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Mitochondrial targeted β-lapachone induces mitochondrial dysfunction and catastrophic vacuolization in cancer cells

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Abstract

Mitochondria play important roles in tumor cell physiology and survival by providing energy and metabolites for proliferation and metastasis. As part of their oncogenic status, cancer cells frequently produce increased levels of mitochondrial-generated reactive oxygen species (ROS). However, extensive stimulation of ROS generation in mitochondria has been shown to be able to induce cancer cell death, and is one of the major mechanisms of action of many anticancer agents. We hypothesized that enhancing mitochondrial ROS generation through direct targeting of a ROS generator into mitochondria will exhibit tumor cell selectivity, as well as high efficacy in inducing cancer cell death. We thus synthesized a mitochondrial targeted version of β-lapachone (XJB-Lapachone) based on our XJB mitochondrial targeting platform. We found that the mitochondrial targeted β-lapachone is more efficient in inducing apoptosis compared to unconjugated β-lapachone, and the tumor cell selectivity is maintained. XJB-Lapachone also induced extensive cellular vacuolization and autophagy at a concentration not observed with unconjugated β-lapachone. Through characterization of mitochondrial function we revealed that XJB-Lapachone is indeed more capable of stimulating ROS generation in mitochondria, which led to a dramatic mitochondrial uncoupling and autophagic degradation of mitochondria. Taken together, we have

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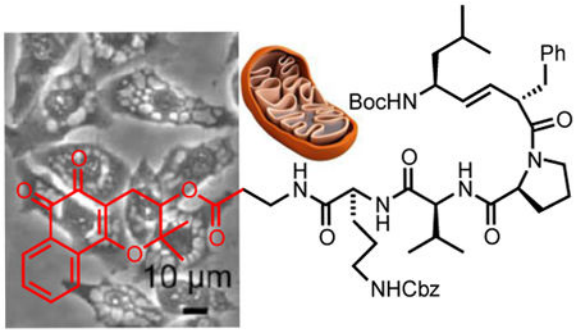
demonstrated that targeting β-lapachone accomplishes higher efficacy through inducing ROS generation directly in mitochondria, resulting in extensive mitochondrial and cellular damage. XJB-Lapachone will thus help to establish a novel platform for the design of next generation mitochondrial targeted ROS generators for cancer therapy.

Graphical abstract



Keywords

Lapachone; mitochondria; ROS; vacuolization; cytotoxicity; mitophagy; XJB-5-131

Mitochondria control many growth and survival pathways in eukaryotic cells, not only as the major energy-producing organelle and a regulator of apoptosis and autophagy, but also as a source of steroids, hemes, amino acids, neurotransmitters, and organic acids. One side product of ATP production in the electron transfer chain (ETC) is the generation of up to 90% of total intracellular reactive oxygen species (ROS), including superoxide radical anion, hydroxyl radical, and reactive nitrogen species (RNS).[1,2,3] ROS bursts can cause oxidative stress levels associated with acute and chronic damage to cellular components, including mitochondrial membrane lipids such as cardiolipin and mitochondrial DNA (mtDNA).[4] Cumulative oxidative damage will result in functional aberrations of cellular metabolism and signaling pathways and various pathological disorders.[5] However, rather than just representing a chemical nuisance and dangerous progenitor of lipid, protein, and DNA oxidation products, leading to apoptosis,[6] ROS also mediate a diverse range of cellular processes such as signaling cascades,[7] cell cycle control, and autophagy.[8] In fact, controlled ROS release can serve as a modulator of redox-homeostasis and cell signaling pathways.[9] Therefore, rather than complete abolition of ROS, controlled inflection of ROS levels may offer treatment options for a large number of diseases such as cancer, diabetes, cardiovascular and neurodegenerative disorders,[10,11,12] where mitochondria have emerged as a key contributing factor.[13]

Cancer cells apparently increase their ROS production relative to normal cells, which is believed to be essential for maintaining oncogenic signaling.[14] Furthermore, disrupting redox homeostasis by either suppressing antioxidant enzymes or enhancing the ROS production in cancer cells has been shown to be able to induce cancer cell death and thus offers an effective strategy for cancer therapy.[15] Directly or indirectly, ROS are also known to play important roles in the anticancer activities of many chemotherapeutic drugs.

Unfortunately, these agents often fail to induce cell death in cancer cells due to alterations in their endogenous cell death signaling such as the p53 pathway.[16] Heat shock proteins compensating for oxidative stress are also frequently upregulated in cancer tissue.[14,17] Therefore, agents that directly target mitochondria to induce mitochondria-initiated cell death are thought to have greater potential in circumventing tumor cell resistance compared to standard chemotherapeutic drugs. Tumor cells are more dependent on mitochondrial energy production under genotoxic conditions and upon radiation damage, relocating mTOR to mitochondria and reversing the Warburg effect.[18] Furthermore, in addition to bioenergetics, other aspects of mitochondrial metabolism are also required for the function of many types of tumors, including melanoma.[19] Induction of mitochondrial dysfunction can thus be considered to be a promising strategy for cancer treatment.[20]

The marked differences between normal and cancer cells in mitochondrial metabolism and function provide support for the hypothesis that selectivity, as well as high therapeutic efficacy can be accomplished by direct targeting of mitochondria.[21,22,23,24] In fact, targeting the electron transfer chain (ETC) in mitochondria with synthetic and natural toxins has attracted significant recent interest.[25,26,27,28,29] Several classes of mitochondrial targeted anticancer agents, known as ‘mitocans’, have been reported and categorized into eight classes depending on their sites of action, i.e. 1) hexokinase inhibitors (**7**); 2) Bcl-2 family protein ligands (**3**, **4**); 3) thiol redox system disruptors (**1**, **2**); 4) mitochondrial membrane transporter/channel inhibitors (**11**); 5) electron transfer chain deregulators (**5**, **6**, **12**); 6) inner mitochondrial membrane disruptors (**9**, **10**); 7) TCA cycle inhibitors (**8**, **12**, **13**); and 8) mtDNA damaging agents (**1**, **2**) (Figure 1).[30,31] Most of these targets are closely correlated to cancer specific alterations of mitochondrial functions and bioenergetics.

Previously, we have reported an alkene peptide isostere segment of the antibiotic gramicidin S (GS), i.e. the XJB-peptide, as a mitochondrial targeting vector.[32,33,34] The presence of a type II′ β-turn in this pentapeptide sequence facilitates membrane permeability since the polar functionality of the backbone is less solvent exposed. After several structural modifications,[35] the mitochondrial targeting antioxidant nitroxide, XJB-5-131, was developed as a first generation lead compound and a promising therapeutic agent.[36,37,38] In parallel to these efforts to generate mitochondrial targeted antineurodegenerative compounds,[34,37,39,40] we have sought to develop a mitochondrial targeted ROS inducer for anticancer therapy.[41,42]

A principal concern with ROS inducers in cancer therapy is whether these compounds have preferential affinities to localize and achieve sufficient concentrations for efficacy within the mitochondrial targets of malignant cells. Due to the distinct features of mitochondrial membranes, major challenges and strategies for a successful treatment rely on the design of effective delivery systems that can penetrate mitochondrial membrane barriers.[43] Toward this goal, various chemistry-based approaches to targeting mitochondria have been reported.[32,44] The most widely used strategy for the delivery of organic molecules targeting mitochondria is the use of lipophilic cationic phosphonium ions, which are attracted by the large inner-mitochondrial membrane potential and accumulate within the negatively charged mitochondrial matrix.[45] Conjugation of bioactive molecules to peptide-based MT (mitochondria) targeting sequence, such as SS-peptides,[46] mitochondria-penetrating

peptides (MPPs),[47] synthetic peptides and amino acid-based transporters[48] are also major strategies for MT delivery. Additionally, the use of targeting systems with nanoparticles and liposomes is expanding.[49] While therapeutic success has been limited,[50] these early results provide support for additional investigations for the design of mitochondrial targeted anticancer drugs.[51,52] For example, a TPP-tagged dichloroacetate showed three orders of magnitude enhanced potency and cancer cell specificity.[53]

In order to generate a highly effective inducer of cell death pathways through direct targeting of mitochondria,[54] we selected an electron-rich *ortho*-quinone, β-lapachone (**1**, Figure 1), for the payload portion of the XJB mitochondrial targeting platform. Quinones are frequently involved in redox cycling and glutathione depletion after reductive activation by enzymes, generating bursts of ROS in addition to serving as electrophilic alkylators.[55,56] Specifically, β-lapachone (ARQ-501) is a naturally occurring *ortho*-naphthoquinone isolated from the lapacho tree (*Tabebuia avellanedae*) that has shown a range of potent biological activities relevant to antibacterial, antifungal, anti-inflammatory, and antitumor pathways.[57] Furthermore, it has entered Phase I/II clinical trials for several types of cancer either as a single agent or in combination with other chemotherapeutics. The cytotoxic effects of β-lapachone are derived from ROS generation dependent on the action of cytosolic NAD(P)H:quinone oxireductase 1 (NQO1) which reduces β-lapachone to a reactive hydroquinone and semiquinone redox-cycler. Since cellular levels of NQO1 vary between cell types, especially between normal and cancer cells, β-lapachone can selectively kill NQO1 overexpressing cancer cells. Besides these aspects, the exact mechanism of action of β-lapachone has yet to be elucidated. Several other molecular targets for β-lapachone have been proposed and apoptosis,[58] necrosis,[59] as well as autophagic cell death have been observed after treatment.[60] Hence, we hypothesized that a targeted delivery of β-lapachone to mitochondria would 1) provide further insight into the mechanism of action of the active species; 2) trigger a specific activation of a more defined molecular cell death mechanism; and, 3) provide a novel mitocan causing mitochondrial dysfunction and therefore potentially broadening its therapeutic profile in cancer treatment.

For the synthesis of the XJB-conjugated β-lapachone (Scheme 1), 3-hydroxy β-lapachone (**14**) was prepared from commercially available lapachol by epoxidation and intramolecular ring opening using *meta*-chloroperoxybenzoic acid (*m*-CPBA).[61] The secondary alcohol in **14** was acylated in 72% yield with the *N*-Boc β-alanine spacer group in the presence of 1,1′-carbonyldiimidazole (CDI) and DBU in DMF. Cleavage of the Boc group in the resulting ester **15** with TFA in CH_2Cl_2 afford amine **16** in quantitative yield.[62] The XJB-derived Boc-Leu-D-Phe-Pro-Val-Orn(Cbz)-OH targeting sequence **17**[63] was coupled to the amine **16** by EDCI in the presence of HOAt, Hünig's base (DIPEA) and DMAP to afford the desired XJB β-lapachone conjugate **18** in 82% yield.

In order to investigate the cellular toxicity of **18** (XJB-Lapachone), we treated A549 non-small cell lung cancer cells with various concentrations of XJB-Lapachone for 24 h, and compared its effect with XJB-OMe (the mitochondrial targeting moiety) and unconjugated 3-hydroxy-β-lapachone (**14**). A CellTiter-Blue based cell survival assay revealed that XJB-Lapachone reduced cell viability in a dose dependent manner (Figure 2A). The cytotoxic effect of XJB-Lapachone is superior compared to unconjugated β-lapachone, while XJB-

OMe has no effect on cell survival (Figure 2A). Furthermore, XJB-Lapachone is also effective against other types of tumor cells, including PEO1 ovarian cancer cells, MDA-MB-231 breast cancer cells, and 983B melanoma cells (Figure 2B). Since MDA-MB-231 breast cancer cells are deficient in NQO1,[64] the high efficacy of XJB-Lapachone in this cell type suggests that the effect of the conjugate, mitochondrial targeted XJB-Lapachone, is no longer solely dependent on NQO1 activity. β-Lapachone is known to have tumor cell-selective toxicity due to higher expression of the enzyme NQO1 in certain tumor cells.[65] We therefore examined if the tumor selectivity of β-lapachone is maintained after XJB conjugation. We compared the cytotoxic effect of XJB-Lapachone in A549 lung cancer and non-transformed human lung fibroblast cells IMR90 by a cell growth/death assay. We found that a four-hour exposure to 10 μM XJB-Lapachone significantly reduced the viability of A549 cells, while the toxic effect was less prominent in IMR90 cells, indicating the tumor selectivity of XJB-Lapachone (Figure 2C). Remarkably, in A549 cells we observed extensive vacuolization after XJB-Lapachone exposure. In contrast, vacuolization was not detected after treatment with XJB-OMe or 3-hydroxy-β-lapachone (Figure 2D). β-Lapachone has been shown to induce apoptosis or programmed necrosis depending on cell types,[66,67] and we therefore investigated the mechanism of cell death induced by XJB-Lapachone. Through annexin V and PI staining, we observed that more annexin V positive cells were induced after treatment with XJB-Lapachone, compared to unconjugated 3-hydroxy-β-lapachone (Figure 2E), indicating extensive apoptosis after XJB-Lapachone exposure.

Prior studies have shown that conjugation to the XJB sequence enhances the mitochondrial localization of small heterocyclic payloads by >100-fold.[36,38,40,68] We therefore focused on the effect of XJB-Lapachone on mitochondrial function. While β-lapachone is known to promote ROS generation after bio-activation by NQO1 and subsequent NQO1-dependent futile cycling in cytoplasm, XJB-Lapachone's activity in MDA-MB-231 cells that lack NQO1 suggests other potential mechanisms.[69] After treating A549 cells with 6 μM XJB-OMe, 3-hydroxy-β-lapachone (**14**), or XJB-Lapachone (**18**) for 16 h, we observed a dramatic increase of ROS generation after in the presence of XJB-Lapachone, as determined by both DCF and the mitochondria-specific superoxide sensor, MitoSox, through flow cytometry analysis. As expected, the effect of the XJB-conjugated, targeted lapachone **18** on ROS generation is stronger than unconjugated 3-hydroxy-β-lapachone (Figure 3A). Through examining the oxygen consumption of mitochondria, we further found that following treatment with XJB-Lapachone, mitochondrial respiration can no longer be inhibited by oligomycin, which blocks mitochondrial complex V ATP synthase. In contrast, oligomycin was efficient in inhibiting the mitochondrial respiration in cells treated with XJB-OMe or 3-hydroxy-β-lapachone. The absence of response to oligomycin administration indicates a substantial uncoupling effect of the mitochondrial targeted lapachone (Figure 3B). Electron microscopy analysis further revealed that after treatment with XJB-Lapachone in A549 cells onion skin-like structures of mitochondria were observed, and cells developed extensive vacuoles (Figure 3C). This finding suggests that XJB-Lapachone exposure leads to autophagic degradation of mitochondria (mitophagy). Consistent with the morphologic changes of mitochondria during autophagy, we observed an increase in an LC3-II immunoblot signal after XJB-Lapachone exposure, in contrast to XJB-OMe and 3-hydroxy-

β-lapachone exposure (Figure 3D), supporting a potential important role of autophagy in XJB-Lapachone induced cell death.

The preferential, dramatic effects of XJB-Lapachone on mitochondrial function, as compared to unconjugated **14**, are conceivably the results of its accumulation in mitochondria leading to the generation of ROS and subsequent ROS-mediated mitochondrial damage, and/or covalent modifications of nucleophilic mitochondrial components. We have previously shown that the targeting sequence in XJB-5-131 can enrich small organic molecule payloads in mitochondria,[35,38,40,68] but we have yet to establish quantitative data for the specific enrichment level of XJB-Lapachone. Furthermore, the results in Figure 2 indicate that XJB-Lapachone induces cell death at least in part independent on NQO1, and therefore the mechanism of action of XJB-Lapachone will require further follow-up investigations. Since the XJB-Lapachone conjugate **18** contains an *ortho*-quinone/catechol substructure, NQO1-independent mechanisms such as a Cu(II) or Fe(III) chelation may play a significant role in its mitochondrial toxicity.[70,71] Combined, these data suggest that mitochondrial targeting of β-lapachone results in the activation of unique signaling pathways leading to a yet to be classified type of cell death as highlighted by an extensive formation of vacuoles, in stark contrast to the phenotype of unconjugated β-lapachone.

In summary, we prepared a new mitochondrial targeted β-lapachone analog and demonstrated its unique effects on cancer cell lines. XJB-Lapachone is able to preferentially and efficiently induce mitochondrial ROS generation and subsequent mitochondrial damage, resulting in enhanced cytotoxicity compared to unconjugated β-lapachone. Due to the altered mitochondrial function and ROS metabolism in cancer cells compared to normal cells, mitochondrial targeted lapachone may yield a higher tumor selectivity than unconjugated lapachone. The remarkable cellular vacuolization is a striking result observed upon treatment of A549 cells with XJB-Lapachone (**18**), but not with XJB-OMe or the control 3-hydroxy-β-lapachone (**14**).

Taken together, these results support the selection of mitochondria as cancer targets. While many tumors have the capacity to produce a large percentage of their ATP through glycolysis, i.e. the Warburg effect, it is clear that tumor cells are metabolically flexible and can adapt to the harsh tumor environment by altering their carbon sources to provide energy and key metabolic intermediates.[72,73,74] In addition, oncogene overexpression, such as Myc, while increasing glycolysis can also increase mitochondrial biogenesis.[75,76] Thus, mitochondrial function is a vital factor in tumor cells, and in fact becomes more significant under tumor radiation and genotoxic stress conditions.[18] In addition, cancer cells have a higher ROS load and are likely saturating their scavenging mechanisms.[25] Their ability to respond to additional mitochondrial stress factors is reduced. Many apoptosis-avoiding mutations are upstream from mitochondria, thus retaining cellular susceptibility to mitochondria-triggered death signals. Ongoing investigations on the mechanism of XJB-Lapachone generation of ROS and its associated cell death pathway will provide a basis for exploring the therapeutic potential of this novel agent and establish a platform for the design of next generation mitochondrial targeted ROS generators for cancer therapy. Mechanism-based combination designs such as simultaneous inhibition of signaling cascades or the use

of radiation therapy will likely further enhance the efficacy of mitochondrial targeted lapachone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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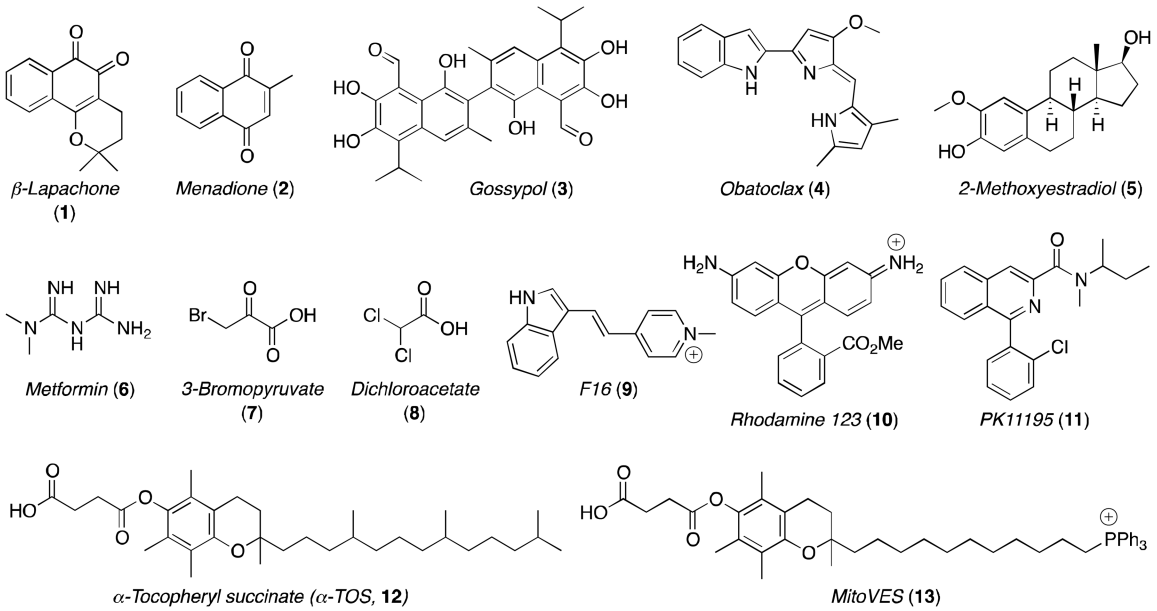


Figure 1.

A selection of mitocans – cytotoxic agents with mitochondrial targeted mechanisms of action.

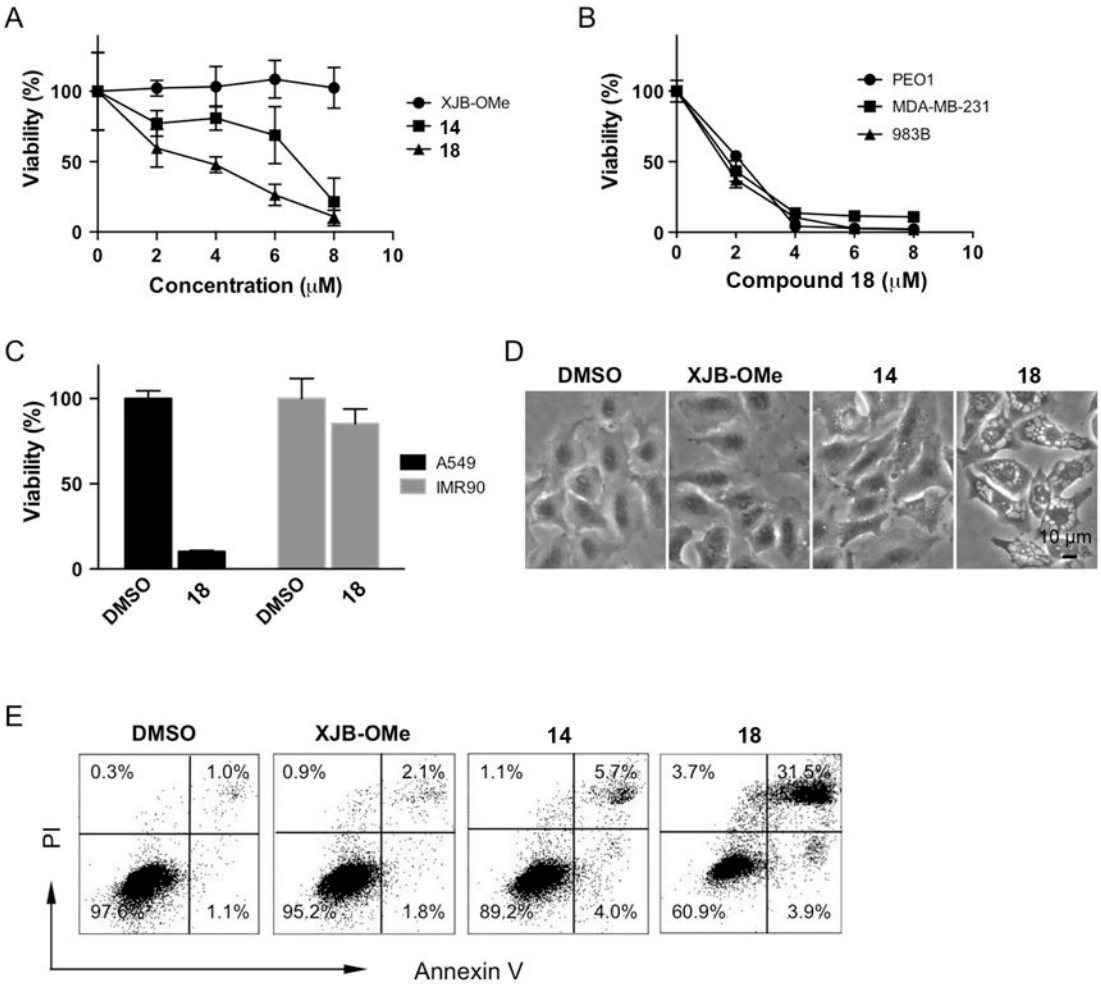


Figure 2.

Mitochondrial targeted β-lapachone (XJB-Lapachone, **18**) shows enhanced efficacy and is tumor cell selective. (A) A549 non-small cell lung cancer cells were treated with XJB-OMe, 3-hydroxy-β-lapachone (**14**), or XJB-Lapachone (**18**) at indicated concentrations for 24 h. Cell viability was determined by a CellTiter-Blue assay. Data represents the mean±SEM. (B) PEO1 ovarian cancer cells, MDA-MB-231 breast cancer cells, and 983B melanoma cells were treated with XJB-Lapachone (**18**) at indicated concentrations for 24 h. Cell viability was determined by a CellTiter-Blue assay. Data represents the mean±SEM. (C) Equal numbers of A549 and IMR90 lung fibroblast cells were treated with 10 μM XJB-Lapachone (**18**) for 4 h. XJB-Lapachone was then washed away and cells were incubated in drug free media for 3 days. Cell viability was determined by a CellTiter-Blue assay. (D) A549 cells were treated with XJB-OMe, 3-hydroxy-β-lapachone (**14**), or XJB-Lapachone (**18**) at 6 μM concentrations for 20 h. The formation of vacuoles was examined by phase contrast microscopy. Representative images are shown. (E) A549 cells were treated as described in D. Apoptotic and necrotic cell death were determined by Annexin V and PI staining. Representative images are shown.

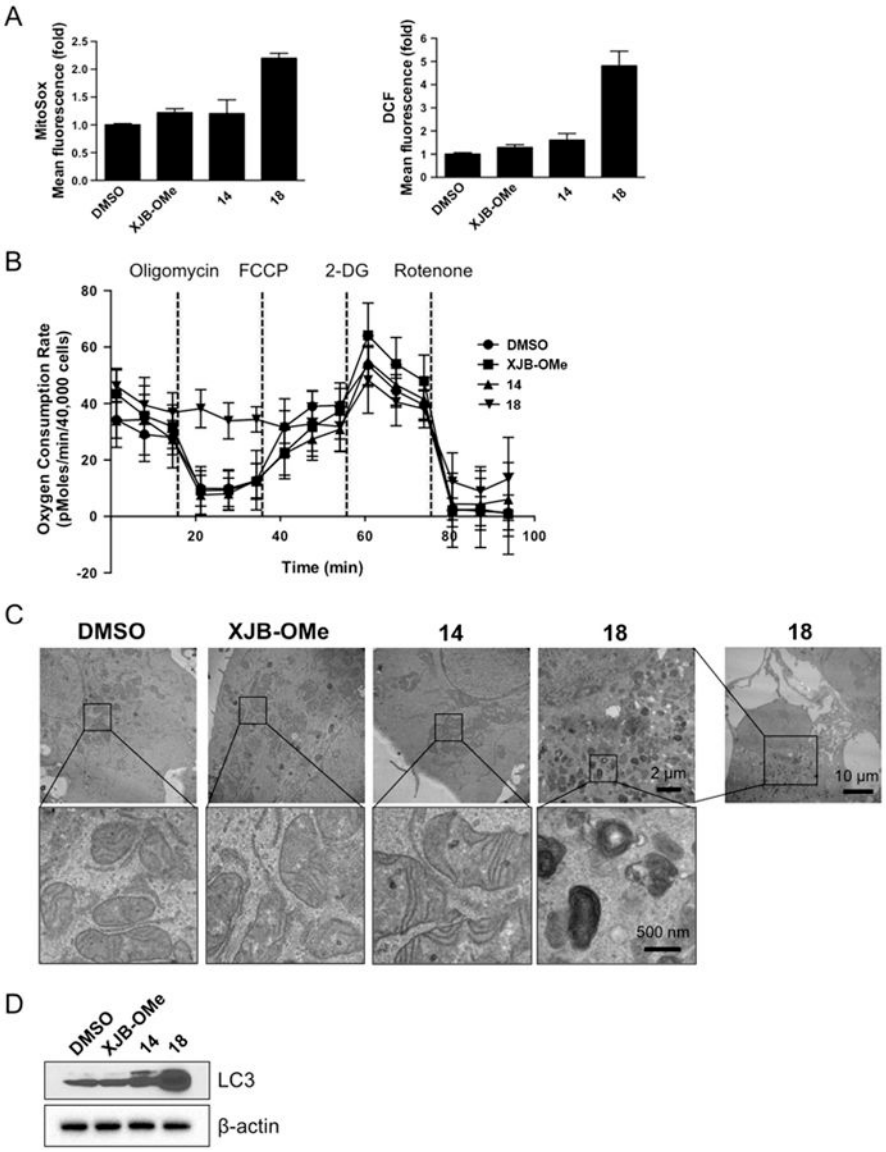
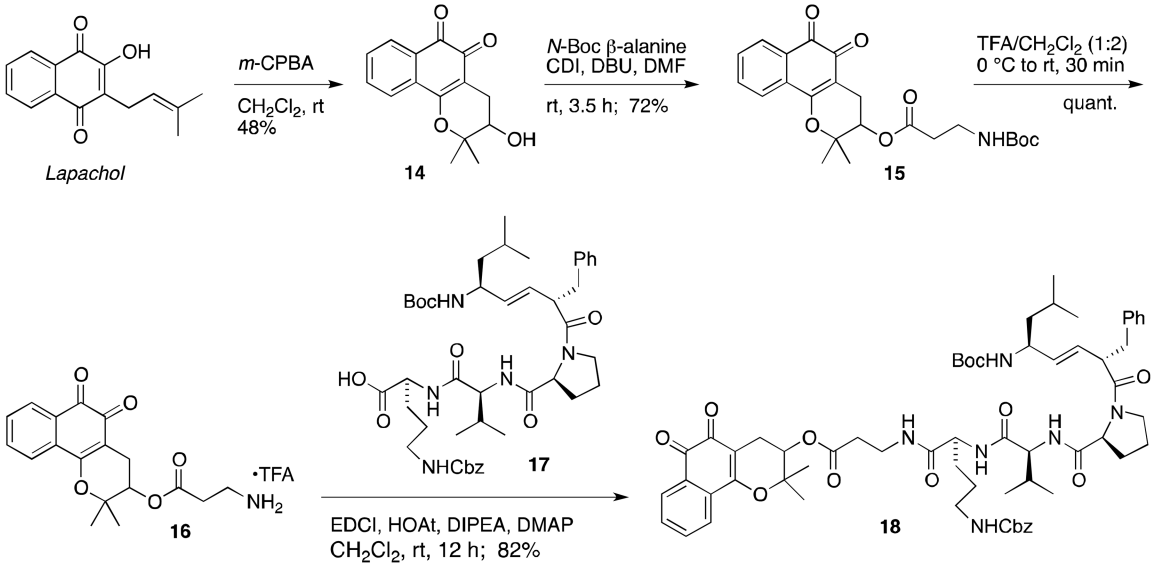


Figure 3.

Mitochondrial targeted β-lapachone (XJB-Lapachone, **18**) induces prominent mitochondrial dysfunction. (A) A549 cells were treated with XJB methyl ester (XJB-OMe), 3-hydroxy-β-lapachone (**14**), or XJB-Lapachone (**18**) at 6 μM for 16 h. The generation of ROS was determined by staining cells with MitoSox and DCFH-DA. The fluorescence intensity was measured by flow cytometry. Data represents the mean±S.D. of triplicates. (B) A549 cells were treated as described in A, and the oxygen consumption rate was determined by a Seahorse extracellular flux analyzer. These data are the mean±S.D. of six wells, and are representative of three experiments. (C) A549 cells were treated as described in A, and mitochondria morphology was examined by electron microscopy. Representative images are shown. (D) A549 cells were treated as described in A. The expression of the autophagy marker LC3 was examined by western blot. β-actin was used as a loading control. Representative images are shown.



Scheme 1.

Synthesis of the mitochondrial targeted β-lapachone conjugate **18**.