Main Manuscript for

β-Lapachone Regulates Mammalian Inositol Pyrophosphate Levels in an NQO1- and Oxygen-dependent Manner

Verena B. Eisenbeis[1], Danye Qiu[1,2], Lisa Strotmann[1], Guizhen Liu[1], Isabel Prucker[1], Kevin Ritter[1], Christoph Loenarz[3], Adolfo Saiardi[4]*, Henning J. Jessen[1,2]*

[1]Institute of Organic Chemistry, Albert-Ludwigs-Universität Freiburg, Albertstrasse 21, 79104 Freiburg im Breisgau, Germany

[2]CIBSS—The Center for Integrative Biological Signaling Studies, Albert-Ludwigs-Universität Freiburg

[3]Institute for Pharmaceutical Sciences, Pharmaceutical and Medicinal Chemistry, Albert-Ludwigs-Universität Freiburg, Albertstrasse 25, 79104 Freiburg im Breisgau, Germany

[4]Medical Research Council, Laboratory for Molecular Cell Biology, University College London, United Kingdom

*Henning Jacob Jessen; Adolfo Saiardi

Email: henning.jessen@oc.uni-freiburg.de; a.saiardi@ucl.ac.uk

Author contributions: V.B.E., A.S., and H.J.J. designed research and wrote the paper. C.L. designed research. V.B.E., L.S., and A.S. performed research and analyzed data. D.Q., G.L., and I.P. performed analytical measurements and analyzed data. K.R. performed chemical synthesis. C.L. and A.S. contributed materials/devices. All authors discussed the paper.
Competing interest statement: The authors declare no conflict of interest.

This PDF file includes:

Main text
Figure 1-5

1 Abstract

Inositol pyrophosphates (PP-InsPs) are energetic signalling 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 involves 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 signalling 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 new aspects of β-lap pharmacology and its impact on PP-InsP levels. Our identification of different quinone drugs as modulators of PP-InsP synthesis will allow to better appreciate their overall impact on cellular function.

2 Significance Statement

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 signalling 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 signalling under oxidative stress. Moreover, β-lap mediated PP-InsP signalling 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.

3 Introduction

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$_6$ and is achieved by two different classes of enzymes: the inositol hexakisphosphate kinases (IP6Ks) and the diphosphoinositol-pentakisphosphate kinases (PPIP5Ks, **Figure 1 A**).[1, 4] Among the biosynthesized PP-InsPs, 5-PP-InsP$_5$ is the most abundant isomer inside mammalian cells while 1-PP-InsP$_5$ and 1,5-(PP)$_2$-IP$_4$ occur in comparably low concentrations.[1] However, very recent data suggests 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,[8, 9] carcinogenesis,[10, 11] and hyperphosphatemia.[12] New therapeutic strategies targeting the PP-InsP pathway are therefore evolving.[12] 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.[13, 14] 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.[15]

As massive oxidative stress is lethal to the cell, chemotherapeutic agents that selectively release ROS in cancers have been identified. The naphthoquinone β-lapachone (β-lap) is an antitumor drug with activity against solid tumours evaluated in several phase I clinical trials.[16, 17] Furthermore, it showed efficacy against pancreatic cancer in a phase II study when applied in combination with gemcitabine.[18] 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).[19] 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, closes the futile cycle, and serves as starting point for the next round (**Figure 1 B**).[20] Using MCF7:WS8 cell extracts as NQO1 source it was demonstrated, that

one mole of β-lap was capable of oxidizing 50-60 mole NADH in 3 min.[20] As various human solid tumors express significantly higher NQO1 levels than normal tissues,[19] β-lap enables selective killing of cancer cells.

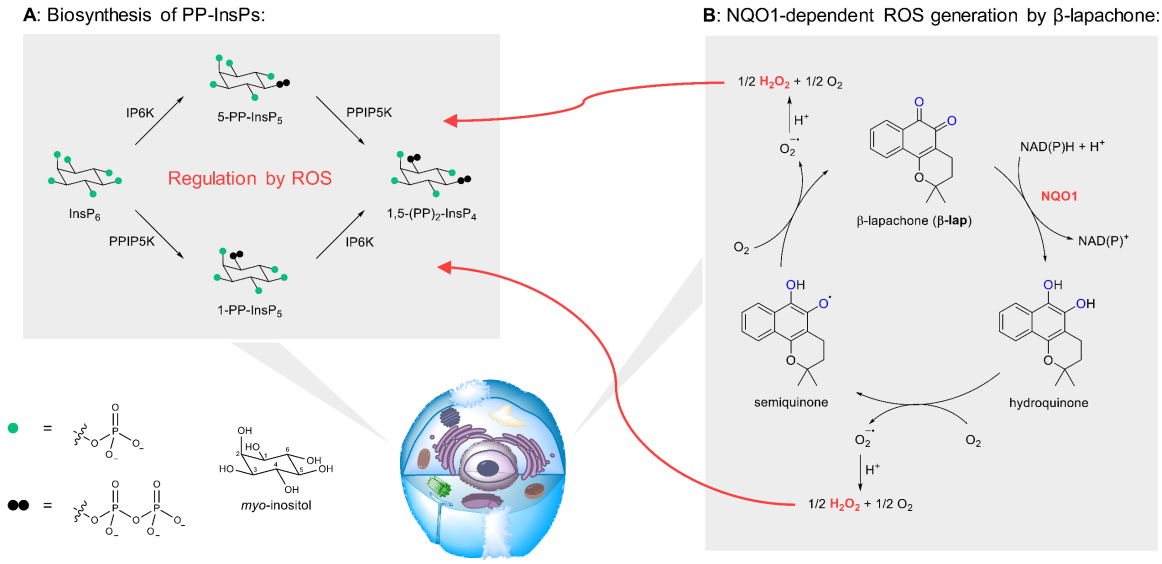

Figure 1 | Exploring the influence of ROS on mammalian PP-InsP levels. *(**A**) Biosynthesis of PP-InsPs from $InsP_6$ via IP6Ks and PPIP5Ks. (**B**) Proposed mechanism of action of β-lap.* β-Lap redox cycles in NQO1 expressing cells between hydroquinone and semiquinone forms thereby generating cytotoxic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

β-Lap mediated cellular ROS generation has been extensively studied[21–25] and various downstream targets have been described. These include hyperactivation of poly(ADP-ribose)polymerase-1 in an NQO1-dependent manner,[23] which leads to dramatic decrease of intracellular ATP.[23, 26, 27] 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.[28]

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 **Figure 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.[29] These cell lines either expressed (HCT116^{UCL} 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,[28] a

deeper understanding of their impact on cell signalling molecules is highly warranted and provided in this study.

4 Results and Discussion

H_2O_2 and bioactivated β-lapachone reduce PP-InsP levels in HCT116^{UCL} cells

To evaluate the impact of exogenous ROS on mammalian PP-InsP levels, the human colon cancer cell line HCT116^{UCL} was used.[30] HCT116^{UCL} cells maintain higher 5-PP-InsP$_5$ as well as 1,5-(PP)$_2$-InsP$_4$ levels than other mammalian cell lines[31] 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 resolution.[7, 32, 33]

Initially, HCT116^{UCL} 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 InsP$_6$ were enriched with TiO_2 beads.[34] The enriched extracts were spiked with stable isotope labelled (SIL) [^{13}C]-PP-InsP and [^{13}C]-InsP$_6$[35, 36] internal standards and analyzed by CE-ESI-MS.[37] 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. (**Figure 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^{UCL} 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 (**Figure 2 B1** and **B2**).

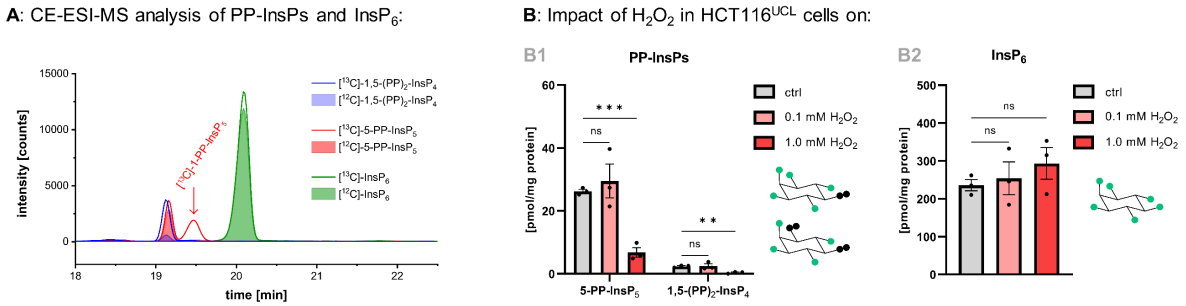

Figure 2 | Exogenous ROS (H_2O_2) decrease mammalian PP-InsP levels. *(**A**) Electropherogram of 1,5-(PP)$_2$-InsP$_4$, 5-PP-InsP$_5$, and InsP$_6$ (filled areas) in untreated HCT116^{UCL} cells and stable isotope labelled [^{13}C]-1,5-(PP)$_2$-InsP$_4$, [^{13}C]-5-PP-InsP$_5$, [^{13}C]-1-PP-InsP$_5$, and [^{13}C]-InsP$_6$ internal standards (unfilled areas).* 1-PP-InsP$_5$ in HCT116^{UCL} 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^{UCL} control cells and HCT116^{UCL} 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.

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.[15] 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.[38] We conclude that extracellular H_2O_2 concentrations in the millimolar range are required to significantly lower PP-InsP levels in mammalian cell lines, particularly $HCT116^{UCL}$. Analysis of methanol-extracted ATP from co-cultured cells revealed unaffected ATP levels in cells incubated with 0.1 mM H_2O_2. In contrast, ATP levels in $HCT116^{UCL}$ cells treated with 1.0 mM H_2O_2 were reduced (ca. 3-fold) confirming reported cellular ATP losses after excessive exposure to oxidative stress (**Figure S1**).[39] 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 signalling and cellular energy dynamics.[2, 3] However, it remains unclear if ATP reduction 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 intracellular ROS. The bioactivatable quinone β-lap was chosen as an intracellular generator of oxidative stress. HCT116 cells are synthesizing functioning NQO1[40, 41] and endogenous ROS production in this cell line can consequently be achieved by β-lap treatment. To test whether PP-InsP levels were altered upon exposure, $HCT116^{UCL}$ cells were subjected to 2.5 µM, 5 µM, or 10 µM β-lap 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 (**Figure 3 A1-A3**). 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 analysed and β-lap mediated depletion of ATP levels was confirmed (**Figure S2 A**).[23, 26, 27] The results obtained from assaying PP-InsPs, $InsP_6$, and ATP levels in β-lap treated $HCT116^{UCL}$ cells supported the idea that mammalian PP-InsPs are responding to intracellularly generated oxidative stress, whereas $InsP_6$ remains unaffected.

In addition to β-lap, other *o*-naphthoquinones, such as (±)-dunnione (dun, chemical structure shown in **Figure 3 B**), are substrates for NQO1 and are able to generate large quantities of ROS inside the cell.[42] In contrast, the *p*-naphthoquinone α-lapachone (α-lap, chemical structure shown in **Figure 3 B**) is known to produce no or only comparably small amounts of intracellular ROS.[43–45] Therefore, the impact of α-lap, β-lap, and dun on PP-InsP levels in $HCT116^{UCL}$ cells was compared with their reported ability to generate cellular ROS. $HCT116^{UCL}$ 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. 2-fold) and 1,5-(PP)$_2$-InsP$_8$ levels (ca. 3-fold) whereas α-lap had no significant impact on PP-InsP levels (**Figure 3 C1**). Neither the two different *o*-quinones nor the *p*-quinone altered InsP$_6$ levels (**Figure 3 C2**). The naphthoquinone-dependent extent of reduction of PP-InsP levels was therefore in alignment with their ability to produce ROS inside the cell.[42–45]

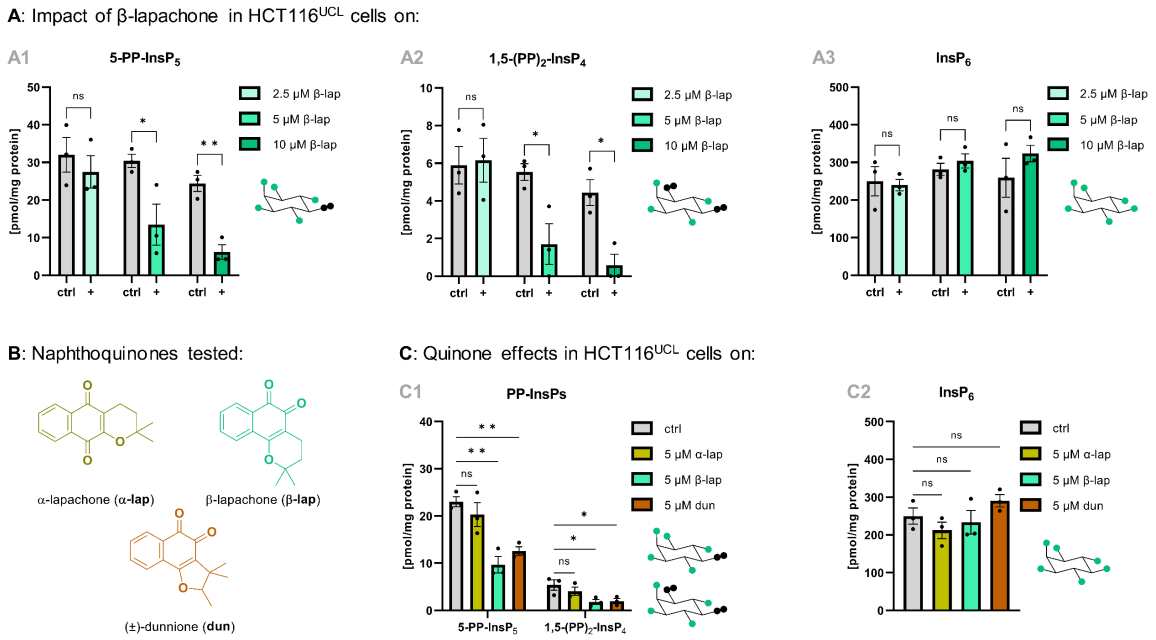

Figure 3 | Mammalian PP-InsP levels fall in response to naphthoquinone-generated endogenous ROS. *(**A**) PP-InsP and InsP$_6$ levels in HCT116^{UCL} control cells and HCT116^{UCL} cells treated with 2.5 μM, 5 μM, or 10 μM β-lap for 2 h. (**B**) Structures of the p-naphthoquinone α-lap and the o-naphthoquinones β-lap and dun used in this study. (**C**) PP-InsP and InsP$_6$ levels in HCT116^{UCL} control cells and HCT116^{UCL} cells treated with 5 μM α-lap, β-lap, or dun for 2 h.* (**A**)+(**C**): 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 (*$P \leq 0.05$; **$P \leq 0.01$). ctrl: control. ns: not significant.

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 % (**Figure S2 B**). These results demonstrate that β-lap could directly target IP6K1, rendering it one new inhibitor of IP6Ks of which only a handful exist.[12, 46–48] However, the *in cellulo* reduction of PP-InsP levels by the quinone was already significant at a concentration as low as 5 μM (**Figure 3 A1** and **A2**) 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 test our hypothesis, we investigated if 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]) (**Figure 1 B**), reduced p[O_2] is expected to diminish β-lap mediated ROS production and consequently lower loss of PP-InsP levels. In initial experiments, basal 5-PP-$InsP_5$, 1,5-$(PP)_2$-$InsP_4$, and $InsP_6$ concentrations in $HCT116^{UCL}$ cells cultured under hypoxia were determined. Cells were initially seeded and pre-incubated 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 $InsP_6$ (**Figure 4 A**). Analyses of cellular ATP extracted from parallel dishes were also performed.

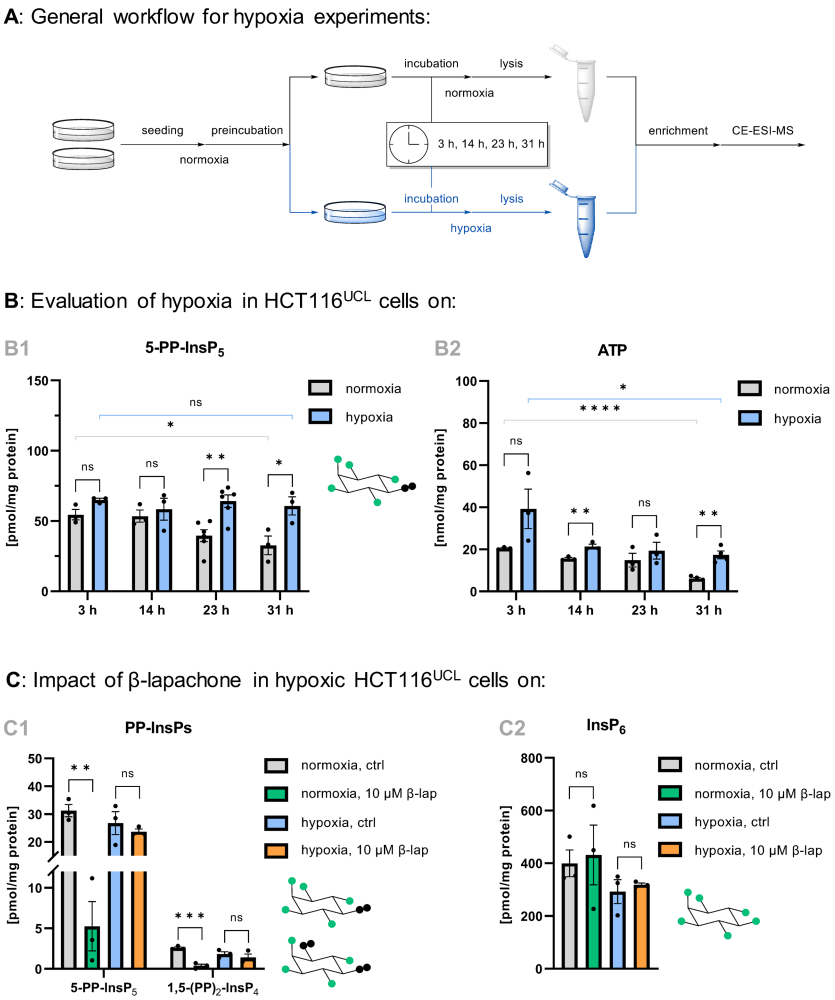

Figure 4 | Oxygen is required for β-lapachone effects on PP-InsP levels. *(**A**) Workflow for hypoxia experiments with subsequent enrichment of PP-InsPs and $InsP_6$ from the cells followed by CE-ESI-MS. (**B**) 5-PP-$InsP_5$ and ATP levels in $HCT116^{UCL}$ cells grown for 3 h, 14 h, 23 h, and 31 h under normoxic and hypoxic conditions.* ATP was extracted from cells grown in parallel to those used for perchloric acid extration of PP-InsPs and $InsP_6$. *(**C**) PP-InsP and InP_6 levels in normoxic and hypoxic control $HCT116^{UCL}$ cells and normoxic and hypoxic $HCT116^{UCL}$ cells treated with 10 µM β-lap for 2 h.* Preincubation time of cells under hypoxia prior to drug addition was 23 h. (**B**)+(**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 (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$). ns: not significant. ctrl: control.

Interestingly, 5-PP-$InsP_5$ levels in normoxic cells declined to about half of the initial value at the endpoint of this study (31 h). In contrast, 5-PP-$InsP_5$ levels in hypoxic cells remained stable over the observed period **Figure 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 **Figure 4 B2**). 1,5-$(PP)_2$-$InsP_4$ and $InsP_6$ levels in hypoxic cells were not significantly lower when compared with those in normoxic cells, while over time, a decrease in 1,5-$(PP)_2$-$InsP_4$ levels was observed under normoxia (**Figure 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)[49]. 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-$InsP_5$/IP_6 ratio in corresponding normoxic cells.[50, 51] 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.[52] Hence, 5-PP-$InsP_5$ might be part of the cellular machinery that mediates the metabolic adaptation to reduced p[O_2].

It is known that cells undergo various metabolic adaptions 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.[53] In this context, 5-PP-$InsP_5$ might have a key role in governing the cellular response to ROS signalling under hypoxia. Decreased cellular ATP utilization under hypoxia[53] explains the stable ATP levels in hypoxic $HCT116^{UCL}$ 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 (**Figure 4 C**).

Next, the effect of reduced p[O_2] on PP-InsP concentrations in β-lap treated cells was evaluated. $HCT116^{UCL}$ 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. 6-fold for 5-PP-$InsP_5$, ca. 7-fold 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 (**Figure 4 C1**). $InsP_6$ levels in neither normoxic nor hypoxic $HCT116^{UCL}$ cells subjected to β-lap

were affected (**Figure 4 C2**). Moreover, consistent with these findings, cellular ATP levels in hypoxic β-lap treated HCT116^{UCL} 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 (**Figure S3 I B**). These results underscore that β-lap reduces PP-InsP levels *via* ROS production, which is prevented in the absence of O_2, and not by direct IP6K binding.

Hypoxia does not influence the effect of other IP6Ks inhibitors

There are a few other known inhibitors of PP-InsP synthesis *in cellulo*, among them the widely-used IP6K inhibitor N^2-(*m*-(trifluoromethyl)benzyl) N^6-(*p*-nitrobenzyl)purine[46] (TNP, structure shown in **Figure S3 II A**) and the natural product Quercetin[47] (Q, structure shown in **Figure S3 II A**), which is an antioxidant.[54] The potency of both, Q and TNP, might also depend on p[O_2]: Q is able to increase intracellular ROS[55] which might affect the biosynthesis of PP-InsPs and the nitro group of TNP could be reduced under hypoxia.[56, 57] Therefore, we took advantage of the hypoxia chamber to investigate, whether the inhibitory effect of Q and TNP on IP6Ks is dependent on p[O_2]. Q was capable of significantly reducing 5-PP-InsP_5 levels under hypoxia as well as normoxia (**Figure 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 **(Figure S3 II B1-B3**).

β-Lapachone mediated modulation of PP-InsP levels requires NQO1

Finally, the role of NQO1 in the response of PP-InsP levels to β-lap was investigated. The human breast cancer cell line MDA-MB-231 proved to be useful for this purpose: MDA-MB-231 cells are homozygous for the NQO1*2 polymorphism[58] caused by a C to T point mutation in the NQO1 complementary DNA.[59, 60] Cell types homozygous for the mutant allele have no measurable NQO1 activity and are consequently resistant to β-lap.[23, 41, 59–61] MDA-MB-231 cells were therefore used to study the function of NQO1 in ROS-dependent PP-InsP reduction caused by β-lap. The absence of the NQO1 protein in MDA-MB-231 cells and its presence in HCT116^{UCL} cells were verified by western blot (**Figure 5 A**, whole PAGE shown in **Figure S4**). Also, NQO1-dependent cytotoxicity of β-lap was evaluated (**Figure 5 B1**): cell viability of HCT116^{UCL} 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 (**Figure 5 B2**). α-Lap only slightly reduced the viability of HCT116^{UCL} cells and had no cytotoxic effect in MDA-MB-231 cells (**Figure 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.[41, 42, 45, 62] Also, the cytotoxicity data for the different quinones mirrors their ability to generate intracellular ROS[42–45]and to reduce PP-InsP levels (**Figure 3 C1**).

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^{UCL} cells after treatment with 10 μM β-lap (4-8fold, see **Figure 3 A1** and **A2**), 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 (**Figure 5 C1**). InsP$_6$ levels in β-lap treated MDA-MB-231 cells were comparable to those in control cells (**Figure 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 (**Figure 5 C3**) which had also been observed in HCT116^{UCL} cells subjected to the naphthoquinone (**Figure 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.[63, 64] Importantly, the significant decrease of ATP in β-lap treated MDA-MD-231 cells had no effect on PP-InsP levels (**Figure 5 C1**). Therefore, the β-lap driven reduction of PP-InsP levels in HCT116^{UCL} is potentially not related to the reduced level of ATP. Instead, it is dependent on a functional NQO1 protein that produces ROS in response to futile cycling of β-lap.

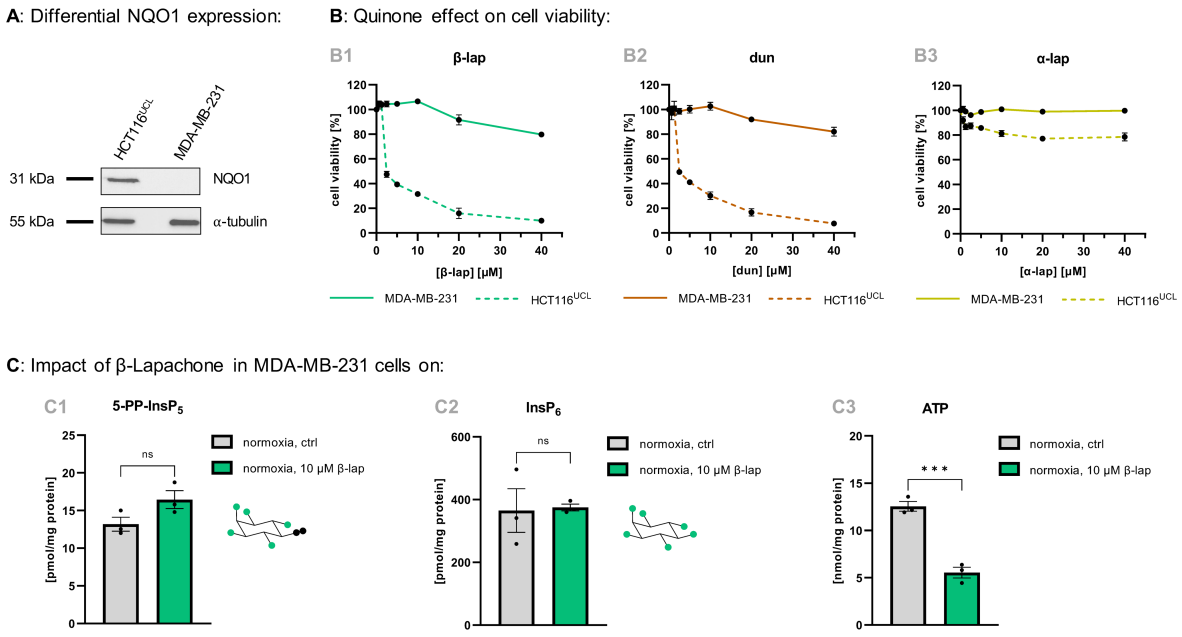

Figure 5 | NQO1 is required for β-lap mediated reduction of PP-InsP levels. *(**A**) NQO1 protein levels in HCT116^{UCL} and MDA-MB-231 cells.* Equal loading was monitored by using α-tubulin. Whole PAGE is shown in Figure S4 *(**B**) Viability assays of MDA-MB-231 cells and HCT116^{UCL} cells treated 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 extration 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.

5 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^{UCL} cells to exogenous and endogenous ROS reduced PP-InsP levels. Moreover, hypoxia completely abolished β-lap mediated reduction of PP-InsP levels in HCT116^{UCL} cells, which suggests that the quinone is indirectly 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 %,[65] ROS-dependent β-lap toxicity shown *in cellulo* might be significantly reduced *in vivo*. With regards to the role of β-lap and other modified *o*-quinones as emerging new 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^{UCL} cells decreased with increasing incubation time while basal 5-PP-InsP$_5$ and ATP levels in co-cultured hypoxic HCT116^{UCL} cells remained stable. This could be explained by the downregulated ATP

utilization in hypoxic cells that finally limits ROS overproduction caused by reduced p[O_2].[53] 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. This demonstrates that the modulation of PP-InsPs *via* β-lap requires both, sufficient O_2 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.

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 the 5-PP-InsP_5 synthesizing enzymes IP6K1/2/3 *via* the oxidation of cysteine residues inside the protein. In fact, the authors of that study identified a specific evolutionarily conserved cysteine residue that might be attacked by cellular ROS.[15] However, the identification of the specific redox sensitive amino acid *in vivo* will require further investigation.

Among many cytotoxic natural products, β-lap is a promising antitumor agent effective against NQO1-expressing cancer cells. Adding disturbed PP-InsP signalling 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 anti-cancer therapies. Our growing ability to manipulate PP-InsP signalling in conjunction with the use of quinone-based drugs could lead to the development of novel anti-cancer approaches.

6 Materials and Methods

Cell culture. $HCT116^{UCL}$ 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. Hypoxic experiments were conducted with 1 % O_2. Details are stated in the SI.

Assays. Manipulations of PP-InsP levels, cell viability determinations, western blotting, and IP6K *in vitro* experiments are described in the SI.

CE-ESI-MS analyses. Procedures are based on ref. 29 and described in detail in the SI.

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 the SI.

7 Data availability

All data, associated protocols, methods, and sources of materials can be accessed in the text or SI Appendix.

8 Acknowledgments

This research was supported by the Deutsche Forschungsgemeinschaft (DFG) under Germany's Excellence Strategy (CIBSS–EXC-2189–Project ID 390939984). H.J.J. additionally acknowledges financial support from the DFG (Grant JE 572/4-1). HJJ and GL acknowledge funding from the Volkswagen Foundation (VW Momentum Grant 98604). C.L. acknowledges support from the German Scholars Organization and Carl Zeiss Foundation (GSO/CZS 20). The authors wish to thank Dorothea Fiedler (Leibniz Forschungsinstitut für Molekulare Pharmakologie, Berlin) and particularly Minh Nguyen Trung und Robert Harmel for providing ^{13}C-labeled reference compounds.

9 References

1. S. G. Thota, R. Bhandari. The emerging roles of inositol pyrophosphates in eukaryotic cell physiology. *J. Biosci.* **40**, 593–605 (2015).

2. S. B. Shears. Intimate connections: Inositol pyrophosphates at the interface of metabolic regulation and cell signaling. *J. Cell. Physiol.* **233**, 1897–1912 (2018).

3. C. Azevedo, A. Saiardi. Eukaryotic phosphate homeostasis: the inositol pyrophosphate perspective. *Trends Biochem. Sci.* **42**, 219–231 (2017).

4. M. S. C. Wilson, T. M. Livermore, A. Saiardi. Inositol pyrophosphates: between signalling and metabolism. *Biochem. J.* **452**, 369–379 (2013).

5. A. Saiardi, R. Bhandari, A. C. Resnick, A. M. Snowman, S. H. Snyder. Phosphorylation of proteins by inositol pyrophosphates. *Science* **306**, 2101–2105 (2004).

6. R. Bhandari *et al.* Protein pyrophosphorylation by inositol pyrophosphates is a posttranslational event. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 15305–15310 (2007).

7. D. Qiu *et al.* Capillary electrophoresis mass spectrometry identifies new isomers of inositol pyrophosphates in mammalian tissues. [Preprint] (2022). DOI:10.1101/2022.09.14.507917.

8. Q. Zhu *et al.* Adipocyte-specific deletion of *Ip6k1* reduces diet-induced obesity by enhancing AMPK-mediated thermogenesis. *J. Clin. Invest.* **126**, 4273–4288 (2016).

9. A. Chakraborty *et al.* Inositol pyrophosphates inhibit Akt signaling, thereby regulating insulin sensitivity and weight gain. *Cell* **143**, 897–910 (2010).

10. R. S. Jadav *et al.* Deletion of inositol hexakisphosphate kinase 1 (IP6K1) reduces cell migration and invasion, conferring protection from aerodigestive tract carcinoma in mice. *Cell. Signal.* **28**, 1124–1136 (2016).

11. F. Rao *et al.* Inositol pyrophosphates promote tumor growth and metastasis by antagonizing liver kinase B1. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1773–1778 (2015).

12. Y. Moritoh *et al.* The enzymatic activity of inositol hexakisphosphate kinase controls circulating phosphate in mammals. *Nat. Commun.* **12**, 4847 (2021).

13. B. H. Morrison *et al.* Inositol hexakisphosphate kinase 2 sensitizes ovarian carcinoma cells to multiple cancer therapeutics. *Oncogene* **21**, 1882–1889 (2002).

14. E. Nagata *et al.* Inositol hexakisphosphate kinase-2, a physiologic mediator of cell death. *J. Biol. Chem.* **280**, 1634–1640 (2005).

15. S. M. N. Onnebo, A. Saiardi. Inositol pyrophosphates modulate hydrogen peroxide signalling. *Biochem. J.* **423**, 109–118 (2009).

16. C. Li *et al.* A phase Ib trial of ARQ 501, a selective checkpoint activator, in combination with docetaxel in patients with advanced solid tumors. *J. Clin. Oncol.* **24**, 13053 (2006).

17. G. I. Shapiro *et al.* Phase I trial of ARQ 501, an activated checkpoint therapy (ACT) agent, in patients with advanced solid tumors. *J. Clin. Oncol.* **23**, 3042 (2005).

18. H. T. Khong *et al.* A phase 2 study of ARQ 501 in combination with gemcitabine in adult patients with treatment naïve, unresectable pancreatic adenocarcinoma. *J. Clin. Oncol.* **25**, 15017 (2007).

19. K. Zhang *et al.* NAD(P)H:Quinone Oxidoreductase 1 (NQO1) as a therapeutic and diagnostic target in cancer. *J. Med. Chem.* **61**, 6983–7003 (2018).

20. J. J. Pink *et al.* NAD(P)H:quinone oxidoreductase activity is the principal determinant of *β*-lapachone cytotoxicity. *J. Biol. Chem.* **275**, 5416–5424 (2000).

21. X. Li *et al.* NQO1 targeting prodrug triggers innate sensing to overcome checkpoint blockade resistance. *Nat. Commun.* **10**, 3251 (2019).

22. M. S. Bentle, K. E. Reinicke, E. A. Bey, D. R. Spitz, D. A. Boothman. Calcium-dependent modulation of poly(ADP-ribose) polymerase-1 alters cellular metabolism and DNA repair. *J. Biol. Chem.* **281**, 33684–33696 (2006).

23. E. A. Bey *et al.* An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by *β*-lapachone. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 11832–11837 (2007).

24. Z. Moore *et al.* NAMPT inhibition sensitizes pancreatic adenocarcinoma cells to tumor-selective, PAR-independent metabolic catastrophe and cell death induced by *β*-lapachone. *Cell Death. Dis.* **6**, e1599 (2015).

25. H. Liu *et al.* UDP-glucuronosyltransferase 1A determinates intracellular accumulation and anti-cancer effect of β-lapachone in human colon cancer cells. *PLoS One* **10**, e0117051 (2015).

26. C. Tagliarino, J. J. Pink, G. R. Dubyak, A. L. Nieminen, D. A. Boothman. Calcium is a key signaling molecule in *β*-lapachone-mediated cell death. *J. Biol. Chem.* **276**, 19150–19159 (2001).

27. Y. Dong *et al.* Prostate cancer radiosensitization through poly(ADP-Ribose) polymerase-1 hyperactivation. *Cancer Res.* **70**, 8088–8096 (2010).

28. L. Dunsmore *et al.* Controlled masking and targeted release of redox-cycling *ortho*-quinones via a C-C bond-cleaving 1,6-elimination. *Nat. Chem.* **14**, 754–765 (2022).

29. D. Qiu *et al.* Analysis of inositol phosphate metabolism by capillary electrophoresis electrospray ionization mass spectrometry. *Nat. Commun.* **11**, 6035 (2020).

30. C. Gu, M. S. C. Wilson, H. J. Jessen, A. Saiardi, S. B. Shears. Inositol pyrophosphate profiling of two HCT116 cell lines uncovers variation in $InsP_8$ levels. *PLoS One* **11**, e0165286 (2016).

31. M. S. C. Wilson, S. J. Bulley, F. Pisani, R. F. Irvine, A. Saiardi. A novel method for the purification of inositol phosphates from biological samples reveals that no phytate is present in human plasma or urine. *Open. Biol.* **5**, 150014 (2015).

32. E. Riemer *et al.* ITPK1 is an $InsP_6$/ADP phosphotransferase that controls phosphate signaling in Arabidopsis. *Mol. Plant* **14**, 1864–1880 (2021).

33. Y. Desfougères *et al.* The inositol pyrophosphate metabolism of *Dictyostelium discoideum* does not regulate inorganic polyphosphate (polyP) synthesis. *Adv. Biol. Regul.* **83**, 100835 (2022).

34. M. S. Wilson, A. Saiardi. Inositol phosphates purification using titanium dioxide beads. *Bio Protoc.* **8**, e2959 (2018).

35. R. K. Harmel *et al.* Harnessing ^{13}C-labeled *myo*-inositol to interrogate inositol phosphate messengers by NMR. *Chem. Sci.* **10**, 5267–5274 (2019).

36. R. Puschmann, R. K. Harmel, D. Fiedler. Scalable chemoenzymatic synthesis of inositol pyrophosphates. *Biochemistry* **58**, 3927–3932 (2019).

37. D. Qiu, V. B. Eisenbeis, A. Saiardi, H. J. Jessen. Absolute quantitation of inositol pyrophosphates by capillary electrophoresis electrospray ionization mass spectrometry. *J. Vis. Exp.* DOI:10.3791/62847 (2021).

38. K. Choi, E. Mollapour, S. B. Shears. Signal transduction during environmental stress: $InsP_8$ operates within highly restricted contexts. *Cell. Signal.* **17**, 1533–1541 (2005).

39. C. Szabó, V. L. Dawson. Role of poly(ADP-ribose) synthetase in inflammation and ischaemia–reperfusion. *Trends Pharmacol. Sci.* **19**, 287–298 (1998).

40. A. Anwar *et al.* Interaction of human NAD(P)H:quinone oxidoreductase 1 (NQO1) with the tumor suppressor protein p53 in cells and cell-free systems. *J. Biol. Chem.* **278**, 10368–10373 (2003).

41. M. A. Silvers *et al.* The NQO1 bioactivatable drug, *β*-lapachone, alters the redox state of NQO1+ pancreatic cancer cells, causing perturbation in central carbon metabolism. *J. Biol. Chem.* **292**, 18203–18216 (2017).

42. J. Bian *et al.* Synthesis and evaluation of (±)-dunnione and its *ortho*-quinone analogues as substrates for NAD(P)H:quinone oxidoreductase 1 (NQO1). *Bioorg. Med. Chem. Lett.* **25**, 1244–1248 (2015).

43. A. Boveris, R. Docampo, J. F. Turrens, A. O. Stoppani. Effect of β-lapachone on superoxide anion and hydrogen peroxide production in *Trypanosoma cruzi*. *Biochem. J.* **175**, 431–439 (1978).

44. D. C. M. Ferreira *et al.* Ex vivo activities of β-lapachone and α-lapachone on macrophages: a quantitative pharmacological analysis based on amperometric monitoring of oxidative bursts by single cells. *Chembiochem* **10**, 528–538 (2009).

45. R. Inagaki, M. Ninomiya, K. Tanaka, M. Koketsu. Synthesis, characterization, and antileukemic properties of naphthoquinone derivatives of lawsone. *ChemMedChem* **10**, 1413–1423 (2015).

46. U. Padmanabhan, D. E. Dollins, P. C. Fridy, J. D. York, C. P. Downes. Characterization of a selective inhibitor of inositol hexakisphosphate kinases: use in defining biological roles and metabolic relationships of inositol pyrophosphates. *J. Biol. Chem.* **284**, 10571–10582 (2009).

47. C. Gu *et al.* Inhibition of inositol polyphosphate kinases by quercetin and related flavonoids: a structure-activity analysis. *J. Med. Chem.* **62**, 1443–1454 (2019).

48. G. Liao *et al.* Identification of small-molecule inhibitors of human inositol hexakisphosphate kinases by high-throughput screening. *ACS Pharmacol. Transl. Sci.* DOI:10.1021/acsptsci.0c00218 (2021).

49. C. Frezza *et al.* Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival. *PLoS One* **6**, e24411 (2011).

50. Z. Zhang *et al.* Inositol pyrophosphates mediate the effects of aging on bone marrow mesenchymal stem cells by inhibiting Akt signaling. *Stem. Cell. Res. Ther.* **5**, 33 (2014).

51. J. Deng *et al.* Inositol pyrophosphates mediated the apoptosis induced by hypoxic injury in bone marrow-derived mesenchymal stem cells by autophagy. *Stem. Cell. Res. Ther.* **10**, 159 (2019).

52. J. L. Sun *et al.* MicroRNA regulation in hypoxic environments: differential expression of microRNAs in the liver of largemouth bass (*Micropterus salmoides*). *Fish Physiol. Biochem.* **46**, 2227–2242 (2020).

53. W. W. Wheaton, N. S. Chandel. Hypoxia. 2. Hypoxia regulates cellular metabolism. *Am. J. Physiol. Cell Physiol.* **300**, C385-93 (2011).

54. W. Wang *et al.* The biological activities, chemical stability, metabolism and delivery systems of quercetin: A review. *Trends Food Sci. Technol.* **56**, 21–38 (2016).

55. L. Gibellini *et al.* Interfering with ROS metabolism in cancer cells: the potential role of quercetin. *Cancers (Basel)* **2**, 1288–1311 (2010).

56. S. L. Collins, J. Saha, L. C. Bouchez, E. M. Hammond, S. J. Conway. Hypoxia-activated, small-molecule-induced gene expression. *ACS Chem. Biol.* **13**, 3354–3360 (2018).

57. A. Skwarska *et al.* Development and pre-clinical testing of a novel hypoxia-activated KDAC inhibitor. *Cell Chem. Biol.* **28**, 1258-1270.e13 (2021).

58. A. Begleiter, M. K. Leith, G. P. Doherty, T. J. Digbya, S. Pan. Factors influencing the induction of DT-diaphorase activity by 1,2-dithiole-3-thione in human tumor cell lines. *Biochem. Pharmacol.* **61**, 955–964 (2001).

59. R. D. Traver *et al.* Characterization of a polymorphism in NAD(P)H: quinone oxidoreductase (DT-diaphorase). *Br. J. Cancer* **75**, 69–75 (1997).

60. R. D. Traver *et al.* NAD(P)H:quinone oxidoreductase gene expression in human colon carcinoma cells: characterization of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity. *Cancer Res.* **52**, 797–802 (1992).

61. D. Siegel, S. M. McGuinness, S. L. Winski, D. Ross. Genotype-phenotype relationships in studies of a polymorphism in NAD(P)H:quinone oxidoreductase 1. *Pharmacogenetics* **9**, 113–121 (1999).

62. E. J. S. Salustiano *et al.* Comparison of the cytotoxic effect of lapachol, α-lapachone and pentacyclic 1,4-naphthoquinones on human leukemic cells. *Invest. New Drugs* **28**, 139–144 (2010).

63. T. Iyanagi. On the mechanism of one-electron reduction of quinones by microsomal flavin enzymes: the kinetic analysis between cytochrome B5 and menadione. *Free Radic. Res. Commun.* **8**, 259–268 (1990).

64. S. Deller, P. Macheroux, S. Sollner. Flavin-dependent quinone reductases. *Cell. Mol. Life Sci.* **65**, 141–160 (2008).

65. A. Carreau, B. El Hafny-Rahbi, A. Matejuk, C. Grillon, C. Kieda. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J. Cell. Mol. Med.* **15**, 1239–1253 (2011).