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Mechanistic studies of cancer cell mitochondria- and NQO1-mediated redox activation of beta-lapachone, a potentially novel anticancer agent

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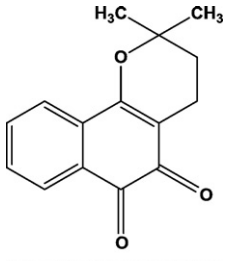
ABSTRACT

Beta-lapachone (beta-Lp) derived from the Lapacho tree is a potentially novel anticancer agent currently under clinical trials. Previous studies suggested that redox activation of beta-Lp catalyzed by NAD(P)H:quinone oxidoreductase 1 (NQO1) accounted for its killing of cancer cells. However, the exact mechanisms of this effect remain largely unknown. Using chemiluminescence and electron paramagnetic resonance (EPR) spin-trapping techniques, this study for the first time demonstrated the real-time formation of ROS in the redox activation of beta-lapachone from cancer cells mediated by mitochondria and NQO1 in melanoma B16–F10 and hepatocellular carcinoma HepG2 cancer cells. ES936, a highly selective NQO1 inhibitor, and rotenone, a selective inhibitor of mitochondrial electron transport chain (METC) complex I were found to significantly block beta-Lp meditated redox activation in B16–F10 cells. In HepG2 cells ES936 inhibited beta-Lp-mediated oxygen radical formation by ~80% while rotenone exerted no significant effect. These results revealed the differential contribution of METC and NQO1 to beta-lapachone-induced ROS formation and cancer cell killing. In melanoma B16–F10 cells that do not express high NQO1 activity, both NOQ1 and METC play a critical role in beta-Lp redox activation. In contrast, in hepatocellular carcinoma HepG2 cells expressing extremely high NQO1 activity, redox activation of beta-Lp is primarily mediated by NQO1 (METC plays a minor role). These findings will contribute to our understanding of how cancer cells are selectively killed by beta-lapachone and increase our ability to devise strategies to enhance the anticancer efficacy of this potentially novel drug while minimizing its possible adverse effects on normal cells.

Introduction



Structure of beta-lapachone

Cancer is one of the major causes of death worldwide. Currently, there is no cure for the large majority of cancers, and as such, development of effective anticancer drugs is of urgent clinical importance. In this context, beta-lapachone (beta-Lp), a quinone compound derived from Lapacho tree, has been shown to be highly effective in treating

Abbreviations: beta-Lp, beta-lapachone; CL, chemiluminescence; DCF, dichlorodihydrofluoroscein; DCIP, dichloroindophenol; DEPMPO, 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; DMEM, Dulbecco's modified Eagle's medium; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; FBS, fetal bovine serum; HRP, horseradish peroxidase; METC, mitochondrial electron transport chain; NQO1, NAD(P)H:quinone oxidoreductase 1; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase.

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various types of cancer in experimental models, including liver cancer and melanoma (Blanco et al., 2010; Brightman et al., 1992; Dong et al., 2009; Pardee et al., 2002; Reinicke et al., 2005). This novel agent is also currently under clinical trials for treating cancer patients (Trachootham et al., 2009).

Although beta-Lp is a promising anticancer agent, its mechanisms of action still need to be fully elucidated. The leading theory holds that beta-Lp reacts with the cellular enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1), which is overexpressed in many cancers, leading to a futile cycling between its quinone and hydroquinone forms, thereby consuming NADPH and generating reactive oxygen species (ROS) (Pink et al., 2000; Siegel et al., 2012). It is suggested that depletion of NADPH may trigger cancer cell apoptosis. In addition, formation of ROS from redox cycling of beta-Lp may also contribute to its cancer cell killing activity (Pink et al., 2000; Siegel et al., 2012). Also, current theories seem to agree on the importance of NQO1 in the anticancer action of beta-Lp, especially in cancer cells that express high NQO1 activity (Pink et al., 2000; Siegel et al., 2012). In most of the experiments verifying NQO1 as the principal cellular factor, the NQO1 inhibitor dicumarol was used (Bey et al., 2007; Li et al., 2011; Pardee et al., 2002). Dicumarol, however, is also a mitochondrial inhibitor (Gonzalez-Aragon et al., 2007), making it difficult to delineate the exact role of NQO1 in redox activation of beta-Lp. Moreover, the involvement of other cellular factors in redox activation of beta-Lp in cancer cells that do not express high NQO1 activity and the subsequent real-time formation of ROS remain unknown. Unlike those in normal cells, mitochondria in cancer cells are extremely inefficient in generating ATP, which provides energy for cellular work, in part because of excessive electron leakage in the electron transport chain (Enns and Ladiges, 2012; Lu et al., 2012). We hypothesized that leaked electrons from mitochondrial electron transport chain (METC) may also play a role in redox activation of beta-Lp and thus the generation of ROS, particularly in cancer cells that do not express high NQO1 activity. Using melanoma and hepatocellular carcinoma cell lines and selective NQO1 and mitochondrial inhibitors as well as purified NQO1 enzyme and isolated mitochondria, we for the first time demonstrated real-time formation of ROS from cancer cell mitochondria- and NQO1-mediated redox activation of beta-Lp and revealed the differential contribution of METC and NQO1 to beta-Lp-induced ROS formation and cancer cell killing. These findings may significantly contribute to our understanding of how beta-Lp becomes redox activated to selectively kill cancer cells and may thus increase our ability to develop strategies to enhance its cancer killing activity while minimizing its potential adverse effects on normal cells.

Materials and methods

Materials. B16–F10 melanoma and hepatocellular carcinoma HepG2 cell lines were from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were from Invitrogen (Carlsbad, CA). Cell culture flasks and other plastic wares were from Corning (Corning, NY). The specific NQO1 inhibitor ES936 was a gift from Dr. David Ross (University of Colorado, Denver). The spin traps 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were from Enzo (Farmingdale, NY). Recombinant human NQO1 was from MyBioSource (San Diego, CA). All other chemicals/agents were from Sigma (St. Louis, MO).

Cell culture. B16–F10 and HepG2 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Isolation of mitochondria. Mitochondria were isolated from freshly harvested B16–F10 cells by differential centrifugation method described before (Zhu et al., 2009). Briefly, cells were washed once with PBS and centrifuged. The cell pellet was resuspended in 5 ml sucrose buffer (0.25 M sucrose, 10 mM Hepes, 1 mM EGTA and 0.5% BSA, pH 7.4), homogenized in a Dounce tissue grinder on ice. The homogenate was centrifuged at 1500 *g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 10,000 *g* for 10 min at 4 °C. The resulting mitochondrial pellet was washed twice with sucrose buffer and then resuspended in ice-cold 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.1% Triton X-100, followed by sonication to lyse the mitochondria. The resulting mitochondrial pellet was washed twice with sucrose buffer and kept on ice for ROS measurement (see below).

NQO1 activity assay. Cellular NQO1 activity was determined using dichloroindophenol (DCIP) as the two-electron acceptor, as described before (Zhu et al., 2009). The dicumarol-inhibitable cellular NQO1 activity was calculated using the extinction coefficient of 21.0 mM^{-1} cm^{-1}, and expressed as nanomoles of DCIP reduced per minute per milligram of cellular protein.

Oxygen consumption assay. The rate of O_2 consumption by cancer cells was measured polarographically with a Clark-type O_2 electrode at 37 °C in 2.5 ml complete PBS, as described before (Li and Trush, 1998). For measuring O_2 consumption stimulated by beta-Lp redox cycle, 0.2 mM KCN was added to inhibit mitochondrial respiration before adding beta-Lp to the cell suspension.

Chemiluminescence (CL) assays. Chemiluminescence (CL) assays are best known for their remarkable sensitivity in detecting cellular ROS in various biological systems as previously described by us and others (Li and Trush, 1998; Li et al., 1998, 1999a,b). In this context, lucigenin-amplified CL assay has been used to sensitively detect biological superoxide generation. On the other hand, luminol-amplified CL in the present of horseradish peroxidase (HRP) has been used to sensitively detect H_2O_2 formation in cells and isolated mitochondria (Li and Trush, 1998; Li et al., 1998, 1999a,b). For detecting cellular ROS formation, CL was continuously measured with a Berthold LB9505 luminometer at 37 °C upon mixing the cells (1×10^6/ml) with lucigenin (20 μM) or luminol (10 μM) plus HRP (10 μg/ml) in complete PBS. For measuring mitochondrial H_2O_2 release, CL was measured in the same manner as described above upon mixing mitochondria (0.05 mg/ml) with 0.5 mM pyruvate and 0.5 mM malate (to provide NADH for the mitochondrial electron transport chain) in respiration buffer.

Electron paramagnetic resonance (EPR) assay. For spin-trapping measurement of oxygen radicals, EPR spectra were recorded at room temperature with a spectrometer (E-Scan, Bruker), operating at X-band with a TM cavity, as described previously (Zang and Misra, 1993). For measuring cellular oxygen radical formation, cells (1×10^6/ml) containing 10 μM Fe^{2+} in complete PBS were incubated with 80 mM DMPO in the presence or absence of beta-Lp at 37 °C for 5 min before being subject to EPR measurement. The EPR spectrometer settings of DMPO spin trapping measurement were: modulation frequency, 100 kHz; X-band microwave frequency, 9.5 GHz; microwave power, 15 mW (milliwatts); modulation amplitude, 1.0 G (gauss); time constant, 160 s; scan time, 200 s; and receiver gain, $1 \times l0^5$. For measuring oxygen radical formation by isolated mitochondria, mitochondria (0.05 mg/ml) were incubated with 25 mM DEPMPO in respiration buffer in the presence or absence of beta-Lp at 37 °C for 5 min before being subject to EPR measurement. For measuring oxygen radical formation from purified NQO1-catalyzed redox cycling of beta-Lp, NQO1 (0.5 μg/ml) was incubated with 0.25 mM NADPH and 25 mM DEPMPO in complete PBS in the presence or absence of beta-Lp at 37 °C for 5 min before being subject to EPR measurement. The EPR spectrometer settings of DEPMPO spin trapping measurement were: modulation frequency, 100 kHz, X band microwave frequency, 9.5 GHz; microwave power, 20 mW