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Preclinical Pharmacokinetic Evaluation of β-Lapachone: Characteristics of Oral Bioavailability and First-Pass Metabolism in Rats

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Abstract

β-Lapachone has drawn increasing attention as an anti-inflammatory and anti-cancer drug. However, its oral bioavailability has not been yet assessed, which might be useful to develop efficient dosage forms possibly required for non-clinical and clinical studies and future market. The aim of the present study was thus to investigate pharmacokinetic properties of β-lapachone as well as its first-pass metabolism in the liver, and small and large intestines after oral administration to measure the absolute bioavailability in rats. A sensitive HPLC method was developed to evaluate levels of β-lapachone in plasma and organ homogenates. The drug degradation profiles were examined in plasma to assess the stability of the drug and in liver and intestinal homogenates to evaluate first-pass metabolism. Pharmacokinetic profiles were obtained after oral and intravenous administration of β-lapachone at doses of 40 mg/kg and 1.5 mg/kg, respectively. The measured oral bioavailability of β-lapachone was 15.5%. The considerable degradation of β-lapachone was seen in the organ homogenates but the drug was quite stable in plasma. In conclusion, we suggest that the fairly low oral bioavailability of β-lapachone may be resulted from the first-pass metabolic degradation of β-lapachone in the liver, small and large intestinal tracts and its low aqueous solubility.

Key Words: β-Lapachone, Preclinical, Pharmacokinetics, Bioavailability, Metabolism

INTRODUCTION

β-Lapachone (3,4-dihydro-2,2-dimethyl-2*H*-naphthol[1,2-b] pyran-5,6-dione, Fig. 1) is one of naphthoquinones generally isolated from the trees of bignoniaceae and verbanaceae families and recently known to have potent anti-cancer and anti-inflammatory effects (Park *et al*., 2011; Byun *et al*., 2013). Specifically, it has clinically been investigated for the treatment of pancreatic cancer, head and neck cancer, and leiomyosarcoma (Savage *et al*., 2008). β-Lapachone has demonstrated anti-inflammatory activities in endotoxin-induced macrophages (Tzeng *et al*., 2003). Although, to date, the anti-inflammatory action of β-lapachone has not still been clearly elucidated, the primary mechanism of its anti-cancer activity is strongly believed to be owing to induction of apoptosis through increase of Bax/Bcl-2 and activation of caspase-3, and a concomitant loss of PGE_2 and telomerase activity by decreasing the COX-2 and hTERT expression (Lee *et al*., 2005).

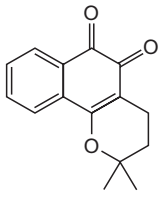

Fig. 1. Chemical structure of β-lapachone.

When considering the research attempts made to reveal the efficacy of β-lapachone as anti-cancer and anti-inflammatory agents it might obviously be expected that the drug could be highly effective in clinical practice. For this reason, an efficient delivery system of β-lapachone is required to maximize its

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therapeutic usefulness. In order to be able to develop clinically acceptable and patient-preferred dosage forms and formulations, one should consider the pharmacokinetic behaviors of the drug along with physico-chemical properties. In case of oral drug administration, the drug is potentially affected by the first-pass metabolism that usually reduces the fraction of unchanged (or unmetabolized) drug reached to systemic circulation. However, to the best of our knowledge, the oral bioavailability and pharmacokinetic properties of β-lapachone has not yet been assessed.

In the present study, therefore, we set the aim to investigate pharmacokinetic properties of β-lapachone after oral administration for evaluating absolute bioavailability in rats as well as its first-pass metabolism in the liver, and small and large intestines. We also report the stability of β-lapachone in plasma to check how the degradation of β-lapachone in plasma affects the pharmacokinetic profiles and bioavailability of the drug tested.

MATERIALS AND METHODS

Materials

β-Lapachone (>98% determined by thin-layer chromatography) and nifedipine were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). HPLC grade methanol and acetonitrile were purchased from Honeywell Burdick & Jackson Company (Morristown, NJ, USA). All other reagents were of the highest analytical grade. Drug-free rat plasma (i.e., blank plasma) was obtained using Sprague-Dawley rats purchased from Hanlim Experimental Animal Laboratory (Hwasung, Korea) following the centrifugation of the whole blood treated with heparin and stored at -80°C until needed.

Animals and drug administration

The present study was reviewed and approved by the Institutional Animal Care and Use Committee of Chung-Ang University (Seoul, Korea). Male Sprague-Dawley rats, 8-week old and weighing 260-300 g were employed for pharmacokinetic study. Rats were fasted for 24 h with free access of water. The rats were anesthetized with chloroform before the cannulation of a polyethylene tube into the femoral artery for blood sampling. β-Lapachone suspended in 1% sodium carboxymethylcellulose was orally given to the rats at a dose of 40 mg/kg and blood samples (approximately 0.3-0.5 ml) were collected into heparinized tubes at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h. Separately, the drug dissolved in 50% dimethyl sulfoxide (DMSO) was administered intravenously through the tail vein at a dose of 1.5 mg/kg and blood samples were collected at 0.03, 0.08, 0.17, 0.25, 0.5, 1, 2, 4, 8 and 12 h. The blood samples were immediately centrifuged at 12,800 rpm for 10 min. Plasma samples were then stored at -80°C prior to analysis.

Stability of β-lapachone in liver and intestinal homogenates

The liver, and small and large intestines were taken up from the rats anesthetized in chloroform chamber and rapidly washed with pH 7.4 phosphate-buffered saline (PBS). Manual washing of the organs was performed to remove remaining blood (liver) and feces (small and large intestines) using PBS and the weight of the organs were recorded. They were cut into small pieces, placed in conical tubes and homogenized at 4°C for 3 min. The homogenates maintained at 4°C were centrifuged at 12,800 rpm for 10 min and the supernatants were carefully withdrawn and stored until the measurement of protein concentrations. Pierce™ BCA Protein Assay Kit was used to determine levels of total protein in the organ homogenates. The protein concentrations were adjusted to be 10 mg/ml with pH 7.4 PBS. The organ homogenates (3.9 ml) were taken and to this 0.1 ml of β-lapachone solution (0.4 mg in 1 ml DMSO) was added. The mixtures were incubated at 37 ± 0.5°C and samples (0.1 ml) were periodically withdrawn at 0, 30, 60, 120, 180 and 300 min after the experiments. Methanol (0.4 ml) was added to the samples to terminate the reaction and the mixtures were centrifuged at 12,800 rpm for 10 min at 37 ± 0.5°C. The supernatants were filtered with RC syringe filters and stored prior to HPLC analysis.

Stability of β-lapachone in plasma

In order to analyze the effect of β-lapachone stability while studying its bioavailability, the degradation of β-lapachone in plasma was evaluated along with in DMSO as the solvent was used to solubilize β-lapachone. The blank plasma (3.9 ml) described in the materials section was placed in glass vials and maintained at 37 ± 0.5°C. The drug solution (0.1 ml) in DMSO (0.4 mg/ml) was added to the plasma and levels of β-lapachone were periodically monitored with HPLC. The same experiment was performed using DMSO instead of the plasma.

Instrument for analysis of β-lapachone

Chromatographic separation was carried out on a Hitachi HPLC system (Tokyo, Japan) equipped with Capcell-Pak C_{18} column (150 x 4.6 mm, Shiseido, Tokyo, Japan) maintained at 35°C. The data acquisition was performed by Ezchrom Elite® (version 3.1.3) data system from Hitachi Company. The mobile phase was a mixture of acetonitrile and water (31:69. v/v) with a flow rate set at 1.5 ml/min. β-Lapachone and nifedipine (internal standard, IS) in eluents were monitored at 254 nm.

Preparation of analytical samples

After thawing the deep-frozen samples at 4°C, an aliquot of each sample (400 μl) was transferred into a polypropylene 2 ml microtube and 40 μl of IS solution (nifedipine 24 μg/ml DMSO) were added and vortexed for 1 min. β-Lapachone and nifedipine were extracted with ethyl acetate (1.6 ml) for 10 min by vortexing mixing and the mixture was centrifuged at 12,800 rpm for 10 min. The supernatant was transferred to another clean microtube and the solvent was evaporated to dryness under nitrogen at 40°C. The residue was reconstituted with 80 μl of methanol and the reconstituted samples (30 μl) were injected into the HPLC system. For the determination of β-lapachone after intravenous administration, an aliquot of each sample (100 μl) was transferred into the microtube and the internal standard solution (20 μl) was added and mixed using vortex mixer for 1 min. β-Lapachone and nifedipine were extracted with ethyl acetate (1.3 ml) and the mixture was centrifuged at 12,800 rpm for 10 min. Then, the same procedure was applied as mentioned above except a 50 μl of the sample was injected into the HPLC.

Pharmacokinetic data analysis

Pharmacokinetic analysis was carried out using BA Calc 2007 program made available by the Korea Food and Drug Administration (KFDA). The area under the plasma concentra-

tion-time curve, $AUC_{(0\text{-last})}$ was computed based on the trapezoidal rule and linearly regressed to infinity to obtain $AUC_{(0\text{-}\infty)}$. The maximum plasma concentration (C_{max}) and the time required to reach the maximum plasma concentration (T_{max}) were determined by the program. The elimination rate constant (k_{el}) was obtained from the terminal slope using a regression analysis, and the calculated elimination half-life ($t_{1/2}$) of the drug was estimated by a relationship of $0.693/k_{el}$. The absolute bioavailability (AB,%) of β-lapachone was calculated using the following equation:

$$AB\ (\%) = \frac{AUC_{oral}/dose_{oral}}{AUC_{iv}/dose_{iv}}$$

where, the subscript oral and iv indicate the parameters were obtained from oral and intravenous drug administration, respectively.

Statistical analysis

All data are presented as mean ± standard deviation. Statistical significance was checked by Student's *t*-test and $p< 0.05$ was considered to be significantly different. Figures were produced with results obtained from three to five independent experiments.

RESULTS

HPLC analysis of β-lapachone in plasma and organ homogenates

The assay specificity was visually assessed with the chromatograms of blank plasma underwent the same extraction procedure mentioned under the heading of "preparation of analytical samples" in comparison to the resulting chromatograms of plasma samples spiked with IS and β-lapachone. There were no interfering peaks with either β-lapachone or IS in the chromatograms. Calibration curves for determination of β-lapachone were linear over the concentration range of 12.5-800 ng/ml in rat plasma. The analytical method developed with HPLC was successfully validated (data not shown).

Stability of β-lapachone in plasma

The chemical stability of β-lapachone was monitored for 24 h in rat plasma and DMSO in order to examine the influence of drug stability on the bioavailability and first-pass metabolism. Fig. 2 obviously demonstrates that β-lapachone was considerably stable for 24 h as judged by the amount remained after 24 h. Indeed, no noticeable decreases in the levels of β-lapachone were observed in the plasma (99.2 ± 0.3) as well as DMSO (99.8 ± 0.1) during 24 h of the experiments, respectively.

Stability of β-lapachone in liver and intestinal homogenates

The degradation of β-lapachone was evaluated in liver, small and large intestine of the rats. The drug was gradually degraded in the organ homogenates and the degradation was the fastest in the liver followed by the small intestine and large intestine (Fig. 3). After 300 min of the experiments, 79.43 ± 0.78% of the initial β-lapachone remained in the liver homogenates. The remaining percent of β-lapachone in the small and large intestine homogenates was not statistically different and

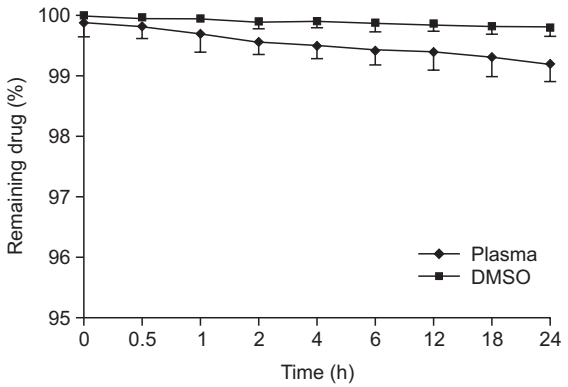

Fig. 2. Stability profiles of β-lapachone in rat plasma and dimethyl sulfoxide (DMSO) used to solubilize the drug. Each data point indicates mean ± SD (n=3).

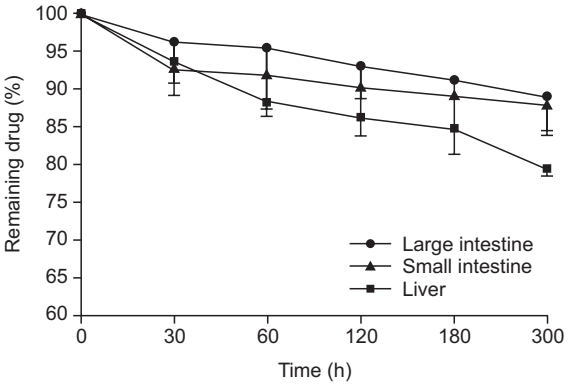

Fig. 3. Metabolic degradation of β-lapachone in liver, small and large intestine homogenates. Each data point indicates mean ± SD (n=3).

measured to be 87.87 ± 3.24% for small intestine and 89.06 ± 4.99% for large intestine homogenates. Before the metabolism experiments, protein levels of the organ homogenates were measured and the rank order of the protein concentrations was liver (71.31 mg/ml) > small intestine (39.74 mg/ml) and large intestine (28.56 mg/ml).

Pharmacokinetics and oral bioavailability of β-lapachone

The average plasma concentration of β-lapachone as a function of time after single oral and intravenous administration in the rats is displayed in Fig. 4. Furthermore, Table 1 demonstrates the pharmacokinetic parameters and absolute bioavailability value of β-lapachone. In case of the intravenous administration of β-lapachone, the plasma concentration of β-lapachone was estimated to be 7.53 μg/ml at time 0 and right after the intravenous administration the concentrations were markedly decreased. Approximately 30 min after the administration, most β-lapachone was disappeared from the systemic circulation with a half-life of 2.48 h. Following oral administration of 40 mg/kg β-lapachone, the greatest plasma concentration of 0.218 μg/ml was achieved at 6 h. The calculated $AUC_{(0\text{-}\infty)}$ was determined to be 4.866 μg·h/ml. Based on AUC and dose administered, the absolute bioavailability of β-lapachone was 15.5%. The calculated elimination half-

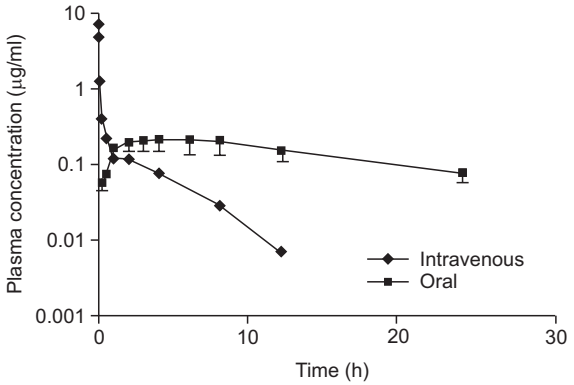

Fig. 4. The profiles of levels of β-lapachone in plasma as a function of time after oral and intravenous administration of β-lapachone to rats at dose of 40 mg/kg and 1.5 mg/kg. Each data point indicates mean ± SD (n=5).

Table 1. Pharmacokinetic parameters of β-lapachone obtained with the oral and intravenous administration of β-lapachone in rats

	$Oral_{(0\text{-}24\ h)}$	$IV_{(0\text{-}12\ h)}$
Dose as β-lapachone (mg/kg)	40	1.5
$AUC_{(0\text{-}last)}$ (μg·h/ml)	3.602	1.149
$AUC_{(0\text{-}\infty)}$ (μg·h/ml)	4.866*	1.174
C_{max} (μg/ml)	0.218	7.133*
T_{max} (h)	6	0
$t_{1/2}$ (h)	11.36*	2.45
AB (%)	15.54	

The values are mean values measured from five repetitive experiments. Asterisk. *Indicates a statistical difference at $p<0.05$ compared to oral or intravenous administration. AB (%) indicates absolute bioavailability.

life ($t_{1/2}$) of β-lapachone given to the oral route was 4.6 times greater than that was measured with the intravenous administration (Table 1).

DISCUSSION

Although β-lapachone is thought to be a promising anti-inflammatory and anti-cancer agent based on the previous research trials, to the best of our knowledge, the value of oral bioavailability of β-lapachone has not been reported, which is regarded as one of the key parameters required to design efficient dosage forms or drug delivery systems (Subramanian *et al*., 2004). Even though cyclodextrin inclusion complexes of β-lapachone have once been reported as a means to enhance solubility and bioavailability, but the authors actually did not measure the bioavailability of β-lapachone alone or its complexes with cyclodextrins (Nasongkla *et al*., 2003). Thus, we attempted to evaluate oral bioavailability of β-lapachone by performing pharmacokinetic studies.

We have successfully developed and validated HPLC methods with UV detection for quantitative determination of β-lapachone in rat plasma. The method employed liquid-liquid extraction of the drug from rat plasma with reverse-phase chromatographic separation. Also, this method has been successfully applied to the pharmacokinetic study of β-lapachone after oral and intravenous administration. The lowest plasma level of β-lapachone in pharmacokinetic study was 0.02 μg/ml, which was equivalent to the limit of detection value.

Prior to the investigation of the pharmacokinetic properties of orally and intravenously given β-lapachone in rats, the stability and degradation profiles of β-lapachone in a variety of conditions were assessed to see any possible effect on pharmacokinetic profiles. The drug stability could be guaranteed in plasma as only less than 1% of the change in drug amount was observed, enabling proper measurement of pharmacokinetic parameters of β-lapachone exposed to the systemic circulation. This result considers being quite similar to that the good stability of β-lapachone has been reported in human plasma for 120 min (Miao *et al*., 2008). The homogenates of metabolic organs have widely been utilized to evaluate the metabolic properties of drugs (Crooke *et al*., 2000; Koo *et al*., 2005). Thus, we used the homogenates obtained from liver, small and large intestines of mice to evaluate the metabolism of drugs in such organs. The degradation profiles of β-lapachone in the liver, small and large intestine homogenates obviously implies that there should be enzymes in the organ homogenates tested responsible for first-pass metabolism. When considering the fact that the protein amount of each organ homogenate was maintained at 10 mg/ml, the drug metabolism of β-lapachone is expected to be fast in the liver and the metabolic effect from each organ might be significantly greater in vivo. Moreover, the metabolic effects in the homogenates of each metabolic organ have to be totally assessed because the orally administered drug passes through the gastrointestinal tract. Therefore, the metabolism of β-lapachone in the metabolic organs may significantly decrease its oral bioavailability.

The possible limitation of the current work is thought to be the lack of the elucidation of mechanisms causing the disappearance of the drug in the homogenates. Phase I and phase II metabolism are largely responsible for the biotransformation of xenobiotics in the body but totally different mechanisms are known for each reaction demanding a considerable amount of research efforts. Even, phase II metabolism frequently plays a critical role in the first-pass metabolism of orally administered drugs. In fact, tanshinone IIA, one of the quinone compounds like β-lapachone was proved to be metabolized by the intestinal glucuronidation after NQO1 mediated reduction and the inhibition of the metabolic pathways was able to increase AUC values leading to an enhanced oral bioavailability (Hao *et al*., 2007). Although the current study could not reveal the mechanism involved in the first-pass metabolism of β-lapachone, further mechanistic research should be done to advance the development of highly bioavailable dosage forms and drug delivery systems.

On the basis of the pharmacokinetic study, the absolute oral bioavailability of β-lapachone was measured to be 15.54% being regarded as low bioavailability. The main reason for this might be due largely to limited solubility and first-pass metabolism of the drug. The aqueous solubility of β-lapachone measured in our laboratory was in the range of 31-35 μg/ml depending on the pH of the media, which might be expected to reduce its oral bioavailability because drugs should be dissolved in gastro-intestinal tracts to be exposed to the systemic circulation (Takano *et al*., 2008). Furthermore, as obviously observed in the Fig. 3, the first-pass metabolism would also

contribute to lower the systemic exposure of β-lapachone. Actually, as listed in Table 1 the T_{max} observed was 6 h and this considerably slow absorption of β-lapachone could be caused by both poor water solubility and quite extensive biodegradation of β-lapachone in the small and large intestinal tracts as well as liver prior to being reached to the systemic circulation.

In terms of the safety of β-lapachone, the unique action mechanism of β-lapachone including its bioactivation through the NQQ1 enzyme makes it act selectively on the tumor tissues that overexpress the enzyme, and there has been a report that demonstrated no systemic side effects of the drug when it was administered to mice (Dong *et al*., 2007). However, another study showed its adverse effects such as a labored breathing and irregular gait in animal models (Blanco *et al*., 2010). Thus, further studies are necessary to examine the side effects of β-lapachone.

In conclusion, the present study first reports the oral bioavailability of β-lapachone obtained with pharmacokinetic profiles of the drug administered orally and intravenously to rats. The fairly low oral bioavailability may stem from the first-pass metabolic destruction of β-lapachone in the liver, small and large intestinal tracts. Our finding would be useful to develop efficient drug delivery systems of β-lapachone in non-clinical and clinical studies or to be marketed in the future. Further research effort should be made to reveal the mechanism involved in the first-pass metabolism to develop highly bioavailable dosage forms of β-lapachone.

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REFERENCES

Blanco, E., Bey, E. A., Khemtong, C., Yang, S. G., Setti-Guthi, J., Chen, H., Kessinger, C. W., Carnevale, K. A., Bornmann, W. G., Boothman, D. A. and Gao, J. (2010) Beta-lapachone micellar nanotherapeutics for non-small cell lung cancer therapy. *Cancer Res.* **70**, 3896-3904.

Byun, S. J., Son, Y., Cho, B. H., Chung, H.-T. and Pae, H.-O. (2013) β-Lapachone, a substrate of NAD(P)H:quinone oxidoreductase, induces anti-inflammatory heme oxygenase-1 via AMP-activated protein kinase activation in RAW264.7 macrophages. *J. Clin. Biochem. Nutr.* **52**, 106-111.

Crooke, R. M., Graham, M. J., Martin, M. J., Lemonidis, K. M., Wyrzykiewiecz, T. and Cummins, L. L. (2000) Metabolism of antisense oligonucleotides in rat liver homogenates. *J. Pharmacol. Exp. Ther.* **292**, 140-149.

Dong, Y., Chin, S. F., Blanco, E., Bey, E. A., Kabbani, W., Xie, X. J., Bornmann, W. G., Boothman, D. A. and Gao, J. (2009) Intratumoral delivery of beta-lapachone via polymer implants for prostate cancer therapy. *Clin. Cancer Res.* **15**, 131-139.

Hao, H., Wang, G., Cui, N., Li, J., Xie, L. and Ding, Z. (2007) Identification of a novel intestinal first pass metabolic pathway: NQO1 mediated quinone reduction and subsequent glucuronidation. *Curr. Drug Metab.* **8**, 137-149.

Koo, T. S., Kim, D. H., Ahn, S. H., Kim, K. P., Kim, I. W., Seo, S. Y., Suh, Y. G., Kim, D. D., Shim, C. K. and Chung, S. J. (2005) Comparison of pharmacokinetics of loxoprofen and its active metabolites after an intravenous, intramuscular, and oral administration of loxoprofen in rats: evidence for extrahepatic metabolism. *J. Pharm. Sci.* **94**, 2187-2197.

Lee, J. H., Cheong, J., Park, Y. M. and Choi, Y. H. (2005) Down-regulation of cyclooxygenase-2 and telomerase activity by β-lapachone in human prostate carcinoma cells. *Pharmacol. Res.* **51**, 553-560

Miao, X. S., Song, P., Savage, R. E., Zhong, C., Yang, R.-Y., Kizer, D., Wu, H., Volckova, E., Ashwell, M. A., Supko, J. G., He, X. and Chan, T. C. (2008) Identification of the in vitro metabolites of 3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione (ARQ 501; β-lapachone) in whole blood. *Drug Metab. Dispos.* **36**, 641-648.

Nasongkla, N., Wiedmann, A. F., Bruening, A., Beman, M., Ray, D., Bornmann, W. G., Boothman, D. A. and Gao J. (2003) Enhancement of solubility and bioavailability of β-lapachone using cyclodextrin inclusion complexes. *Pharm. Res.* **20**, 1626-1633.

Park, M.-T., Song, M.-J., Lee, H., Oh, E.-T., Choi, B.-H., Jeong, S.-Y., Choi, E.-K. and Park, H.J. (2011) β-lapachone significantly increases the effect of ionizing radiation to cause mitochondrial apoptosis via JNK activation in cancer cells. *PLoS One* **6**, e25976.

Savage, R. E., Tyler, A. N., Miao, X.-S. and Chan, T. C. K. (2008) Identification of a novel glucosylsulfate conjugate as a metabolite of 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione (ARQ 501, β-lapachone) in mammals. *Drug Metab. Dispos.* **36**, 753-758.

Subramanian, N., Subhabrata, R., Ghosal, S. K., Bhadra, R. and Moulik, S. P. (2004) Formulation design of self-microemulsifying drug delivery systems for improved oral bioavailability of celecoxib. *Biol. Pharm. Bull.* **27**, 1993-1999.

Takano, R., Furumoto, K., Shiraki, K., Takata, N., Hayashi, Y., Aso, Y. and Yamashita, S. (2008) Rate-limiting steps of oral absorption for poorly water-soluble drugs in dogs; prediction from a miniscale dissolution test and a physiologically-based computer simulation. *Pharm. Res.* **25**, 2334-2344.

Tzeng, H. P., Ho, F. M., Chao, K. F., Kuo, M. L., Lin-Shiau, S. Y. and Liu, S. H. (2003) β-Lapachone reduces endotoxin-induced macrophage activation and lung edema and mortality. *Am. J. Respir. Crit. Care Med.* **168**, 85-91.