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# β-lapachone regulates mammalian inositol pyrophosphate levels in an NQO1- and oxygen-dependent manner

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**Inositol pyrophosphates (PP-InsPs) are energetic signaling molecules with important functions in mammals. As their biosynthesis depends on ATP concentration, PP-InsPs are tightly connected to cellular energy homeostasis. Consequently, an increasing number of studies involve PP-InsPs in metabolic disorders, such as type 2 diabetes, aspects of tumorigenesis, and hyperphosphatemia. Research conducted in yeast suggests that the PP-InsP pathway is activated in response to reactive oxygen species (ROS). However, the precise modulation of PP-InsPs during cellular ROS signaling is unknown. Here, we report how mammalian PP-InsP levels are changing during exposure to exogenous ($H_2O_2$) and endogenous ROS. Using capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS), we found that PP-InsP levels decrease upon exposure to oxidative stressors in HCT116 cells. Application of quinone drugs, particularly β-lapachone (β-lap), under normoxic and hypoxic conditions enabled us to produce ROS in cellulo and to show that β-lap treatment caused PP-InsP changes that are oxygen-dependent. Experiments in MDA-MB-231 breast cancer cells deficient of NAD(P)H:quinone oxidoreductase-1 (NQO1) demonstrated that β-lap requires NQO1 bioactivation to regulate the cellular metabolism of PP-InsPs. Critically, significant reductions in cellular ATP concentrations were not directly mirrored in reduced PP-InsP levels as shown in NQO1-deficient MDA-MB-231 cells treated with β-lap. The data presented here unveil unique aspects of β-lap pharmacology and its impact on PP-InsP levels. The identification of different quinone drugs as modulators of PP-InsP synthesis will allow the overall impact on cellular function of such drugs to be better appreciated.**

β-lapachone | ROS | inositol pyrophosphates | hypoxia | NQO1

## Significance

Inositol pyrophosphates (PP-InsPs) are messenger molecules regulating various functions in mammals. They are associated with the oxidative stress response, but the underlying mechanism is unclear. We investigate PP-InsP signaling in mammalian cells subjected to reactive oxygen species (ROS). Applying the quinone β-lapachone (β-lap) generated intracellular ROS resulting in decreased PP-InsP levels. This indicates a key role of PP-InsPs in cellular signaling under oxidative stress. Moreover, β-lap-mediated PP-InsP signaling required oxygen and the enzyme NAD(P)H:quinone oxidoreductase-1 (NQO1). Since quinone drugs are cytotoxic, our data provide a basis for further investigations into the role of PP-InsPs during quinone-dependent chemotherapies. This is of special relevance since a phase II clinical trial demonstrated β-lap efficacy in a combination chemotherapy against pancreatic cancer.

*myo*-Inositol pyrophosphates (PP-InsPs hereafter) are intracellular messengers implicated in a wide range of physiological processes in eukaryotes. Particularly, they have been referred to as "metabolic messengers" as their concentration is bound to ATP levels (1–4). They are composed of phosphate esters and either one or two pyrophosphate groups attached to the six-carbon *myo*-inositol ring resulting in PP-$InsP_5$ or $(PP)_2$-$InsP_4$, respectively (1). Apart from regular protein binding (2), PP-InsPs are also capable of nonenzymatic protein pyrophosphorylation (5, 6). Mammalian PP-InsP synthesis mainly starts from the inositol phosphate (InsP) $InsP_6$ and is achieved by two different classes of enzymes: the inositol hexakisphosphate kinases (IP6Ks) and the diphosphoinositol- pentakisphosphate kinases (PPIP5Ks, Fig. 1*A*) (1, 4). Among the biosynthesized PP-InsPs, 5-PP-$InsP_5$ is the most abundant isomer inside mammalian cell lines, while 1-PP-$InsP_5$ and 1,5-$(PP)_2$-$InsP_4$ occur in comparably low concentrations (1). However, very recent data suggest that large fluctuations in concentrations of the distinct isomers can occur in mammalian tissues and that additional isomers are also present (7).

To date, a variety of studies have connected PP-InsPs and their kinases with diseases, such as type 2 diabetes (9, 10), carcinogenesis (11, 12), and hyperphosphatemia (13). New therapeutic strategies targeting the PP-InsP pathway are therefore evolving (13). Mammals express three different IP6K paralogs: IP6K1/2/3 (2). Among those, IP6K2 has attracted significant attention since several experiments demonstrated that this isoform sensitizes mammalian cells to apoptosis caused by stressors, such as reactive oxygen species (ROS), γ-irradiation, and cisplatin (14, 15). Moreover, it was found that ROS decreased PP-$InsP_5$, as well as $(PP)_2$-$InsP_4$ levels in yeast cells suggesting that PP-InsPs are directly involved in the yeast oxidative stress response (16).

While ROS have historically been considered to be dangerous by-products of aerobic metabolism, the past 20 y have witnessed a reassessment of their biological significance. It is now well known that these species regulate multiple signaling pathways, including mitochondrial function and consequently cellular bioenergetics (17, 18). Nevertheless, since massive oxidative stress is lethal to the cell, the toxic nature of ROS is exploited in cancer

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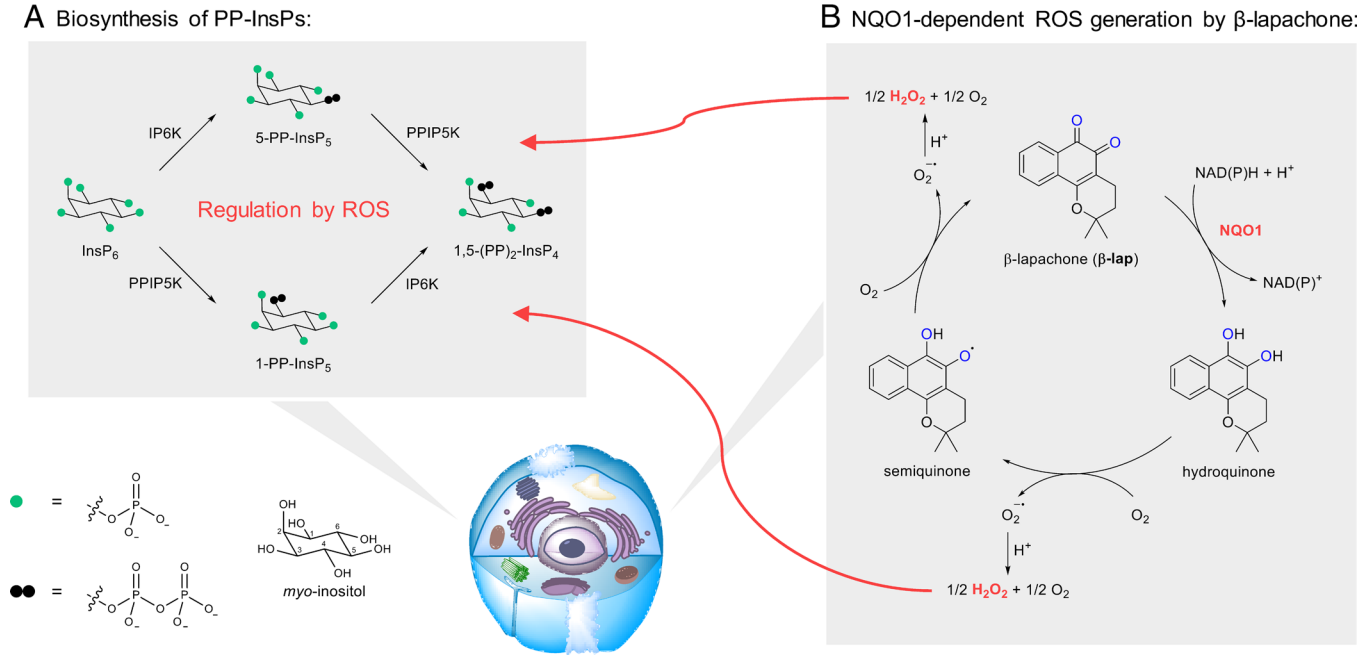


**Fig. 1.** Exploring the influence of ROS on mammalian PP-InsP levels. (*A*) Biosynthesis of PP-InsPs from $InsP_6$ via IP6Ks and PPIP5Ks. (*B*) Proposed mechanism of action of β-lap. β-lap redox cycles in NQO1 expressing cells between hydroquinone and semiquinone forms thereby generating cytotoxic ROS. Spontaneous hydroquinone and semiquinone oxidation occur in the presence of oxygen. Mechanism based on Pink et al. (8)

treatment and chemotherapeutic agents that selectively release ROS in cancers have been identified. The naphthoquinone β-lapachone (β-lap) is such an antitumor drug with activity against solid tumors evaluated in several phase I clinical trials (19, 20). Furthermore, it showed efficacy against pancreatic cancer in a phase II study when applied in combination with gemcitabine (21). β-lap is capable of releasing ROS in cells expressing the two-electron reductase NAD(P)H:quinone oxidoreductase 1 (NQO1, EC 1.6.5.2) (22). According to the mechanism proposed by Pink et al., NQO1 reduces the drug to an unstable hydroquinone thereby consuming one molecule NAD(P)H per reaction. The hydroquinone autoxidizes through a semiquinone intermediate to the parent compound. This spontaneous oxidation produces ROS and closes the futile cycle. (Fig. 1*B*) (8). Using MCF7:WS8 cell extracts as NQO1 source, it was demonstrated that one mole of β-lap was capable of oxidizing 50 to 60 mol NADH in 3 min (8). As various human solid tumors express significantly higher NQO1 levels than normal tissues (22), β-lap enables selective killing of cancer cells.

β-lap-mediated cellular ROS generation has been thoroughly studied (23–27), and various downstream targets have been described. These include hyperactivation of poly(ADP-ribose) polymerase-1 in an NQO1-dependent manner (25), which leads to dramatic decrease of intracellular ATP (25, 28, 29). A recently developed prodrug-based delivery technique that improves tumor targeting demonstrates that systemic toxicity can be significantly reduced, rendering *o*-quinones a candidate compound family for targeted therapeutics (30).

In this work, we explore the levels of mammalian PP-InsPs in response to oxidative stress, particularly brought about by quinone reagents, such as β-lap, in an NQO1-dependent manner under normoxic and hypoxic conditions (overview shown in Fig. 1). We therefore add another layer to our understanding of *o*-quinone action on cells. Application of highly sensitive capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) allowed the absolute quantitation of PP-InsP levels in two different mammalian cell lines (31). These cell lines either expressed (HCT116 cells) or did not express NQO1 (MDA-MB-231 cells). We demonstrate the importance of NQO1 for the modulation of PP-InsP levels under hypoxic and normoxic conditions. With the emergence of targeted *o*-quinones as cytotoxic drugs (30), a deeper understanding of their impact on cell signaling molecules is highly warranted and provided in this study.

## Results and Discussion

**$H_2O_2$ and Bioactivated β-Lapachone Reduce PP-InsP Levels in HCT116 Cells.** To evaluate the impact of exogenous ROS on mammalian PP-InsP levels, the human colon cancer cell line HCT116 was used (32). HCT116 cells maintain higher 5-PP-$InsP_5$ as well as 1,5-$(PP)_2$-$InsP_4$ levels than other mammalian cell lines (33) and are consequently ideally suited to study changes in PP-InsP concentrations. These analytes can be readily detected using CE-ESI-MS with very high sensitivity (7, 34, 35).

Initially, HCT116 cells were oxidatively stressed via exposure to 0.1 mM or 1.0 mM $H_2O_2$ for 30 min. Then, the cellular metabolism was directly quenched with perchloric acid, and PP-InPs and InsPs were enriched with $TiO_2$ beads (36). The enriched extracts were spiked with stable isotope labeled (SIL) [$^{13}$C]-PP-InsP and [$^{13}$C]-$InsP_6$ (37, 38) internal standards and analyzed by CE-ESI-MS (39). Detection of 1,5-$(PP)_2$-$InsP_4$, 5-PP-$InsP_5$, and $InsP_6$ was feasible, whereas 1-PP-$InsP_5$ levels were below the detection limit in several samples and therefore not further considered for quantitation (Fig. 2*A*).

Normalization by protein content revealed decreased 5-PP-$InsP_5$ and 1,5-$(PP)_2$-$InsP_4$ levels after treatment with 1.0 mM $H_2O_2$. PP-InsP levels in HCT116 cells subjected to 0.1 mM $H_2O_2$ as well as $InsP_6$ levels in cells treated with either 0.1 mM or 1.0 mM $H_2O_2$ were not affected (Fig. 2, *B1* and *B2*). These results are in agreement with earlier studies in yeast revealing reduced PP-InsP levels after incubation with 1 mM $H_2O_2$ for 20 min (16). However, Choi et al. described unaffected $(PP)_2$-$InsP_4$ levels in $DDT_1$ $MF_2$ cells after treatment with 0.15 mM or 0.3 mM $H_2O_2$ for 30 min (40). We

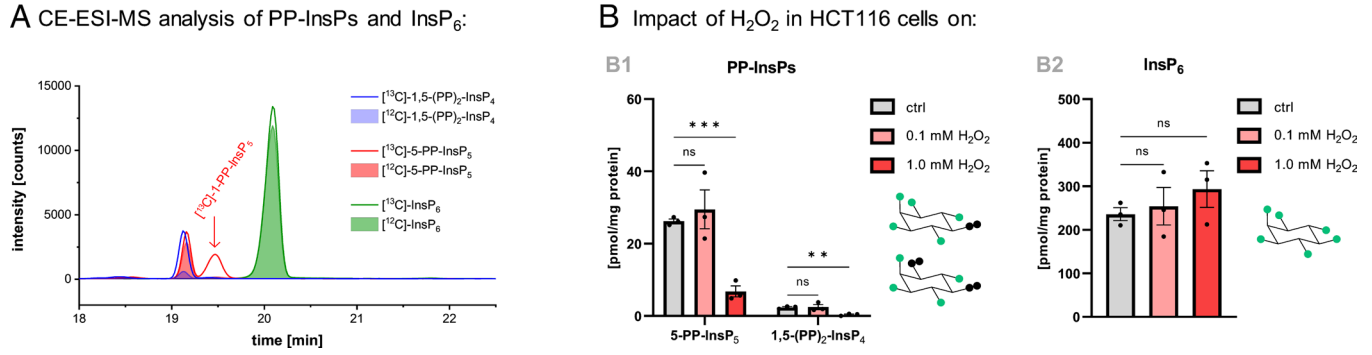


**Fig. 2.** Exogenous ROS ($H_2O_2$) decrease mammalian PP-InsP levels. (*A*) Electropherogram of 1,5-$(PP)_2$-$InsP_4$, 5-PP-$InsP_5$, and $InsP_6$ (filled areas) in untreated HCT116 cells and stable isotope labeled [$^{13}$C]-1,5-$(PP)_2$-$InsP_4$, [$^{13}$C]-5-PP-$InsP_5$, [$^{13}$C]-1-PP-$InsP_5$, and [$^{13}$C]-$InsP_6$ internal standards (unfilled areas). 1-PP-$InsP_5$ in HCT116 control cells was below the detection limit in several samples and therefore not considered for quantitation. (*B*) PP-InsP and $InsP_6$ levels in HCT116 control cells and HCT116 cells treated with 0.1 mM or 1.0 mM $H_2O_2$ for 30 min. Data are means ± SEM from three replicates. Statistical analyses to compare treated cells with control cells were performed using an unpaired two-tailed Student's *t* test (**$P \leq 0.01$, ***$P \leq 0.001$). ns: not significant. ctrl: control.

conclude that extracellular $H_2O_2$ concentrations in the millimolar range are required to significantly lower PP-InsP levels in mammalian cell lines, particularly HCT116. Analysis of methanol-extracted ATP from cocultured cells revealed unaffected ATP levels in cells incubated with 0.1 mM $H_2O_2$. In contrast, ATP levels in HCT116 cells treated with 1.0 mM $H_2O_2$ were reduced (ca. threefold) confirming reported cellular ATP losses after excessive exposure to oxidative stress (*SI Appendix*, Fig. S1) (41). The oxidative stress-dependent reduction of PP-InsP levels as a potential response to reduced ATP levels might be the result of an interplay between PP-InsP signaling and cellular energy dynamics (42). However, it remains unclear whether a loss of ATP or other effects of ROS are critically required for PP-InsP reduction upon $H_2O_2$ exposure.

Therefore, to get a deeper understanding of ROS-dependent PP-InsP regulation, we next investigated whether $H_2O_2$-mediated PP-InsP changes had been caused by endogenous ROS. As an intracellular generator of oxidative stress, the bioactivatable quinone β-lap was chosen. HCT116 cells are synthesizing functioning NQO1 (43, 44), and endogenous ROS production in this cell line can consequently be achieved by β-lap treatment. Application of an $H_2O_2$-sensitive live cell dye (BioTracker Green $H_2O_2$) confirmed significantly increased levels of $H_2O_2$ in HCT116 cells upon incubation with 10 μM β-lap for 2 h (Fig. 3, *A1*). This was in accordance with former studies reporting elevated cellular ROS concentrations in various NQO1-expressing cell lines exposed to the quinone (24, 25, 27, 45). As futile cycling of β-lap also suggests enhanced oxygen consumption in cells subjected to the drug (Fig. 1*B*), we additionally used Seahorse metabolic flux analysis to measure oxygen consumption rates (OCR) in β-lap-treated HCT116 cells (Fig. 3, *A2*). While 1 μM β-lap was already sufficient to induce significantly enhanced respiration in HCT116 cells, a massive burst of OCR was observed upon treatment with 5 μM and 10 μM of the drug (up to ~2.5-fold). Moreover, suppressing the electron transport chain with antimycin A (AA) did not prevent β-lap-enhanced OCR (Fig. 3, *A3*), demonstrating that increased cellular oxygen consumption was independent of mitochondrial ATP production. The results derived from seahorse assays were in line with previous reports (46, 47) and indicated that micromolar doses of β-lap were able to affect cellular respiration and increase ROS production in HCT116 cells.

To test whether PP-InsP levels were altered upon exposure to β-lap, and consequently by intracellular $H_2O_2$ generation, HCT116 cells were subjected to 2.5 μM, 5 μM, or 10 μM of the quinone for 2 h. Cellular metabolism was then quenched, and 5-PP-$InsP_5$, 1,5-$(PP)_2$-$InsP_4$, and $InsP_6$ levels were analyzed by CE-ESI-MS. β-lap dose dependently decreased PP-InsP levels, while $InsP_6$ levels remained stable (Fig. 3, *B1–B3*). Treatment with 10 μM of the naphthoquinone for 2 h almost completely depleted cellular PP-InsP levels. In addition, ATP from cells grown on parallel dishes and subjected to 5 μM or 10 μM of β-lap was analyzed, and β-lap-mediated depletion of ATP levels was confirmed (*SI Appendix*, Fig. S2*A*) (25, 28, 29). The loss of the energy status of the cell might largely arise from poly(ADP-ribose)polymerase-1 hyperactivation (41) as described above. Nevertheless, as further studies also reported that β-lap perturbs glycolysis and downstream metabolism (26, 44), multiple effects caused by the treatment with the quinone might be responsible for the loss of ATP in β-lap-exposed cells. Overall, the results obtained from assaying PP-InsPs, $InsP_6$, and ATP levels in β-lap-treated HCT116 cells supported the idea that mammalian PP-InsPs are responding to intracellularly generated oxidative stress, whereas $InsP_6$ remains unaffected.

As ROS can oxidize redox-sensitive cysteine residues inside proteins (48), we hypothesized that the ROS-dependent reduction of PP-InsP levels had been caused by inactivation of the 5-PP-$InsP_5$ synthesizing kinases IP6K1/2/3. These enzymes contain several cysteine residues within their amino acid sequence, and it has been shown that the homologous yeast enzyme KCS1 can be inactivated by $H_2O_2$ treatment (16). To study $H_2O_2$-dependent IP6K regulation, we incubated in vitro reactions of purified IP6K1 and IP6K2, the dominant IP6K types in mammalian tissues (49), with 1 mM $H_2O_2$ and analyzed the reaction outcomes by PAGE (*SI Appendix*, Fig. S2*B*). Similarly to yeast KCS1, the enzymatic activity of the mammalian $InsP_6$ kinases, i.e., transformation of $InsP_6$ to 5-PP-$InsP_5$, was significantly impaired, demonstrating a direct inactivation of IP6K1/2 by $H_2O_2$.

To verify whether β-lap-dependent ROS production directly inhibits IP6K1 activity in cellulo, we generated an endogenously Flag-tagged IP6K1 (Flag-IP6K1) HCT116 cell line. Flag-IP6K1 cells were treated with 1 μM of β-lap for 24 h, and IP6K1 was subsequently immunoprecipitated and examined by an in vitro kinase assay. Analyses of the reaction products by PAGE gel showed that IP6K1 immunoprecipitated from β-lap-treated cells was unable to synthesize 5-PP-$InsP_5$ from $InsP_6$ (*SI Appendix*, Fig. S2*E*).

In addition to β-lap, other *o*-naphthoquinones, such as (±)-dunnione (dun, chemical structure shown in Fig. 3*C*), are substrates for NQO1 and are able to generate large quantities of ROS inside the cell (45, 50). In contrast, the *p*-naphthoquinone α-lapachone (α-lap, chemical structure shown in Fig. 3*C*) is known to produce no or only comparably small amounts of intracellular

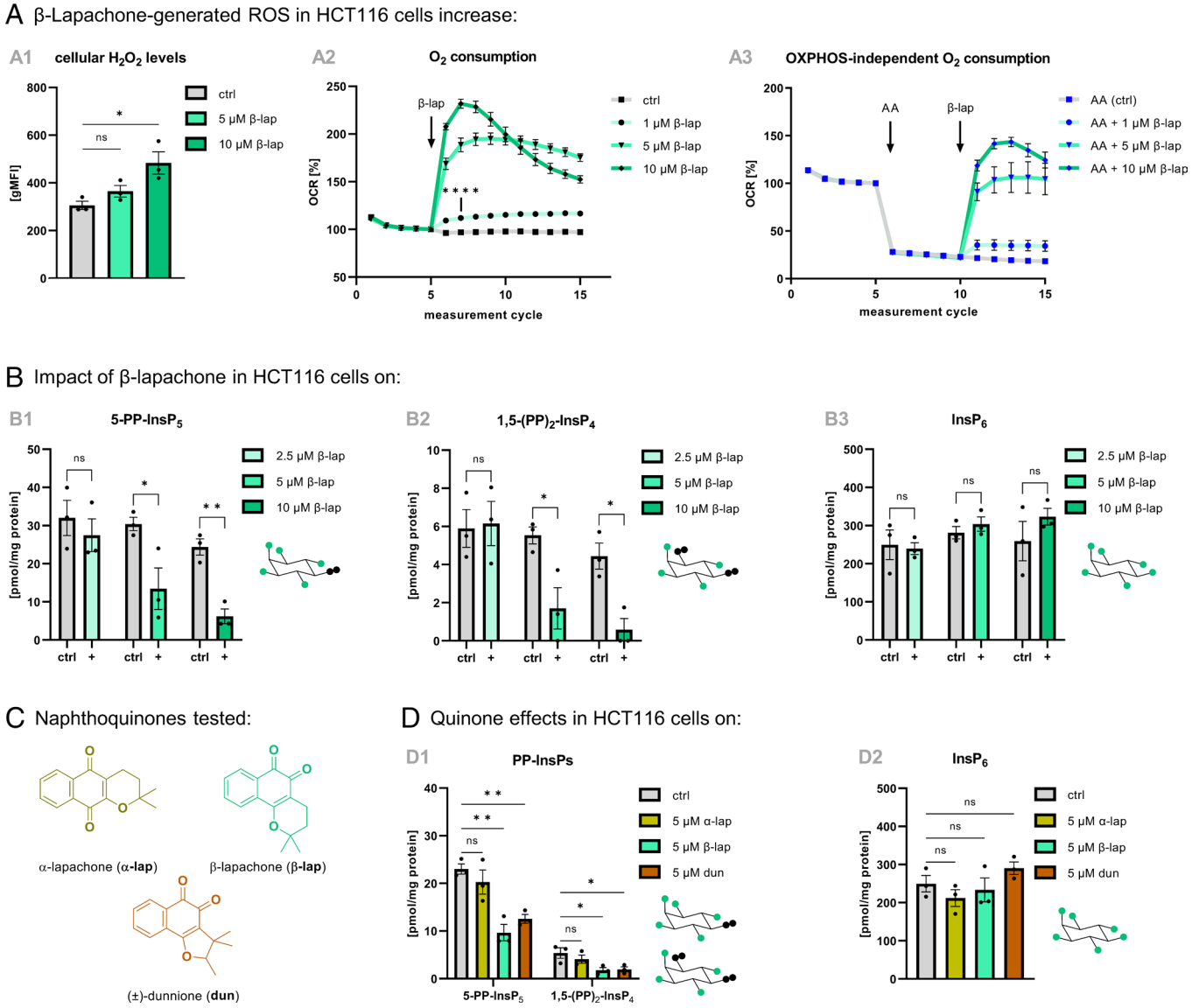


**Fig. 3.** Mammalian PP-InsP levels fall in response to naphthoquinone-generated endogenous ROS. (*A*) $H_2O_2$ levels and OCR in HCT116 control cells and HCT116 cells treated with β-lap. (*A1*) $H_2O_2$ levels were determined 2 h after exposure to β-lap via fluorescence measurement of an $H_2O_2$-sensitive dye (BioTracker Green $H_2O_2$). (*A2* and *A3*) OCR was monitored by Seahorse real-time metabolic flux analysis while β-lap and antimycin A (AA) were added at the indicated time points. (*B*) PP-InsP and $InsP_6$ levels in HCT116 control cells and HCT116 cells treated with 2.5 µM, 5 µM, or 10 µM β-lap for 2 h. (*C*) Structures of the p-naphthoquinone α-lap and the o-naphthoquinones β-lap and dun used in this study. (*D*) PP-InsP and $InsP_6$ levels in HCT116 control cells and HCT116 cells treated with 5 µM α-lap, β-lap, or dun for 2 h. (A)/(B)/(D): Data are means ± SEM from three to four replicates. Statistical analyses to compare treated cells with control cells were performed using an unpaired two-tailed Student's *t* test (*$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$). ctrl: control. gMFI: geometric mean of fluorescence intensity. ns: not significant. OXPHOS: oxidative phosphorylation.

ROS (50–52). Therefore, the impact of α-lap, β-lap, and dun on PP-InsP levels in HCT116 cells was compared with their reported ability to generate cellular ROS. HCT116 cells were incubated with 5 µM α-lap, β-lap, or dun for 2 h, and PP-InsPs and $InsP_6$ were extracted from the cells as described above. CE-ESI-MS analysis revealed that β-lap and dun were equally capable of reducing 5-PP-$InsP_5$ (ca. twofold) and 1,5-$(PP)_2$-$InsP_4$ levels (ca. threefold), whereas α-lap had no significant impact on PP-InsP levels (Fig. 3, *D1*). Neither the two different *o*-quinones nor the *p*-quinone altered $InsP_6$ levels (Fig. 3, *D2*). The naphthoquinone-dependent extent of reduction of PP-InsP levels was therefore in alignment with their ability to produce ROS inside the cell (45, 50–52).

The in cellulo studies with β-lap and its isomers indicated an oxidative stress-dependent effect of the quinone on PP-InsP levels. However, a potential direct binding of β-lap to mammalian IP6Ks resulting in reduced PP-InsP synthesis could not be ruled out. Hence, β-lap was screened against purified IP6K1 serving as representative paralog for the IP6K family. Interestingly, the in vitro experiments revealed a decrease in IP6K1 activity with an $IC_{50}$ value of ca. 10 µM and a maximal inhibition of 70% (*SI Appendix*, Fig. S2*C*). These results demonstrate that β-lap could directly target IP6K1, rendering it one inhibitor of IP6Ks of which only a handful exist (13, 53–56). However, the in cellulo reduction of PP-InsP levels by the quinone was already significant at a concentration as low as 5 µM (Fig. 3, *B1* and *B2*) showing that apart from minor contributions of direct inhibition, β-lap was likely affecting cellular PP-InsP levels through generation of ROS.

**Hypoxia Reveals That β-Lapachone Acts through ROS Production.** To further test our hypothesis, we investigated whether PP-InsP levels are reduced as a result of oxidative stress triggered by futile cycling of β-lap. Since the autoxidation of β-lap is dependent on partial $O_2$ pressure ($p[O_2]$) (Fig. 1*B*), reduced $p[O_2]$ is expected

to diminish β-lap-mediated ROS production and consequently reduce loss of PP-InsP levels. In initial experiments, basal 5-PP-$InsP_5$, 1,5-$(PP)_2$-$InsP_4$, and $InsP_6$ concentrations in HCT116 cells cultured under hypoxia were determined. Cells were initially seeded and preincubated under normoxia. Then, the dishes were placed in a hypoxia chamber and incubated at 1% $O_2$ (referred to as hypoxic conditions) for different time intervals. Subsequently, cells were lysed followed by enrichment as well as CE-ESI-MS analysis of PP-InsPs and $InsP_6$ (Fig. 4*A*). Analyses of cellular ATP extracted from parallel dishes were also performed.

Interestingly, 5-PP-$InsP_5$ levels in normoxic cells declined to about half of the initial value at the end point of this study (31 h). In contrast, 5-PP-$InsP_5$ levels in hypoxic cells remained stable over the observed period (Fig. 4, *B1*). Consistent with this finding, ATP levels in hypoxic cells were only slightly reduced, while ATP levels in normoxic cells decreased with increasing incubation time (Fig. 4, *B2*). 1,5-$(PP)_2$-$InsP_4$ and $InsP_6$ levels in hypoxic cells were not significantly lower when compared to those in normoxic cells, while over time, a decrease in 1,5-$(PP)_2$-$InsP_4$ levels was observed under normoxia (*SI Appendix*, Fig. S3 *I*, *A1* and *A2*). As the data demonstrate, ATP and PP-InsP levels are dependent on p[$O_2$] and also on the duration of the experiment. Such time-dependent adaptations of cellular ATP levels to low $O_2$ levels have been observed in earlier studies as for instance by Frezza et al. who found decreased ATP levels in hypoxic HCT116 cells (36 h under 1% $O_2$) (57). Moreover, hypoxic adaptation of PP-$InsP_5$ has also been observed in bone marrow-derived mesenchymal stem cells (BM-MSCs): When serum-deprived BM-MSCs were exposed to hypoxia (1% $O_2$), the PP-$InsP_5$/$IP_6$ ratio was higher than the PP-$InsP_5$/$InsP_6$ ratio in corresponding normoxic cells (58, 59). Low p[$O_2$] might therefore substantially contribute to alterations of PP-$InsP_5$ levels inside serum-deprived hypoxic cells. Moreover, another study discovered increased IP6K gene expression in largemouth bass exposed to hypoxic conditions (60). Hence, 5-PP-$InsP_5$ might be part of the cellular machinery that mediates the metabolic adaptation to reduced p[$O_2$].

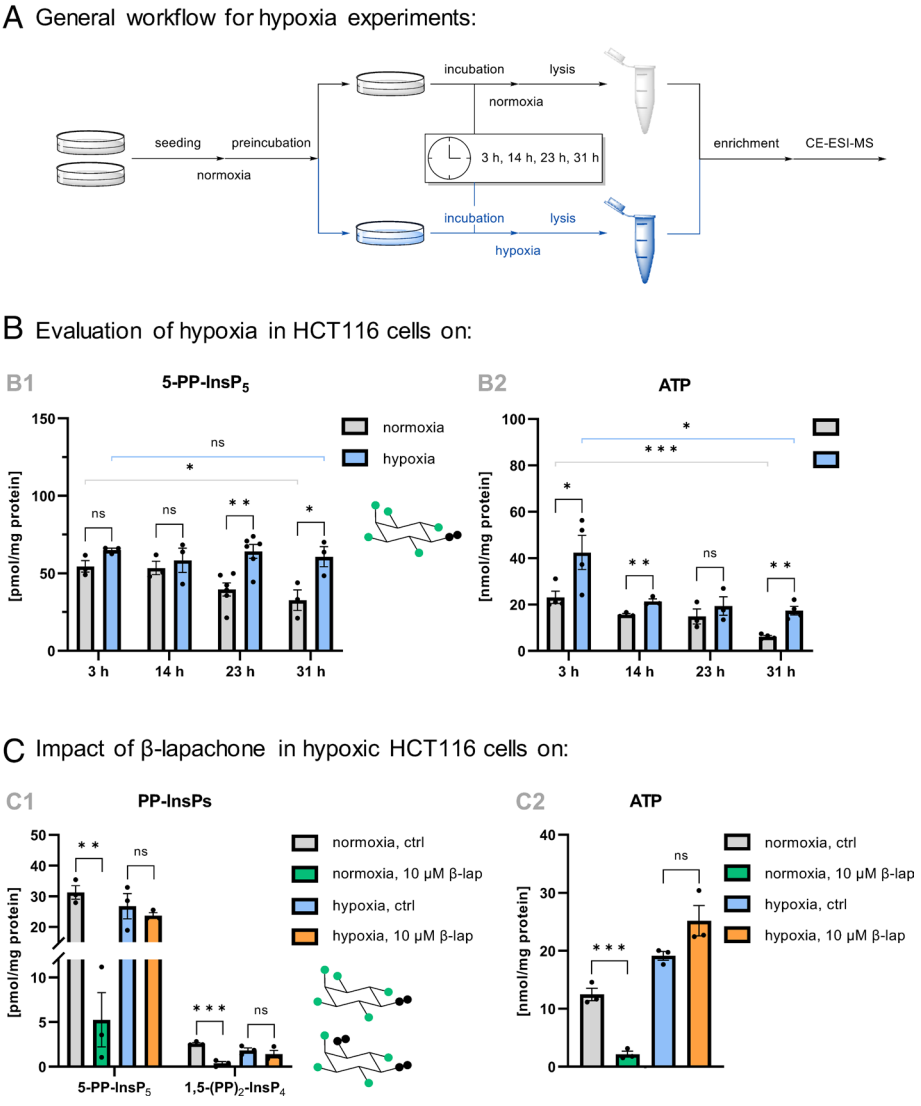


**Fig. 4.** Oxygen is required for β-lapachone effects on PP-InsP levels. (*A*) Workflow for hypoxia experiments with subsequent enrichment of PP-InsPs and $InsP_6$ from the cells followed by CE-ESI-MS. (*B*) 5-PP-$InsP_5$ and ATP levels in HCT116 cells grown for 3 h, 14 h, 23 h, and 31 h under normoxic and hypoxic conditions. ATP was extracted from cells grown in parallel to those used for perchloric acid extraction of PP-InsPs and $InsP_6$. (*C*) PP-InsP and ATP levels in normoxic and hypoxic control HCT116 cells and normoxic and hypoxic HCT116 cells treated with 10 µM β-lap for 2 h. Preincubation time of cells under hypoxia prior to drug addition was 23 h. ATP was extracted from cells grown in parallel to those used for perchloric acid extraction of PP-InsPs and $InsP_6$. (*B* and *C*) Data are means ± SEM from three to six replicates. Statistical analyses to compare hypoxic with normoxic cells, cells cultured for 31 h under normoxia/hypoxia with cells cultured for 3 h under normoxia/hypoxia, and normoxic/hypoxic β-lap-treated cells with normoxic/hypoxic control cells were performed using an unpaired two-tailed Student's *t* test (*$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$). ns: not significant. ctrl: control.

It is known that cells undergo various metabolic adaptations during hypoxia, for instance, upregulation of glycolysis, in order to diminish the respiratory rate. This action prevents ROS overproduction and $O_2$ depletion when its levels are limited (61). In this context, 5-PP-$InsP_5$ might have a key role in governing the cellular response to ROS signaling under hypoxia. Decreased cellular ATP utilization under hypoxia (61) explains the stable ATP levels in hypoxic HCT116 cells observed over a period of 31 h. In contrast, corresponding normoxic cells maintain their regular energy metabolism likely leading to decreased ATP availability with increasing cell growth (Fig. 4, *B2*).

Next, the effect of reduced p[$O_2$] on PP-InsP concentrations in β-lap-treated cells was evaluated. HCT116 cells were incubated for 23 h under hypoxia, and 10 μM of β-lap was then added to the cells. The cells were incubated with the naphthoquinone for another 2 h under reduced p[$O_2$]. Whereas PP-InsP levels in β-lap-treated normoxic cells were reduced (ca. sixfold for 5-PP-$InsP_5$; ca. sevenfold for 1,5-$(PP)_2$-$InsP_4$), PP-InsP levels in β-lap-treated cells grown under 1% $O_2$ were not changed, clearly highlighting the $O_2$ dependence of β-lap action (Fig. 4, *C1*). $InsP_6$ levels in neither normoxic nor hypoxic HCT116 cells subjected to β-lap were affected (*SI Appendix*, Fig. S3 *I*, *B*). Moreover, consistent with these findings, cellular ATP levels in hypoxic β-lap-treated HCT116 cells did not change in response to the drug. In contrast, ATP levels in corresponding normoxic cells subjected to 10 μM of β-lap were decreased (Fig. 4, *C2*). These results further underscore that β-lap reduces PP-InsP levels via ROS production in the presence of sufficient $O_2$, and not by direct IP6K binding.

**Hypoxia Does Not Influence the Effect of Other IP6Ks Inhibitors.** As β-lap is a regulator of PP-InsP levels in cellulo with a previously unknown p[$O_2$] dependence, we were interested in studying this effect also for other known inhibitors of PP-InsP synthesis. Among them is the widely used IP6K inhibitor $N^2$-(*m*-(trifluoromethyl) benzyl) $N^6$-(*p*-nitrobenzyl)purine (53) (TNP, structure shown in *SI Appendix*, Fig. S3 *II*, *A*) as well as the natural product Quercetin (54) (Q, structure shown in *SI Appendix*, Fig. S3 *II*, A), which is an antioxidant (62). The potency of both, Q and TNP, might also depend on p[$O_2$]: Q is able to increase intracellular ROS (63) which might affect the biosynthesis of PP-InsPs and the nitro group of TNP could be reduced under hypoxia (64, 65). Therefore, we wanted to understand if the inhibitory effect of Q and TNP on IP6Ks was also dependent on p[$O_2$], which might restrict the applicability of the drugs during cell culture experiments to distinct $O_2$ levels. However, Q was capable of significantly reducing 5-PP-$InsP_5$ levels under hypoxia as well as normoxia (*SI Appendix*, Fig. S3 *II*, *B1* and *B2*). Consequently, the redox activity of Q might only marginally contribute to a reduction of cellular PP-InsP synthesis. Regarding TNP, the results demonstrate that its activity was not affected by reduction of $O_2$ levels. Of note, under the applied experimental conditions, TNP reduced PP-InsP levels only to a very minor extent. Therefore, we normalized PP-InsP levels by $InsP_6$, which revealed significant but only slight reduction of 5-PP-$IP_5$ levels in TNP-treated normoxic and hypoxic cells (*SI Appendix*, Fig. S3 *II*, *C1*–*C3*). Thus, we conclude that in contrast to β-lap, Q and TNP are not dependent on p[$O_2$].

**β-Lapachone-Mediated Modulation of PP-InsP Levels Requires NQO1.** To conclusively establish that β-lap controls PP-InsP levels via ROS production, we investigated the role of NQO1. For this purpose, we employed the triple-negative (lacking the estrogen, progesterone, and epidermal growth factor receptors) breast cancer cell line MDA-MB-231. This aggressive cancerous cell line is not

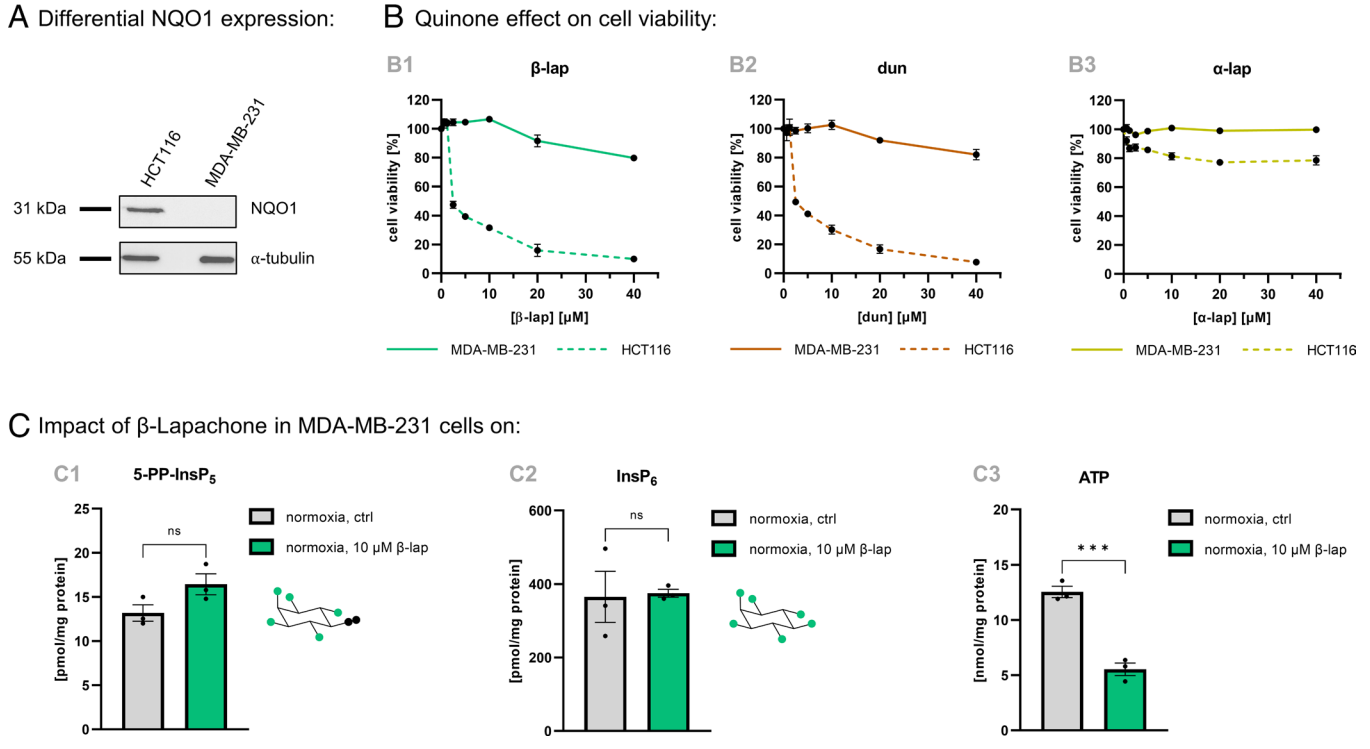


**Fig. 5.** NQO1 is required for β-lap-mediated reduction of PP-InsP levels. (*A*) NQO1 protein levels in HCT116 and MDA-MB-231 cells. Equal loading was monitored by using α-tubulin. Whole PAGE is shown in *SI Appendix*, Fig. S4 (*B*) Viability assays of MDA-MB-231 cells and HCT116 cells treated with different doses of β-lap, dun, and α-lap for 2 h. After exposure, cells were cultivated for 2 d in compound-free medium before viability was assessed. Data are means ± SEM from three to four replicates. (*C*) 5-PP-$InsP_5$, $InsP_6$, and ATP levels in normoxic control MDA-MB-231 cells and normoxic MDA-MB-231 cells treated with 10 μM β-lap for 2 h. ATP was extracted from cells grown in parallel to those used for perchloric acid extraction of 5-PP-$InsP_5$ and $InsP_6$. Data are means ± SEM from three replicates. Statistical analyses to compare treated cells with ctrl cells were performed using an unpaired two-tailed Student's *t* test (***$P \leq 0.001$). ns: not significant. ctrl: control.

only unresponsive to hormonal treatment, but it is also insensitive to quinoid chemotherapeutic agents (66, 67). MDA-MB 231 are a commonly used model since these cells are homozygous for the NQO1*2 polymorphism (68) caused by a C to T point mutation in the NQO1 complementary DNA (69, 70). Cell types homozygous for the mutant allele have no measurable NQO1 activity and are consequently resistant to β-lap (25, 44, 69–71). MDA-MB-231 cells were therefore used to study the function of NQO1 in ROS-dependent PP-InsP reduction caused by β-lap, with the caveat that other genetic differences between MDA-MB-231 and HCT116 cell lines might have unsuspected effects on our analysis. The absence of the NQO1 protein in MDA-MB-231 cells and its presence in HCT116 cells were verified by western blot (Fig. 5*A*, whole PAGE shown in *SI Appendix*, Fig. S4).

Also, NQO1-dependent cytotoxicity of β-lap was evaluated (Fig. 5, *B1*): Cell viability of HCT116 significantly decreased when β-lap was applied to the cells in the low micromolar range. In contrast, MDA-MB-231 cells were almost resistant to the naphthoquinone. When dun was tested, comparable results as for β-lap were obtained (Fig. 5, *B2*). α-lap only slightly reduced the viability of HCT116 cells and had no cytotoxic effect in MDA-MB-231 cells (Fig. 5, *B3*). These findings are in agreement with the literature describing NQO1-dependent lethality for β-lap as well as for dun and minor cytotoxicity for α-lap (44, 45, 50, 72). Also, the cytotoxicity data for the different quinones mirror their ability to generate intracellular ROS (45, 50–52) and to reduce PP-InsP levels (Fig. 3, *D1*).

Next, the impact of β-lap on PP-InsPs in NQO1-deficient MDA-MB-231 cells was evaluated. MDA-MB-231 cells were incubated with 10 μM β-lap for 2 h, and the cells were then lysed and processed and analyzed as described above. In contrast to the substantially decreased PP-InsP levels in HCT116 cells after treatment with 10 μM β-lap (fourfold to eightfold, see Fig. 3, *B1* and *B2*), 5-PP-InsP$_5$ levels in MDA-MB-231 were not affected by the drug, demonstrating again that, in cellulo, IP6K is not directly inhibited by β-lap (Fig. 5, *C1*). InsP$_6$ levels in β-lap-treated MDA-MB-231 cells were comparable to those in control cells (Fig. 5, *C2*). 1,5-(PP)$_2$-InsP$_4$ concentrations in this cell line were below the limit of detection. Conversely, ATP levels from MDA-MB-231 cells exposed to β-lap were significantly reduced (Fig. 5, *C3*), which had also been observed in HCT116 cells subjected to the naphthoquinone (*SI Appendix*, Fig. S2*A*). As MDA-MB-231 cells are not able to express NQO1, loss of ATP levels in β-lap-treated MDA-MB-231 cells may be a result of oxidative stress production caused by other one-electron oxidoreductases (73, 74). Importantly, the significant decrease of ATP in β-lap-treated MDA-MD-231 cells had no effect on PP-InsP levels (Fig. 5, *C1*). Therefore, the β-lap-driven reduction of PP-InsP levels in HCT116 is potentially not related to the reduced level of ATP. Instead, it is dependent on a functional NQO1 protein producing ROS in response to futile cycling of β-lap.

## Conclusion

This study provides insights into how mammalian PP-InsPs are affected by oxidative stress. It was shown here that exposure of NQO1-expressing HCT116 cells to exogenous and endogenous ROS reduced PP-InsP levels. Moreover, hypoxia completely abolished β-lap-mediated reduction of PP-InsP levels in HCT116 cells, which suggests that the quinone is affecting PP-InsP levels via ROS. The loss of potency of β-lap under hypoxia needs to be generally considered when results gained from regular cell culture experiments conducted under normoxia are applied to clinical trials: As physiological $O_2$ concentrations typically range from 1 to 10% (75), ROS-dependent β-lap toxicity shown in cellulo might be significantly reduced in vivo. With regard to the role of β-lap and other modified *o*-quinones as emerging antitumor agents, we reported that these drugs largely reduced cellular PP-InsP levels while they did not affect InsP$_6$. The data obtained from hypoxia experiments showed that basal 5-PP-InsP$_5$ and ATP levels in normoxic HCT116 cells decreased with increasing incubation time while basal 5-PP-InsP$_5$ and ATP levels in cocultured hypoxic HCT116 cells remained stable. This could be explained by the down-regulated ATP utilization in hypoxic cells that finally limits ROS overproduction caused by reduced p[$O_2$] (61). Hence, 5-PP-InsP$_5$ might have a key regulatory function during the cellular adaptation to low $O_2$ levels. Further studies are now required to elucidate this hypothesis in more detail. Additionally, it was shown that β-lap did not alter 5-PP-InsP$_5$ concentrations inside NQO1-deficient MDA-MB-231 cells. While we cannot exclude that other phenotypic aspects of the MDA-MB-231 cells may contribute to the observed phenotype, our data are supportive of the notion that the modulation of PP-InsPs via β-lap requires both, sufficient O2 and functioning NQO1 protein. Despite a direct in vitro inhibition of IP6K by β-lap, the data provide evidence that, in vivo, PP-InsP reductions rely on the NQO1-dependent futile redox cycle of β-lap eventually producing large amounts of ROS.

Our in vitro experiments with mammalian IP6K1 and IP6K2 demonstrate that the activity of the enzymes decreases upon treatment with $H_2O_2$, strongly suggesting that the 5-PP-InsP$_5$ synthesizing kinases are modulated by redox signaling. In addition, the isolation of inactive Flag-tagged IP6K1 from β-lap-treated HCT116 cells further strengthens this hypothesis. Nevertheless, it remains to be solved how precisely ROS target PP-InsP synthesis on a molecular level. As suggested by Onnebo et al. ROS might inactivate IP6Ks via the oxidation of a specific evolutionarily conserved cysteine residue inside the catalytic site of the proteins (16). The identification of the particular redox-sensitive amino acid in vivo using proteomics will be the goal of future studies.

Among many cytotoxic natural products, β-lap is a promising antitumor agent effective against NQO1-expressing cancer cells. Adding disturbed PP-InsP signaling to its various downstream effects now enables a deeper understanding of its lethality. This offers the potential to design quinone derivatives with increased toxicity and fewer side effects, eventually resulting in more effective and tolerable anticancer therapies. Our growing ability to manipulate PP-InsP signaling in conjunction with the use of quinone-based drugs could lead to the development of anticancer approaches.

## Materials and Methods

**Cell Culture.** HCT116 and MDA-MB-231 cells were cultured in DMEM or DMEM/F12, respectively, each supplemented with 10% FBS at 37 °C with 5% $CO_2$, unless stated otherwise. Hypoxic experiments were conducted with 1% $O_2$ using a HypoxyLab hypoxia Workstation. Details are stated in *SI Appendix*.

**Assays.** To determine PP-InsP and InsP$_6$ levels, mammalian cells were treated as indicated, and analytes were extracted from the cells using $TiO_2$ beads as described in refs. 33 and 36. ATP was extracted from the cells with methanol. Absolute quantitation of analytes was performed by CE-ESI-MS as described below. $H_2O_2$ levels in HCT116 cells were assessed using the Biotracker green $H_2O_2$ dye. OCR was measured by Seahorse metabolic flux analysis. For cell viability determinations and western blotting, standard techniques were used. IP6K in vitro experiments were performed with mammalian IP6K1 purified from *Escherichia coli*. Details on in cellulo and in vitro assays can be found in *SI Appendix*.

**CE-ESI-MS Analyses.** Absolute quantitation was achieved via spiking of samples with [$^{13}$C]-PP-InsP and [$^{13}$C]-InsP$_6$ standards based on ref. 31. Details are described in *SI Appendix*.

**Statistics.** Statistical significance was assessed by unpaired two-tailed Student's *t* tests; $P \leq 0.05$ is considered significant. For more information, refer to *SI Appendix*.

**Data, Materials, and Software Availability.** The raw data are publicly available through the University College London Research Data Repository (https://doi.org/10.5522/04/23804505) (76). All data, associated protocols, methods, and sources of materials can be accessed in the text or *SI Appendix*.

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