known as *in vitro* TRAP assay) the reactions were treated with boldine in two distinct steps to differentiate between its effects on the enzymes. Two different incubation times were applied; 0 (for adding boldine immediately) and 30 min before the correlated enzyme starts its activity. Briefly, a master mix of q-TRAP reaction including MCF-7 cell lysate was prepared and aliquoted to four sets on ice (A to D). Samples in each set were treated with different concentrations of boldine (10, 100 and 160 μM) at the specified step and for the defined incubation time. In two sets boldine was added before telomerase activity; one set treated immediately before enzyme activity (B) and another set were incubated 30 min in presence of boldine on ice (A); in these two sets, both telomerase and Taq polymerase are exposed to boldine. Then all samples were incubated 20 min at 25 °C for extending TS primer by telomerase. All four sets were put back on ice and

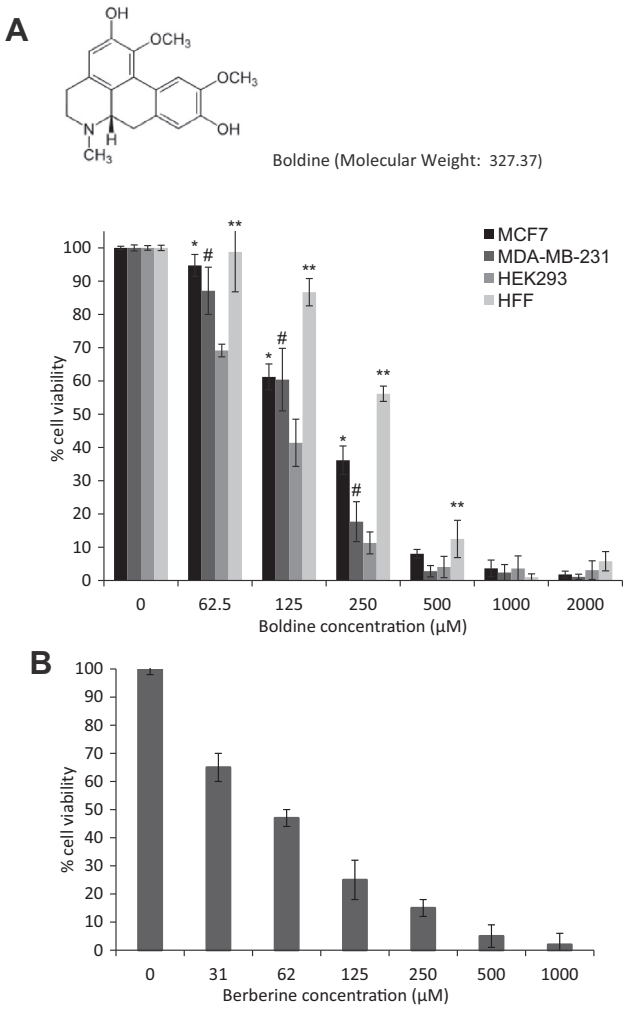

Fig. 1. Viability of different cell cultures MCF-7, MDA-MB-231, HEK293, HFF incubated for 48 h with various concentrations of boldine using MTT assay (A). Cell viability of MCF-7 under treatment of berberine was shown in (B). The mean values ± SD have been represented; *p* values for *, ** and # are 0.001, 0.05 and 0.02 respectively.