Biochemical Pharmacology 93 (2015) 266–276

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Cellular mechanisms of the cytotoxicity of the anticancer drug elesclomol and its complex with Cu(II)

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ARTICLE INFO

Article history:
Received 5 November 2014
Accepted 1 December 2014
Available online 27 December 2014

Chemical compounds studied in this article:
Elesclomol (PubChem CID: 300471)
Cu(II)–elesclomol (PubChem CID: 45273878)
Etoposide (PubChem CID: 36462)

Keywords:
Elesclomol
Copper
JC-1
Cell cycle
Oxidative stress
Efflux

ABSTRACT

The potent anticancer drug elesclomol, which forms an extremely strong complex with copper, is currently undergoing clinical trials. However, its mechanism of action is not well understood. Treatment of human erythroleukemic K562 cells with either elesclomol or Cu(II)–elesclomol caused an immediate halt in cell growth which was followed by a loss of cell viability after several hours. Treatment of K562 cells also resulted in induction of apoptosis as measured by annexin V binding. Elesclomol or Cu(II)–elesclomol treatment caused a G_1 cell cycle block in synchronized Chinese hamster ovary cells. Elesclomol and Cu(II)–elesclomol induced DNA double strand breaks in K562 cells, suggesting that they may also have exerted their cytotoxicity by damaging DNA. Cu(II)–elesclomol also weakly inhibited DNA topoisomerase I (5.99.1.2) but was not active against DNA topoisomerase IIα (5.99.1.3). Elesclomol or Cu(II)–elesclomol treatment had little effect on the mitochondrial membrane potential of viable K562 cells. NCI COMPARE analysis showed that Cu(II)–elesclomol exerted its cytotoxicity by mechanisms similar to other cytotoxic copper chelating compounds. Experiments with cross-resistant cell lines overexpressing several ATP-binding cassette (ABC) type efflux transporters showed that neither elesclomol nor Cu(II)–elesclomol were cross-resistant to cells overexpressing either ABCB1 (Pgp) or ABCG2 (BCRP), but that cells overexpressing ABCC1 (MRP1) were slightly cross-resistant. In conclusion, these results showed that elesclomol caused a rapid halt in cell growth, induced apoptosis, and may also have inhibited cell growth, in part, through its ability to damage DNA.

1. Introduction

Elesclomol is a highly novel anticancer drug that has completed phase 3 clinical trials for patients with advanced melanoma [1] and is currently undergoing Phase 1 and 2 trials for the treatment of a variety of other cancers (http://www.clinicaltrials.gov) [2–4]. Elesclomol and Cu(II)–elesclomol (Fig. 1) are both extremely potent in vitro and typically inhibit cancer cell growth at low nanomolar concentrations [2,5–8]. It has been proposed that elesclomol is cytotoxic through the induction of oxidative stress that is mediated through its Cu^{2+} complex [2,3,5]. The development of copper complexes as anticancer agents has recently been reviewed [9]. A recent report using an HClO-specific fluorescent probe has shown that elesclomol can induce formation of the highly reactive and strongly oxidizing HClO in breast cancer MCF7 cells [10]. However, it is not known whether this is a direct or an indirect effect. Elesclomol strongly binds both Cu^{2+} [2,3,6,8,11] (Fig. 1) and Cu^{+} [5]. Elesclomol can scavenge copper from the culture medium and selectively transport it to the mitochondria where it induces oxidative stress [2,3]. It has also been shown that the elesclomol was subsequently effluxed from the cell after it had transported copper into the cell, and was then free to shuttle more copper into the cell [2]. MCF7 cells with a compromised ability to repair oxidative DNA damage have increased sensitivity to elesclomol [7], which suggests that elesclomol may also exert some of its cytotoxicity through DNA-damaging mechanisms. Interestingly, it

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http://dx.doi.org/10.1016/j.bcp.2014.12.008
0006-2952/

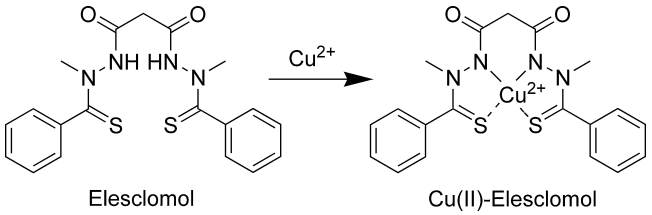

Fig. 1. Structure of elesclomol and its reaction with Cu^{2+} to form the neutral Cu(II)–elesclomol complex.

has been shown that elesclomol-treated patients with normal serum lactate dehydrogenase levels had improved outcomes compared to patients with high lactate dehydrogenase levels [1]. Yeast gene deletion mutant studies suggested that elesclomol does not work through a specific cellular protein target and is unlike any other currently approved anticancer drugs [3].

In previous studies we showed that elesclomol forms an extremely strong 1:1 neutral complex with Cu^{2+} (stability constant of $10^{24.2}\,M^{-1}$; conditional stability constant at pH 7.4 of $10^{17.1}\,M^{-1}$) and also forms a 1:1 complex with Cu^{+} [5,6]. We also showed that ascorbic acid, but not glutathione or NADH, reduces the Cu(II)–elesclomol complex to produce hydrogen peroxide [5]. Electron paramagnetic resonance (EPR) spin trapping experiments showed that the ascorbic acid-reduced Cu(II)–elesclomol complex, in comparison to ascorbic acid-reduced Cu^{2+}, does not directly generate damaging hydroxyl radicals. We also showed that depletion of glutathione levels in K562 cells by treatment with buthionine sulfoximine sensitizes cells to both elesclomol and Cu(II)–elesclomol. Consistent with a role for copper in the cytotoxicity of elesclomol, the highly specific copper chelators tetrathiomolybdate and triethylenetetramine greatly reduce the cytotoxicity of both elesclomol and Cu(II)–elesclomol complex toward K562 cells [5]. These results showed that elesclomol indirectly inhibited cancer cell growth through Cu(II)-mediated oxidative stress.

In order to further characterize the cell growth inhibitory effects of elesclomol and Cu(II)–elesclomol we designed experiments to measure their effects on: (1) cell cycle; (2) induction of apoptosis; (3) mitochondrial membrane potential; (4) formation of DNA double strand breaks; and (5) inhibition of DNA topoisomerase I (5.99.1.2) and DNA topoisomerase IIα (5.99.1.3). We also looked for cross-resistance in cells lines overexpressing several ATP-binding cassette (ABC) type efflux transporters in order to determine if either elesclomol or Cu(II)–elesclomol were substrates for these transporters. Finally, GI_{50} results obtained from submission of Cu(II)–elesclomol for testing in the NCI-60 cell line screen were used to conduct NCI COMPARE analyses in order to determine which compounds in the NCI database had a similar mechanism of action. These results further delineate the mechanisms of the unique cancer cell growth inhibitory effects of elesclomol and Cu(II)–elesclomol.

2. Material and methods

2.1. Materials, cell culture and growth inhibition assays

Elesclomol and Cu(II)–elesclomol were synthesized and characterized as we previously described [6]. Unless specified, all other reagents were obtained from Sigma-Aldrich (Oakville, Canada). Human leukemia K562 cells, obtained from the American Type Culture Collection (Manassas, VA), and the acquired etoposide-resistant K/VP.5 subline (containing decreased levels of topoisomerase IIα mRNA and protein) [12,13] were maintained as suspension cultures in αMEM (minimal essential medium alpha; Life Technologies, Burlington, Canada) containing 10% fetal calf serum and 20 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) (pH 7.2). The spectrophotometric 96-well plate cell (5×10^4 cell/ml, 0.1 ml/well) growth inhibition 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) CellTiter 96 AQueous One Solution Cell Proliferation® assay (Promega, Madison, WI), which measures the ability of the cells to enzymatically reduce MTS after drug treatment, has been described previously [5,6,14]. The compounds tested were dissolved in DMSO and the final concentration of DMSO did not exceed an amount (typically 0.5% or less) that had any detectable effect on cell growth. The cells were incubated with the drugs for 72 h and then assayed with MTS. The effect of elesclomol or Cu(II)–elesclomol on the 72 h growth inhibition of other cell lines that were used to test for the role of efflux transporters were assayed with an 3-[4,5-dimethylthiazol-2-yl]-2,5-tetrazolium bromide (MTT) assay essentially as we have described [14,15]. The IC_{50} values for cell growth inhibition were measured by fitting the absorbance-drug concentration data to a four-parameter logistic equation as we described [14]. The errors that were calculated from these four-parameter non-linear least squares fits to the data are the S.E.M.s.

2.2. Cell growth and viability, cell cycle synchronization, cell cycle analysis and annexin V flow cytometry

Total cell density and viability using a trypan blue assay were determined on a Bio-Rad (Mississauga, Canada) TC10™ automated cell counter. In these experiments erythroleukemic K562 cells at a density of 50,000 cells/ml in 96-well plates were continuously treated with 200 nM elesclomol or Cu(II)–elesclomol for various times. The cell cycle synchronization experiments were carried out as we previously described [14]. A measure of cells that are necrotic is also obtained since necrotic cell membranes are permeable to propidium iodide yielding high red fluorescence. For the synchronization experiments Chinese hamster ovary (CHO) cells were grown to confluence in α-MEM supplemented with 10% fetal calf serum. Following serum starvation with α-MEM-0% fetal calf serum for 48 h, the cells that were seeded at 2×10^4 cells/ml, were repleted with α-MEM-10% fetal calf serum. Directly after repletion they were continuously treated with DMSO vehicle control or 50 nM of either elesclomol or Cu(II)–elesclomol in 35-mm diameter dishes for different periods of time. Cells were fixed in 75% ethanol and stained with a solution containing 20 μg/ml propidium iodide, 100 μg/ml RNase A in 0.1% (v/v) Triton X-100 at room temperature for 30 min. Flow cytometry was carried out on a BD FACSCanto™ II flow cytometry system (BD Biosciences, Mississauga ON, Canada) and analyzed with FlowJo software (Tree Star, Ashland OR) for the proportion of cells in sub-G_0/G_1, G_0/G_1, S, and G_2/M phases of the cell cycle.

The fraction of apoptotic cells induced by treatment of K562 cells with elesclomol and Cu(II)–elesclomol were quantified by two-color flow cytometry by simultaneously measuring integrated green (annexin V-FITC) fluorescence, and integrated red (propidium iodide) fluorescence as we previously described [14]. The annexin V-FITC binding to phosphatidylserine present on the outer cell membrane was determined using an Apoptosis Detection Kit (BD Biosciences, Mississauga, Canada). Briefly, K562 cells in suspension were untreated or treated with elesclomol or Cu(II)–elesclomol at the concentrations indicated at 37 °C for 5 h. The cells were collected by centrifugation at $1000 \times g$ for 3 min and the pooled cells were washed with the manufacturer-supplied binding buffer. Approximately 2.5×10^5 cells were resuspended in 500 μl of manufacturer-supplied binding buffer, and mixed with 5 μl of annexin V-FITC and 5 μl of propidium iodide at a final concentration of 1 μg/ml. After 15 min of incubation in the dark, the cells were analyzed using flow cytometry.