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Novel mechanism of cellular DNA topoisomerase II inhibition by the pyranonaphthoquinone derivatives α-lapachone and β-lapachone

Abstract Purpose: The mechanisms of intracellular topoisomerase II inhibition by the pyranonaphthoquinone derivatives α-lapachone and β-lapachone were studied. Methods: Cell-based mechanistic studies were designed based on the in vitro mechanisms [17] and primarily involved the use of cultured KB (nasopharyngeal tumor cells) cells and the etoposide-resistant sub-line KB-7d. Results: The KB-7d cells exhibited collateral sensitivity to α-lapachone; this supports the possibility of catalytic inhibition of topoisomerase II in the cells. Interestingly, both compounds induced an increase (two- to threefold) in reversible double-stranded DNA breaks in cell lines with a reduced expression of topoisomerase II. However, these drug-induced DNA breaks became irreversible at treatment times greater than 1 h. Studies showed that DNA breaks in KB-7d cells were not caused by endonucleases. Use of antioxidants abolished the appearance of cellular DNA breaks; this suggests involvement of the oxidation-reduction cycle of pyranonaphthoquinones in topoisomerase II inhibition; however, irreversible DNA breaks were not a result of drug-induced oxidative stress. Conclusions: On the basis of the findings, it is proposed that the compounds, on longer incubation with cells, induce abortive dissociation of topoisomerase II from the DNA, leading to an irreversible accumulation of high molecular weight DNA fragments. In addition to establishing topoisomerase II as an intracellular target of α-lapachone, the results suggest that both compounds can be classified as neither typical poisons nor as typical catalytic inhibitors of the enzyme. In summary, both compounds are members of a new inhibitor class, and α-lapachone, in particular, can be considered a potential lead for the development of drugs to treat multidrug-resistant cell lines with lower expression of topoisomerase II.

Key words Catalytic inhibitors · DNA topoisomerase II · Naphthoquinones

Introduction

Type II DNA topoisomerases are essential enzymes that resolve topological problems of DNA during replication, recombination, transcription and chromosome segregation [31]. Inhibitors of the enzyme are classified as “poisons” and “catalytic inhibitors”. In cells, formation of reversible protein-linked DNA complexes is a marker for stabilization of topoisomerase II–DNA covalent complexes by enzyme poisons [2]. Study of catalytic inhibition in mammalian cells is more complicated and the only widely applicable method is limited since it involves an induced enzyme in a virus-infected cell system [26]. Knowledge of in vitro mechanisms of inhibition can therefore help the design and interpretation of cell-based experiments.

The in vitro mechanisms of irreversible catalytic inhibition of topoisomerase II by β-lapachone and α-lapachone (Fig. 1) were recently reported [17]. β-Lapachone induced the enzyme to re-ligate the DNA breaks in the presence of ATP, and dissociated the enzyme from the DNA. α-Lapachone inhibited initial non-covalent binding of topoisomerase II to DNA and, in addition, induced re-ligation of DNA breaks before dissociating the enzyme from the DNA. This report is on a continuation of the mechanistic studies but with a focus on cell-based experiments.

The most common form of drug resistance is due to decreased accumulation of cytotoxic compounds in the cells and is mediated by alterations in the expression of drug efflux pumps including P-glycoprotein and the multidrug-resistance-related protein, MRP [27]. Alterations other than membrane-associated drug transport can also confer a multidrug-resistant (MDR) phenotype.
Another form of MDR is associated with reduced expression or activity of topoisomerase II, and is termed atypical MDR (at-MDR). Reduction in nuclear topoisomerase II levels confers resistance to enzyme poisons due to a decreased amount of enzyme that can be trapped in ternary complexes. However, such cell lines are expected to exhibit collateral sensitivity to catalytic inhibitors [7, 32]. For example, at-MDR cell lines were not cross-resistant to the bis-dioxopiperazine derivative, ICRF-159, but they displayed hypersensitivity [4]. This concept is utilized in the present work and the majority of intracellular mechanistic studies reported here utilize KB-7d, an MDR sub-line of KB (nasopharyngeal tumor cells) with reduced levels of topoisomerase II. Interestingly, a recent study demonstrated that β-lapachone and related 1,2-naphthoquinones were active against fourteen diverse tumor-drug-resistant cell lines; however, none exhibited collateral sensitivity to the agents [6].

Due to the presence of a quinone moiety, the intracellular effects of β-lapachone have been attributed to hydrogen peroxide and free radical production [3, 5, 11]. α-Lapachone being a para-quinone was reported to have lower activities as either an anti-tumor or an anti-parasitic agent presumably due to a decreased production of reactive oxygen species [25]. The redox-cycling properties of pyronaphthoquinones and the resulting oxidation of thiol enzymes is also one of the proposed mechanisms of cellular toxicity [21, 25]. This increased redox cycling by ortho-quinones as compared to para-quinones was clearly differentiated by Neder et al. in experiments demonstrating reactivity of pyronaphthoquinones with 2-mercaptoethanol (a biomimetic model of thiol enzymes) [22].

In addition, β-lapachone can also induce apoptosis in prostate cancer cell lines, breast cancer cells and human leukemia cell lines [19, 24, 33]. An effect of the drug on topoisomerase I/II or any other enzyme as a cause for apoptosis has not yet been demonstrated. So, the molecular mechanism(s) leading to induction of apoptosis remains unknown although Wuerzberger et al. speculated that a direct activation of proteases in MCF-7 cells was involved, through a non-nuclear signaling mechanism [33].

In this report, the intracellular effects of α-lapachone and β-lapachone have been studied with respect to inhibition of topoisomerase II. In the course of the work, some aspects of free radical formation, oxidative inactivation of thiol-enzymes and induction of apoptosis by pyronaphthoquinones have also been addressed. Based on current findings, α-lapachone acts as a novel catalytic inhibitor of cellular topoisomerase II and is a potential lead molecule for developing drugs against MDR tumors with lower levels of the enzyme.

**Materials and methods**

The cell lines used in this study include KB cells (nasopharyngeal tumor cells) and the KB-7d sub-clone (a pleiotropic multidrug-resistant cell line resistant to topoisomerase II poisons) [8]. Some experiments used SV28, baby hamster kidney cells transformed with the SV40 virus, and two sub-lines SV-20ER and SV-5V5ER, developed for resistance to etoposide, with twofold reduction and undetectable levels of topoisomerase II α-isomorph, respectively [12]. All cell lines were propagated in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 100 μg/ml kanamycin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. α-Lapachone and β-lapachone were synthesized from lapachol according to the methods published by Hooker [14]. Etoposide was generously provided by the Natural Products Laboratory, UNC-CH. 9-Ethoxycarbonyl berberine was a generous gift of Dr. K. Iwasa (Kobe Pharmaceutical University, Japan) [15]. The calcium ionophore (A23187) and z-tocopherol were purchased from Sigma (St. Louis, Mo.). The pBR322 DNA was prepared by standard techniques. All other chemicals were reagent grade.

**Clonogenic assay**

Clonogenic survival assays are based on the colony-forming ability of cells after short treatment with the test compounds α-lapachone, β-lapachone and etoposide [1]. Ten thousand cells were treated with the compounds for 90 min. The samples were then diluted to concentrations below inhibitory levels. A fraction of the cells were plated in 12-well plates and incubated for 10 days, at the end of which colonies were fixed and stained with 0.8% (w/v) crystal violet in 50% (v/v) ethanol, and counted by eye. Over the course of the experiments, plating efficiency of untreated KB or KB-7d cells was similar at 40 ± 4% (n = 3). The concentration of compounds that inhibited colony formation by 25 and 50%, the LD₂₅ and LD₅₀ values, respectively, were interpolated from the dose-response graphs.

**Quantitative estimation of intracellular covalent protein/DNA cross-links**

KB cells were seeded at 10⁴ cells/cm² and labeled with [³H] thymidine (1 μCi/ml; 60 Ci/mmol) for 24 h. At 1 h prior to drug treatment, the label was removed, and the cells were then washed with PBS (phosphate-buffered saline) and incubated in fresh medium. The cells were treated with compounds, alone, or in combination with etoposide for 3 h. Treatment schedules included (a) co-treatment of cells with α-lapachone/β-lapachone and etoposide; (b) pretreatment of cells with α-lapachone/β-lapachone for 1 h followed by etoposide; (c) post-treatment of etoposide-treated cells with α-lapachone/β-lapachone after 1 h. After the drug treatments, the protein-DNA complexes were collected by the method described by Culdecott et al. [2]. The protein-DNA complexes were dissolved in 200 μl water and added to 2 ml of scintillant (Ecocint H, National Diagnostics). Radioactivity was measured with a TRI-CARB 2100 liquid scintillation analyzer with 64% counting efficiency. Results were analyzed by a two-tailed Student’s t test with a software package from GraphPad Prism (San Diego, Calif.).

![Fig. 1 Structures of the pyronaphthoquinone derivatives α-lapachone and β-lapachone](image-url)