Quinones and Deubiquitinases

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Harnessing the Oxidation Susceptibility of Deubiquitinases for Inhibition with Small Molecules**

Shimrit Ohayon, Maya Refua, Adi Hendler, Amir Aharoni, and Ashraf Brik**

Abstract: *Deubiquitinases (DUBs) counteract ubiquitination by removing or trimming ubiquitin chains to alter the signal. Their diverse role in biological processes and involvement in diseases have recently attracted great interest with regard to their mechanism and inhibition. It has been shown that some DUBs are regulated by reactive oxygen species (ROS) in which the catalytic Cys residue undergoes reversible oxidation, hence modulating DUBs activity under oxidative stress. Reported herein for the first time, the observation that small molecules, which are capable of generating ROS efficiently, inhibit DUBs by selective and nonreversible oxidation of the catalytic Cys residue. Interestingly, the small molecule beta-lapachone, which is currently in clinical trials for cancer, is among the potent inhibitors, thus suggesting possible new cellular targets for its therapeutic effects. Our study describes a novel mechanism of DUBs inhibition and opens new opportunities in exploiting them for cancer therapy.*

The attachment of monoubiquitin or polyubiquitin chains to a protein substrate, so called ubiquitination, is involved in a wide range of cellular process in eukaryotes.[1] A family of about 80 enzymes, known as deubiquitinases (DUBs), counteracts this posttranslational modification by removing or trimming the ubiquitin chain to alter the signal.[2] The rapidly emerging roles of DUBs in various biological processes, ranging from cell-cycle progression, tissue development and differentiation, to their involvement in various diseases has recently attracted great interest with regard to the DUBs' mechanism, structures, specificities, and inhibition.[3] Such studies are crucial to better understanding DUBs and their role in health and disease as well as their exploitation as drug targets.[3d,e,4] However, our understanding as to how to target DUBs is still not mature compared to, for example, that of proteases which cleave backbone amide bonds (e.g., caspases).

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Mechanistically, DUBs belong to either zinc metalloproteases or Cys proteases (majority) and are known to recognize their substrates by different modes of interaction.[2] Members from the ubiquitin-specific proteases (USPs), which constitutes the majority of DUBs, have been implicated in various diseases such as in cancer and several of these have emerged as potential drug targets.[3d,e,4,5] Recently, three different research groups have shown that DUBs are regulated by exogenous hydrogen peroxide, as a reactive oxygen species (ROS),[6] which selectively oxidizes the catalytic Cys residue in a reversible manner and it is suggested that this oxidation might be a possible mechanism for modulating the function of DUBs under oxidative stress.

Herein we report that small molecules, which are capable of generating ROS efficiently, inhibit DUBs such as USP2 by a novel mechanism of action involving selective and nonreversible oxidation of the catalytic Cys residue. Interestingly, the small molecule beta-lapachone, which is currently in clinical trials for cancer,[7] is among the list of the most potent inhibitors and possibly suggests a new mechanism for its therapeutic effect involving DUBs.

USP2 has been shown to play a critical role in prostate cancer cell survival.[5a,8] This DUB has been shown to have oncogenic behavior mainly by preventing the proteasomal degradation of proteins which are involved in numerous cellular processes.[9] For example, USP2 stabilizes fatty acid synthase (FAS), which associates with aggressive prostate cancer[5a,8] and it has been shown that silencing USP2 leads to apoptosis in several human cancer cell lines.[5a] Hence, the inhibition of USP2 could be a potential approach in cancer therapy.

Recently, novel chemical approaches have been developed to prepare homogeneous ubiquitin-based conjugates (reviewed in Ref. [10]) assays, and probes[11] to support various structural and functional analyses of the ubiquitin system components such as DUBs.[3a,12] Our group reported an assay, based on synthetic ubiquitinated peptides, labeled with a fluorescence quenching pair, to report on the activity of UCH-L3/L1 and thus enabled the screening libraries of small molecules for the identification of inhibitors for DUBs.[11b] Using this substrate we determined the kinetic parameters of USP2, which values of $K_M = 11.14\ \mu M$ and $k_{cat} = 1.01\ s^{-1}$ (see Figure S1 in the Supporting Information).[13]

With this in hand, we proceeded with the search for new inhibitors against USP2. Initially, we tested the most potent compounds which we previously identified for UCH-L3.[11b] Surprisingly, these molecules showed better inhibition toward USP2 than UCH-L3 (see Figure S2), thus hinting at a unique role of the quinone motif. These results motivated us to screen an additional library of commercially available small mole-

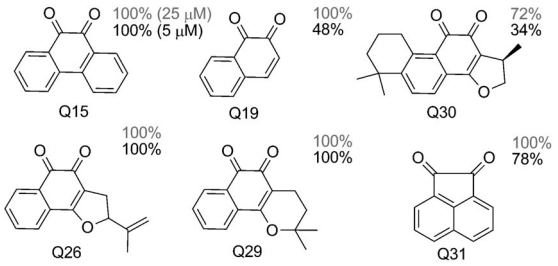


Figure 1. The structures and measured inhibition values of some of the potent quinones against USP2.

cules featuring the quinone scaffold (see Figure S3). Notably, the *ortho*-quinones Q15, Q26, and Q29 exhibited complete inhibition at 5 μM (Figure 1), and prompted us to further characterize these inhibitors and study their mechanism of action.

Interestingly, among these *ortho* quinones is the natural product beta-lapachone (Q29), isolated from the lapacho tree (*T. avellanedae*) and known as ARQ 501, which is currently in advanced clinical trials for the treatment of pancreatic cancer.[7] Time- and dose-dependent inhibition was observed for these molecules with a near linear fit and a rate of inactivation of 1700, 1327, 1500 $M^{-1}s^{-1}$ for Q15, Q26, and beta-lapachone, respectively, and 313 $M^{-1}s^{-1}$ for the less potent Q19 inhibitor (Figures 2A and B and Figure S4). To test whether these *ortho* quinones covalently modify the enzyme we analyzed the change in the molecular weight of USP2 before and after treatment with these inhibitors.

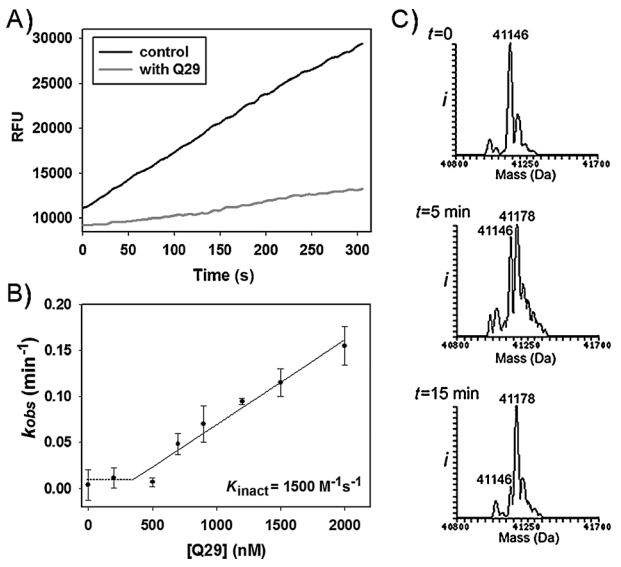


Figure 2. Beta-lapachone mode of inhibition. A) Representative inhibition measurement of USP2 with beta-lapachone (500 nM, after 20 min treatment). B) Plot of the inactivation rate constants (k_{obs}) versus beta-lapachone concentrations to obtain the maximal rate of enzyme inactivation (k_{inact}). Each value represents the mean ± SE of two independent experiments. C) Mass spectrometry analysis of USP2 treated with beta-lapachone (500 nM). Shown here the deconvoluted mass of the USP2 before (calculated mass 41148 Da) and after (calculated mass 41178 Da) treatment with beta-lapachone at different times of incubation.

Surprisingly, in all cases we observed an increase of 32 Da rather than an increase in weight equal to the entire molecular weight of the inhibitors, which are also known to act as Michael acceptors for Cys proteases[14] (Figure 2C). This weight increase suggested a different mechanism of inhibition, possibly by oxidation of one or two Cys residues in USP2.

The oxidation level of a Cys residue could vary from sulfenic acid (SOH), to sulfinic acid (SO_2H), to sulfonic acid (SO_3H) upon exposure to ROS.[15] However, recent studies using exogenous hydrogen peroxide concluded that DUBs are oxidized mainly to the SOH form, where the overall architecture of the catalytic active site and the nearby nitrogen protects Cys from being oxidized to the higher and more stable oxidation states (i.e., SO_2H and SO_3H).[6] Importantly, under a reducing environment the SOH is reversed to the SH form, thus leading to restoration of DUBs activity and suggesting that DUBs can be regulated under oxidative stress conditions.[6] Based on this previous knowledge, we hypothesized that in our study the catalytic Cys side chain of USP2 is oxidized to the SO_2H state, as indicated by the addition of 32 Da (Figure 3). The previous

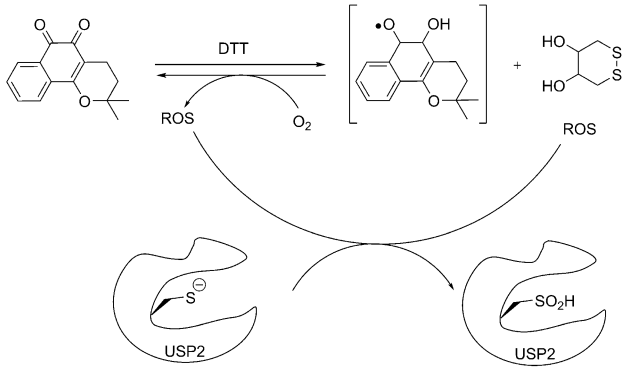


Figure 3. Proposed mechanism for beta-lapachone inhibition.

studies have shown that the H_2O_2 concentration required for deactivation of DUBs is in the high micromolar range.[6a–c] For USP2, we found that 500 μM of H_2O_2 was required to achieve complete inhibition, while only 1 μM of beta-lapachone was sufficient to achieve this level of inhibition. This difference can be a result of various factors such as the rapid generation of ROS, possible binding of the small molecule to the enzyme, which could lead to effective oxidation through more localized ROS, and the type of ROS which may have greater oxidizing properties than H_2O_2, thus leading to the formation of the nonreversible SO_2H. This aspect, however, remains to be tested. It is also interesting to observe that variations around the quinone core (Figure 1) lead to differences in the activity of these molecules, which will also be a subject for future studies.

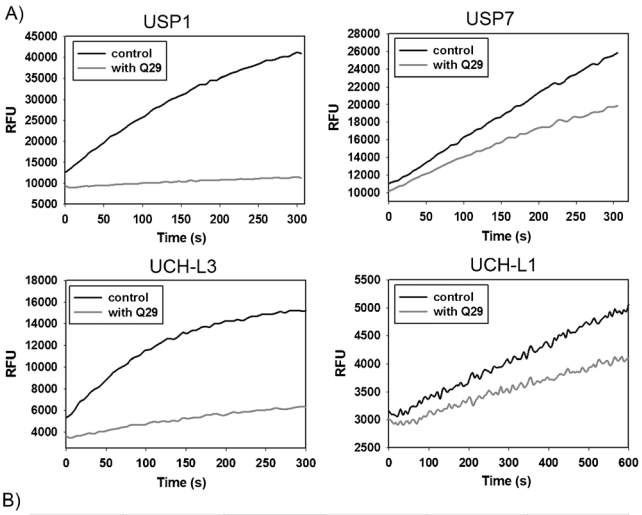
To confirm the proposed mechanism of inhibition and the form of the oxidation state, we performed 1) the reaction in the presence of catalase, an enzyme which decomposes hydrogen peroxide to water and oxygen and 2) dilution with DTT (DTT = dithiothreitol; see Figure S5). The latter is used

to test the reversibility of the inhibition where DTT reduces only SOH to SH. When catalase and beta-lapachone were preincubated together, no inhibition was observed. However, the addition of catalase after the preincubation of USP2 with beta-lapachone did not significantly affect its inhibition by beta-lapachone (see Figure S5A). After dilution with DTT, we observed only minor recovery of USP2 activity (see Figure S5B), thus highlighting the nonreversible nature of USP2 inhibition with beta-lapachone. Moreover, USP2 inhibition with beta-lapachone depends on the presence and amount of DTT, which serves as the "initiator" for the redox cycle reaction of beta-lapachone. At low concentrations of DTT (1 mM) USP2 is fully inhibited while at higher concentrations (5–10 mM) DTT strongly interferes with beta-lapachone inhibition (see Figure S6).

It is known that the active site Cys exhibits a lower pK_a value than other Cys residues present in the enzyme, and thus makes it more prone to oxidation. USP2 has 12 Cys residues which are distributed throughout various regions in the catalytic domain.[16] To test whether the catalytic Cys is the oxidized residue several independent experiments were conducted. We first tested the active site directed probe ubiquitin vinyl methyl sulfone (Ub-VMS),[11d–g,12] which is known to selectively react with the catalytic Cys of DUBs, and observed no labeling of the treated USP2 with beta-lapachone (see Figure S7). Furthermore, beta-lapachone inhibited the cleavage of the K48-linked di-ubiquitin chain by USP2, which depends on the catalytic C223 (Figure S7). Incubation of beta-lapachone with USP2 which has the active site mutation C223A did not lead to change in the molecular weight of the enzyme (see Figure S8). Together, these experiments support that the catalytic Cys is the site of oxidation.

Next, we wondered whether beta-lapachone exhibited any selectivity toward different Cys-based DUBs (UCH-L1, UCH-L3, USP1, USP2, and USP7). For this we incubated each of these DUBs with beta-lapachone and followed its activity. Our analyses indicated different levels of inhibition where beta-lapachone similarly inhibited UCH-L3, USP1, and USP2, but inhibited UCH-L1 and USP7 to a lesser extent (Figure 4). These results could reflect the differences in the pK_a value of the active Cys under the reaction conditions. For example, USP7 and UCH-L1 were reported to be less prone to oxidation when treated with exogenous H_2O_2 and required further activation by binding to other substrates.[6b] In our case, USP7 and UCH-L1 were much less affected by beta-lapachone compared to USP1/2.

Beta-lapachone has been shown to exhibit high cytotoxic activity against various cancer cell lines, prostate and leukemia cancer being among them.[7,17] Several mechanisms of action have been proposed, including activation of topoisomerase,[18] induction of apoptosis,[17a,d,e,19] inhibition of topoisomerase I[17d] and II-α,[20] and suppression of NF-k B activation.[21] Nevertheless, the exact mechanism of action and intracellular target(s) of beta-lapachone remained ambiguous. Recent studies supported the fact that the NAD(P)H:-quinone oxidoreductase (NQO1), which is known to be overexpressed in many human cancer cells, is a key enzyme in activating beta-lapachone to generate ROS.[17b,c,22] This action could lead to rapid depletion of the electron-donor NADH or



B)

	USP1	USP2	USP7	UCH-L1	UCH-L3
% inhibition	92	82	28	36	78

Figure 4. Beta-lapachone inhibition profile with different DUBs. A) The susceptibility of several DUBs to oxidation by beta-lapachone at 500 nM. B) A summary of percent of inhibition toward the different DUBs. Each value represents the mean ±SE of two independent experiments.

NADPH in the futile cycling of beta-lapachone, thus triggering tumor cells to undergo a unique apoptotic pathway.[17b] In addition, other downstream effects to ROS generation were also proposed, such as single-strand DNA breaks and poly(-ADP-ribose)polymerase-1 (PARP1) hyperactivation.[17b,c] However, beta-lapachone was never implicated in affecting DUBs to induce cell apoptosis.

The importance of beta-lapachone in different kinds of cancers[17a–e] triggered us to initiate preliminary studies in cellular context and examine its link to USP2. High levels of USP2 are known to protect cancer cells from apoptosis[5a,8] and as such its inhibition increases the apoptotic level in cancer cell lines. Incubation of beta-lapachone with prostate cancer cell line DU-145, which is known to have higher USP2 expression,[5a] led to increased levels of apoptosis in a dose-dependent manner (Figure 5A). In contrast, incubation of beta-lapachone with U2OS, a human bone osteosarcoma cell line, did not lead to a significant increase in the apoptotic level (Figure 5C). To examine whether the potency of beta-lapachone in these cell lines correlates with the USP2 level, we determined the USP2 expression in DU-145 and U2OS using real time PCR analysis. Our results showed a threefold increase in USP2 levels in DU-145 relative to U2OS (Figure 5B), and is in agreement with the observed beta-lapachone apoptotic effect. To examine whether there is a correlation between the in vitro (Figure 1) and cellular potency of the different inhibitions we treated DU-145 cells with Q15, Q19, Q26, and beta-lapachone. Notably, Q15, Q26, and beta-lapachone showed strong and comparable levels of apoptosis while the less potent inhibitor Q19 exhibited much lower level of cellular potency (Figure 5D).

In summary, we have shown, for the first time, that small molecules, such as beta-lapachone, which are capable of generating ROS, can be used to efficiently and nonreversibly

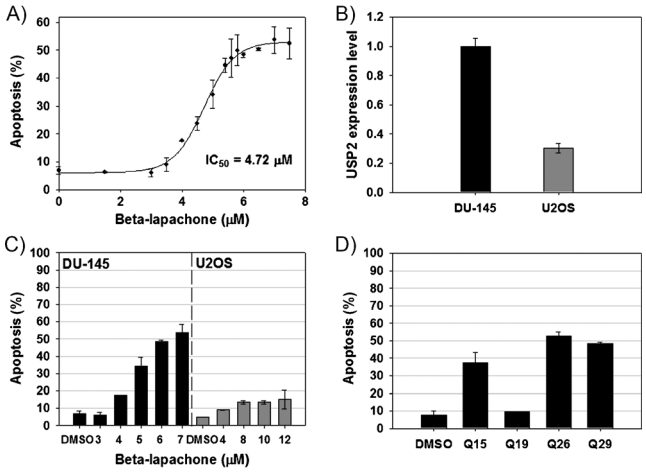


Figure 5. Cellular studies of Q15, Q19, Q26, and beta-lapachone. A) Apoptosis induced by beta-lapachone measured in DU-145 prostate cancer cell line. B) USP2 expression levels in DU-145 and U2OS cell lines as determined by real-time PCR. C) Apoptosis level in DU-145 and U2OS cell lines as response for beta-lapachone treatment in different concentrations. D) Comparison of the apoptosis level in DU-145 cell line as response to treatment by Q15, Q19, Q26, and beta-lapachone at concentration of 6 μM. In (A), (C), and (D), each value represents the mean ± SE of two independent experiments, while in (B) of four independent experiments.

inhibit DUBs, and thus adds DUBs to the list of enzymes which are targeted by ROS, for example, phosphatases.[23] Our results suggest that the therapeutic effect of beta-lapachone could be due to multiple pathways, with USP2 oxidation and inhibition being among them. Additional cellular studies such as determining the ubiquitination level of the USP2 substrates and profiling cellular DUBs upon beta-lapachone treatment with activity-based probes are currently being conducted to further validate our hypothesis.

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