Dienone Compounds: Targets and Pharmacological Responses

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ABSTRACT: The biological responses to dienone compounds with a 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore have been studied extensively. Despite their expected general thiol reactivity, these compounds display considerable degrees of tumor cell selectivity. Here we review *in vitro* and preclinical studies of dienone compounds including b-AP15, VLX1570, RA-9, RA-190, EF24, HO-3867, and MCB-613. A common property of these compounds is their targeting of the ubiquitin−proteasome system (UPS), known to be essential for the viability of tumor cells. Gene expression profiling experiments have shown induction of responses characteristic of UPS inhibition, and experiments using cellular reporter proteins have shown that proteasome inhibition is associated with cell death. Other mechanisms of action such as reactivation of mutant p53, stimulation of steroid receptor coactivators, and induction of protein cross-linking have also been described. Although unsuitable as biological probes due to widespread reactivity, dienone compounds are cytotoxic to apoptosis-resistant tumor cells and show activity in animal tumor models.
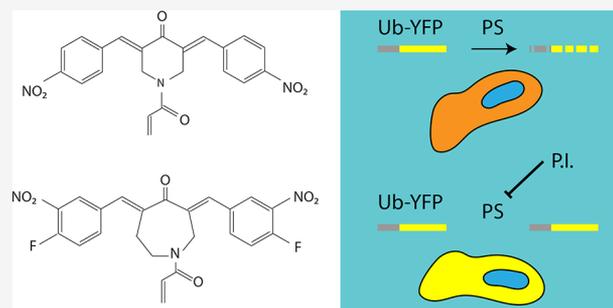
■ INTRODUCTION

A considerable number of anticancer drugs have been discovered and developed using phenotypic screening.[1] Large numbers (in the order of 10^3−10^6) of molecules can be screened for identification of compounds that induce a desired biological response.[2] Screens can be set up using different strategies, using tumor cells that are resistant to conventional therapy, using cells with defined genetic makeups, using cellular reporter systems, etc.[3] A disadvantage of this approach is that identified compounds may be associated with extensive polypharmacology. Furthermore, it is generally difficult to define precise mechanisms of action of screening hits. The revolution of molecular biology has, however, resulted in substantially improved possibilities for elucidation of targets and of molecular mechanisms of action. Thus, methods such as gene expression profiling[4] (CMap), various proteomic techniques,[5−7] and knock-down and gene deletion technologies can be used to generate hypotheses with regard to molecular mechanisms of action, to identify and validate potential targets.[3]

Investigators performing phenotypic screening are faced with the problem of how to manage projects where hit compounds display interesting features in terms of generating desired biological responses but are unattractive from a medicinal chemistry point of view. Should such projects be discontinued since, according to experience, such compounds can rarely be developed into useful drugs? Or is this a prejudiced view? Cancer is a disease characterized by the occurrence of a large number of genetic and epigenetic alterations.[8] There is a considerable medical need for efficient

drugs for improved treatment of diseases with dismal outcomes such as pancreatic cancer and glioblastoma. The clinical efficacy of many anticancer drugs is hampered by the development of clones expressing variant target proteins, resulting in treatment failure.[9,10] Perhaps no stone should be left unturned?

Here we review the reported biological activities of substances containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophores such as 3,5-diarylidene-4-piperidones and dibenzylidene-acetone (DBA) (Figure 1). These compounds are electrophiles, primarily reacting with thiols,[11] and will be referred to as "dienones". Dienone compounds with completely different structures such as prostaglandins of the J-series will generally not be considered here. The general assumption is that molecules containing 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore will show similar mechanisms of action in biological systems by acting as Michael acceptors. This assumption is supported by the finding of common patterns of response in cells exposed to different compounds (see below).
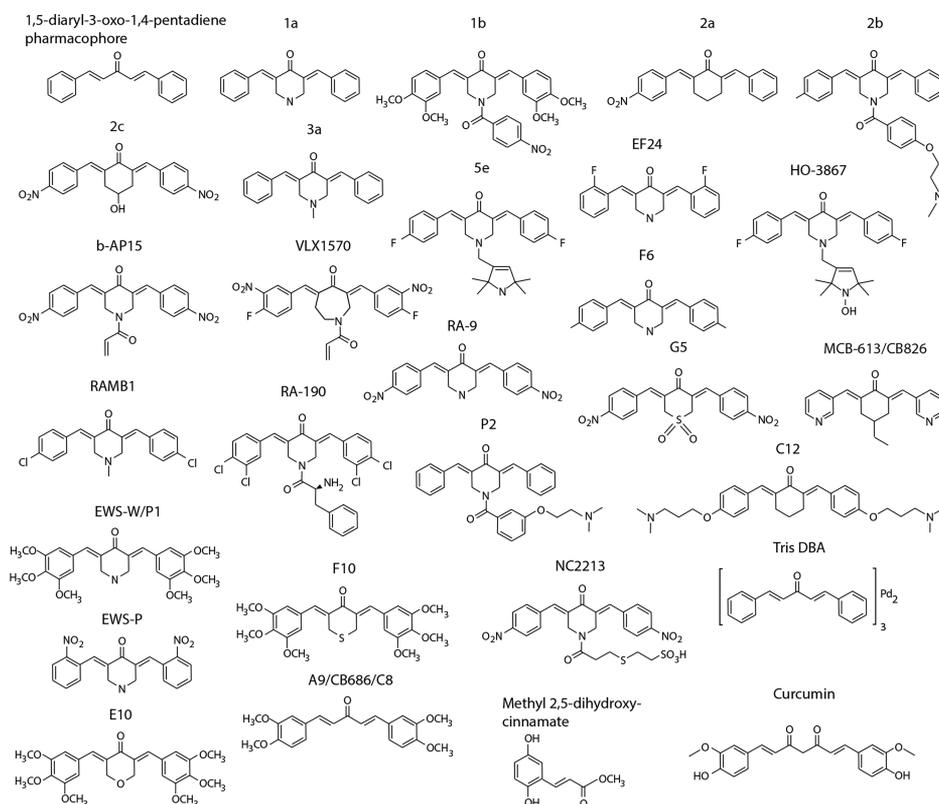
Figure 1. Chemical structures of some of the compounds discussed in the text.

CANCER CELLS ARE SELECTIVELY SENSITIVE TO DIENONE COMPOUNDS

Early studies of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore were performed by Dimmock and colleagues at the University of Saskatchewan.[12−15] These investigators documented interesting biological responses to these compounds, stimulating further interest. One of the early compounds described by Dimmock et al. was **1a**[13] (Figure 1), a compound that inhibited the proliferation of cancer cells in the low μM range. Dimmock and colleagues have over the years studied a respectable number of compounds belonging to this class and have established structure−activity relationships (reviewed in refs 11 and 16). The dienone has been found to be required for optimal cytotoxicity, and as expected, electron drawing groups on the aryls increase the antiproliferative effect.[17]

An interesting property of the compounds described by Dimmock and co-workers is their preferential cytotoxic to tumor cells.[18−21] The Dimmock group reported selective indices (sensitivity of tumor cells relative to noncancerous cells) of >10-fold[19] (see Figure 2). Selective antiproliferative activity to tumor cells has been verified by other laboratories[21−26] (Figure 2, Supplementary Table 1). Analyses have not been restricted only to slowly proliferating primary cells but also to established immortalized noncancerous cell lines such as MCF-10A (a human breast cell line with a reported population doubling time of ∼19 h[27]) and hTERT-RPE1 (an immortalized human epithelial cell line with a doubling time of ∼14 h[28]). The proliferation rates of these cells are in the same range as those of many of the cancer cells listed in Supplementary Table 1. It is therefore unlikely that the preferential cytotoxicities of dienones to tumor cells are solely due to their differences in proliferation rates.

EF24 (3,5-bis(2-fluorobenzylidene)piperidin-4-one) (Figure 1) was identified in a screen of different α,β-unsaturated ketones for their activities against cancer cell lines.[15] EF24 was also suggested to exhibit antiangiogenic properties based on inhibition of the proliferation of endothelial cells.[15] EF24 induces apoptosis as evidenced by activation of caspase-3, externalization of phosphatidylserine, and depolarization of mitochondrial membranes.[29] Inhibition of the proliferation of colon and gastric adenocarcinoma cells was observed at a concentration of 1 μM, whereas mouse embryo fibroblasts were not affected at 5 μM.[26] Selective cytotoxicity to tumor cells by EF24 was also reported by Santiago-Vazquez and co-workers (Figure 2, Supplementary Table 1).[20] Daily ip administration of EF24 suppressed the growth of colon cancer tumor xenografts in nude mice.[26] Administration of 100 mg/kg EF24 did not cause harmful side effects to animals.[15] These findings have been repeated in later studies of EF24 using hepatocellular carcinoma,[30] adrenocortical tumors,[31] colorectal cancer,[32] cholangiocellular carcinoma,[33] oral squamous cell carcinoma[34] and pancreatic cancer.[35]

H-4073 is a para-fluorinated variant of EF24 described to be more potent to ovarian cancer cells than the parental compound.[36] Yet another related compound, named HO-3867, was subsequently developed (Figure 1).[37] HO-3867 showed a higher level of growth inhibitory activity to ovarian cancer cells compared to untransformed human ovarian surface epithelial cells.[37] HO-3867 was effective in an ovarian cancer xenograft model using a schedule of oral administration. Further studies have shown that HO-3867 induces apoptosis of breast, colon, and liver cells whereas normal breast fibroblasts showed limited sensitivity.[38] HO-3867 was also reported to show *in vitro* activity on pancreas cancer cell lines[39,40] and endometrial cancer cell lines.[41] An EF24 analogue was recently
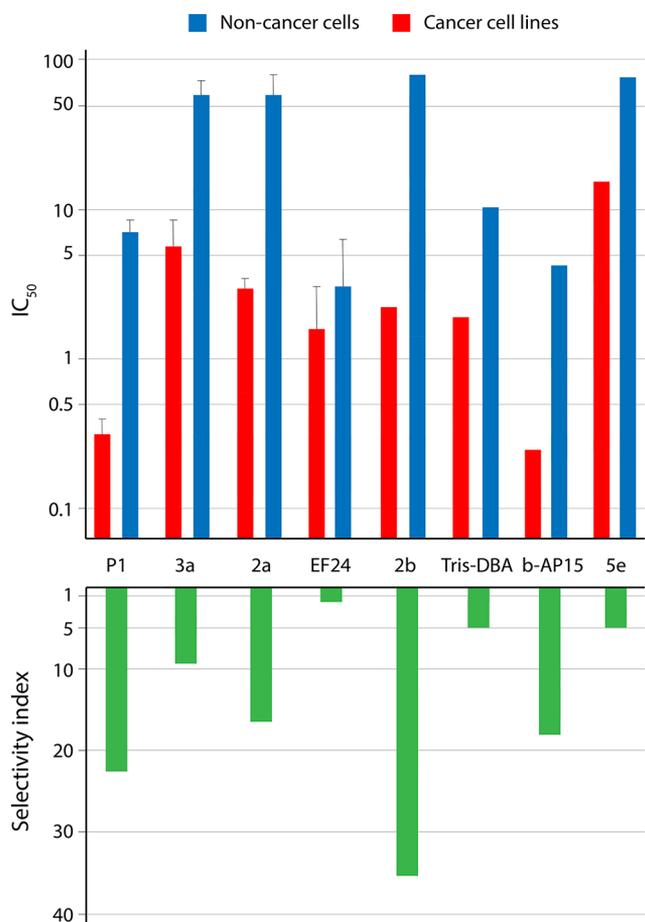
Figure 2. Tumor cell selectivity of dienone compounds. The $\log_{10}$ IC$_{50}$ values are shown on the y-axis in the upper graph and selectivity indices on the y-axis in the lower graph. Shown are the mean ± SD values where possible to calculate (i.e., where data from three or more cell lines were reported). The figure is based on the data in presented Supplementary Table 1.

described that showed a similar antiproliferative activity toward cancer cells as the parental compound but lower activity to normal cells.[42] For a recent review on the biological activities of EF24, see ref 43.

The group of Brancolini and co-workers at the University of Udine identified two compounds, F6 (NSC632839) and G5 (NSC144303) (Figure 1), as capable of triggering caspase activation in cells with a mutated caspase-9.[44] A prodrug version of G5 (2c-PE) that can be activated by esterases was developed and shown to suppress the growth of A549 lung carcinoma xenografts in mice.[45]

The compound NSC687852 (later named b-AP15) was originally identified in a screen for compounds that induce apoptosis of colon cancer cells independently of p53.[46] The compound was later found to elicit the lysosomal apoptosis pathway[47] and to show antineoplastic activity in different tumor models including lung cancer and acute myelocytic leukemia.[22] Nontransformed hTERT-RPE1 epithelial cells showed limited sensitivity to b-AP15[22] (Figure 2). VLX1570 is an analogue of b-AP15 with a central azepane ring (Figure 1) that induces an indistinguishable cellular response from b-AP15 while having a slightly improved solubility and potency.[48,49] VLX1570 has been shown to have antitumor

activity in multiple myeloma,[49] Ewings sarcoma,[50] Waldenströms macroglobulinemia,[51] and also other diseases (see[52]).

Bazzaro and colleagues[53,54] demonstrated cytotoxicity of a series of 3,5-diarylidene-4-piperidones (RA-1 to RA-9, RAMB1 to RAMB4) toward three cervical cancer cell lines, whereas the viability of primary human keratinocytes was only marginally affected. The compound RA-9 (Figure 1) induced apoptosis of ovarian cancer cell lines but not of primary ovarian surface epithelial cells.[24] A related compound named RA-190 was later shown to preferentially kill HPV-transformed cells.[55] RA-190 reduced the growth of both multiple myeloma and ovarian cancer xenografts and the growth of HPV-16 positive cells in a syngeneic mouse model.[55]

Ewing sarcoma is a bone malignancy that affects children.[56] Patients with metastatic disease have dismal outcomes despite aggressive treatment. Shukla et al. screened a library of >300 000 small molecules to identify compounds that selectively inhibit the growth of Ewing sarcoma cell lines.[50] The screen identified two benzyl-4-piperidone compounds, EWS-P (NSC666038) and EWS-W (NSC669441) (Figure 1).

Wang et al.[23] showed that the compound MCB-613 (Figure 1) induced ER stress and reactive oxygen species (ROS) in tumor cell lines. The potency of the compound in proliferation assays was 3−6 μM on tumor cell lines, whereas primary hepatocytes and mouse embryo fibroblasts were less sensitive. MCB-613 was also identified in a screen for UPS inhibitors (as CB826) and was shown to be cytotoxic to melanoma, colon cancer, and multiple myeloma cells.[57]

A number of additional laboratories have synthesized and examined the biological responses to various dienone compounds, generally 3,5-bis(arylidene)-4-piperidones. Two different groups have reported activity of dienones on estrogen receptor negative breast cancer cells.[58,59] Stronger antiproliferative activities were observed toward breast cancer cells compared to the noncancerous human breast cell line MCF-10A. Kalai et al. reported the antiproliferative activities of a number of 3,5-bis(arylidene)-4-piperidones, including 5e, on two cancer cell lines and a noncancerous cardiomyoblast cell line[60] (Figure 1, Figure 2, Supplementary Table 1). Phosphonate derivatives of 3,5-bis(arylidene)-4-piperidones were found to display inhibitory properties toward four different human cancer cell lines with IC$_{50}$ values in the range of 2.5−8.5 μM.[61] α,β-unsaturated piperidones were described that were active at <1 μM when tested on five cancer cell lines.[62] One of the compounds (1b) (Figure 1) displayed marked inhibitory effects in a lung carcinoma xenograft model with no apparent toxicity. Wei et al. synthesized 61 symmetrical dienone compounds and examined their anticancer activity toward three different cancer cell lines.[63] Two compounds containing sulfur or oxygen heteroatoms in the 4-position of the central six-carbon ring (E10, F10; Figure 1) exhibited antiproliferative activities at concentrations below 1 μM. Youssef et al. reported that amino acid conjugates of 3,5-bisarylmethylene-4-piperidones display improved cytostatic activities compared to compounds without N-substitutions.[64] Finally, Cheng et al. showed that the 3,5-bis(arylidene)-4-piperidone 7g decreased PSA promoter activity and lowered viability of LNCaP prostate cancer cells.[65]

Molecules lacking the central ring structure (dibenzylideneacetone, DBA, and related molecules, Figure 1) have also been described to display antineoplastic activities. These compounds were shown to be less active than cycloketones.[15] A possible explanation for this difference is the larger flexibility
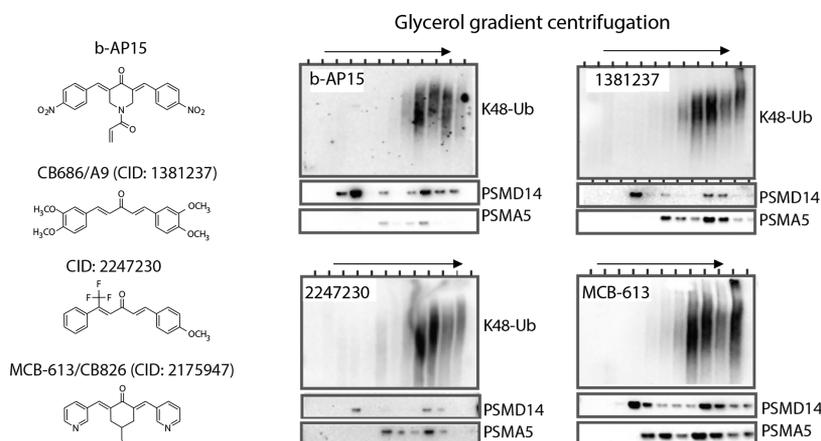
Figure 3. b-AP15 and structurally related compound induce accumulation of polyubiquitinated proteins on proteasomes. Lysates from cells exposed to different compounds were fractionated by glycerol gradient centrifugation, and fractions were collected and processed for immunoblotting. Compounds CID1381237, CID2247230, and CID2175947 were identified in a cell-based screen for compounds stimulate accumulation of Ub^{G76V}-YFP in exposed cells.[57] CID 2175947 (CB826 in ref 57) is also known as MCB-613 and has been described to stimulate steroid receptor coactivator activity and to be preferentially cytotoxic to cancer cell lines.[23] CID1381237 is also known as A9[76] (Figure 1) and C8.[63] Data reproduced from ref 57, which licensed under CC BY 4.0.

of acyclic dienones compared to compounds with rigid piperidine rings.[16] The Arbiser laboratory at Emory University has examined the effects of Tris dibenzylideneacetone dipalladium (Tris DBA) (Figure 1) in a number of studies. Tris DBA showed significant antiproliferative activity against melanoma cells, and ip injection of Tris DBA resulted in a 97% decreased tumor volume in the B16 murine melanoma model.[66] Tris DBA reduces cell proliferation and induces apoptosis in multiple myeloma cells while not exerting toxicity to normal peripheral blood mononuclear cells.[25] Antineoplastic activity of Tris DBA was also documented in an uveal melanoma xenograft model.[67] Finally and interestingly, considering the severity of the disease, Tris DBA was found to be effective in an orthotopic model of pancreatic cancer.[68] Similar results have been reported using similar compounds.[69,70] Human mucoepidermoid carcinoma cell lines and tumor xenografts were found to be sensitive to DBA without reported systemic toxicity.[69]

We conclude from this overview that there is considerable evidence from different laboratories that dienone compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore are cytotoxic to tumor cells and that a number of these compounds are less cytotoxic to nontransformed cells (Figure 2).

■ OTHER ACTIVITIES OF DIENONES

In addition to the cytotoxic/antiproliferative activities to tumor cells, dienone compounds have been reported to have other biological effects, including anti-inflammatory and antiparasitic activity.

Anti-Inflammatory and Antiviral Activity. Zhao et al. showed that compound C12 (Figure 1) showed low cytotoxicity to macrophages but potently inhibited the stimulation of proinflammatory cytokines such as TNF-α and IL-6 by LPS.[71] The activity of C12 was associated with inhibition of ERK/JNK phosphorylation and NF-κB activation.[72] C12 suppresses proinflammatory cytokine production in LPS-exposed mice. The compound was administered by iv injection prior to injection of LPS. EF24 has also been reported to display anti-inflammatory activities, related to NF-κB inhibition.[73,74]

3,5-Bis(arylidene)-4-piperidones have been reported to inhibit the Dengue virus NS3 protease.[75] Only *in vitro* biochemical data were reported, and the results should be viewed with some caution due to the PAINS nature of this class of compounds.

Antiparasitic Activity. Independent research groups have reported that dienones are active against the malaria parasite *Plasmodium falciparum*. The dibenzylidene-acetone A9 (Figure 1) was found to display an IC$_{50}$ of 1.7 μM against a chloroquine-resistant isolate of *P. falciparum*.[76] Similar results were reported using b-AP15, shown to inhibit the growth of intraerythrocytic *P. falciparum* parasites.[77] b-AP15 induced the accumulation of lysine-48-linked polyubiquitinylated proteasome substrates in the treated parasites. The data suggested effects on the ubiquitin−proteasome system, although not at the level of the 20S proteasome or by general inhibition of deubiquitinases.[77] DBA was reported to arrest the growth of *Leishmania donovani* parasites and induce dissipation of mitochondrial membrane potential.[78]

■ TARGETS DESCRIBED FOR DIENONE COMPOUNDS

3,5-Bis(arylidene)-4-piperidones and related compounds are often PAINS, and using these compounds in *in vitro* assays biochemical assays is potentially problematic.[79] Reactive compounds will score positive also in different bioassays,[80] but problems such as inhibition of enzyme activity due to coprecipitation with insoluble compounds are likely to be less common. In the following we have primarily focused on data that have been validated using cellular assays.

Inhibition of the Ubiquitin−Proteasome System (UPS). A number of laboratories have reported that compounds with the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore induce the accumulation of lysine-48-linked polyubiquitinated proteins in cells.[21−23,44,53,55] Lysine-48-linked polyubiquitinated proteins bind to, and are degraded by, the proteasome, and the accumulation of such proteins in cells is a characteristic feature of proteasome inhibitors. Experiments using glycerol gradient centrifugation[49,57] and affinity purification of proteasomes[22] have demonstrated that polyubiquiti-

nated proteins are associated with proteasomes in exposed cells. Further evidence of proteasome inhibition includes the accumulation of a proteasome-degradable reporter (Ub[G76V]-YFP) and proteins such as p53 and p21[Cip1].[22,30,37,45,55,81] These findings are interesting considering that the proteasome is a validated target of anticancer drugs and inhibitors of the 20S proteasome are in clinical use.[82] The compounds discussed here generally do not affect 20S proteasome activity, and different mechanisms have been proposed to explain the phenomenon of accumulation of proteasome substrates.

Inhibition of Deubiquitinases (DUBs). Brancolini et al. showed that compounds G5 and F6 (Figure 1) induced strong accumulation of polyubiquitinated proteins in exposed cancer cells.[44] The same group[83] used a biotin conjugate as a probe to identify the cellular targets of the dienone compound 2c (Figure 1). Importantly, the biotin modification did not alter the biological activity of 2c. The 2c-biotin probe was found to bind to a number of DUBs, USP1 and USP33 showing a higher affinity compared to USP14, USP18, and UCHL5.[83] USP1 and USP33 were reported to be rare proteins, and their contribution to total cellular DUB activity may be limited. Other proteins were found to be modified by 2c-biotin, including AKT and a phosphorylated form of cofilin-1, and it was suggested that these targets contribute to the pharmacological effects of 2c.[83] Thus, cells exposed to 2c show alterations of the cytoskeleton that may be functionally linked to cofilin-1.

Inhibition of Proteasome-Associated DUBs. D'Arcy et al. showed that b-AP15 (Figure 1) induces strong accumulation of polyubiquitin and inhibition of the degradation of a proteasome-degradable reporter in exposed cancer cells.[22] Polyubiquitinated proteins were found to be associated with proteasomes[22,57] (Figure 3). b-AP15 does not inhibit 20S proteasome activity but inhibits the activity of the proteasome-associated DUBs USP14 and UCHL5, both *in vitro* and in cells.[22] Knock-down of both USP14 and UCHL5 is known to result in polyubiquitin accumulation and cytotoxicity,[49,84,85] and inhibition of proteasome-associated DUBs represents an alternative strategy for inhibition of proteasome function.[22] Finally, Didier et al. demonstrated that adenoviral-mediated overexpression of USP14 results in decreased sensitivity to b-AP15.[81]

Since the USP14 and UCHL5 DUBs are not structurally related, it would appear unlikely that a small molecule will inhibit both these enzymes. Inhibition of USP14 and UCHL5 has, however, been demonstrated in cells exposed to pharmacological concentrations of b-AP15.[22,86] Most studies have shown that USP14 is inhibited at lower concentrations of b-AP15 (or VLX1570) than UCHL5.[49,50,81,86,87] VLX1570 binds to USP14 and to proteasomes in surface plasmon resonance (SPR) experiments.[49] Binding of VLX1570 to the 26S proteasome was inhibited by pretreatment with ubiquitin vinylsulfone (an active site-specific DUB probe[88]), consistent with binding to proteasome DUBs.[49] Furthermore, as discussed above, USP14 binds to biotin-conjugated 2c.[83] Incubation of recombinant USP14 with b-AP15 results in molecular weight shifts using MALDI.[57] In these *in vitro* experiments, the degree of binding (i.e., one or two bound b-AP15 molecules) depends on incubation time. Somewhat surprisingly, an apparent thermal stabilization of USP14 at ~53 °C was observed in cells exposed to b-AP15/VLX1570.[49,50,57] The proteasome is known to unfold this temperature,[89] and "thermal stabilization" is likely to result from a partial

dissociation of USP14 from the proteasome, leading to an enrichment in the soluble fraction after heating. This effect has been observed under CETSA conditions (heating); USP14 does not dissociate from the proteasome at other conditions.[49]

UCHL5 appears to be less sensitive than USP14 to inhibition by dienones.[49,81,87] Both G5 and RA-190 do, however, inhibit the activity of recombinant UCHL5.[45,90] UCHL5 binds to a biotin-conjugated 2c probe,[83] and RA-190 binds to the catalytic domain of UCHL5/UCH37.[90] In the latter study, four adducts were observed after incubation of His-UCHL5 with 20-fold excess of RA-190 for 8 h at 37 °C.

RA-9 (Figure 1) was shown to induce accumulation of polyubiquitinated proteins in ovarian cancer and breast cancer cells.[24,91] RA-9 inhibits proteasome-associated DUB activity, whereas total cellular DUB activity is not inhibited by it.[24] Similar to b-AP15, RA-9 induces autophagic flux and inhibition of autophagy increases RA-9-mediated cytotoxicity.[91]

Since USP14 and UCHL5 are members of different families of DUB enzymes, it appears likely that compounds that inhibit the activities of both these enzymes will be general DUB inhibitors. Dienones have indeed been shown to inhibit DUB and also deSUMOylase activities.[92] As discussed above, Ciotti et al. demonstrated widespread reactivity of compound 2c to cellular DUBs.[83] Inhibition of UCHL1, UCHL5, USP2, and USP18 was demonstrated by G5 and 2c.[45] These results are in apparent conflict with the results from other laboratories showing that total cellular DUB activity is not inhibited by b-AP15/RA-9 at concentrations where proteasome DUB activity is inhibited.[22,24,77] A possible explanation for this discrepancy is that the contribution of different DUBs to the total cleavage activity in total cell lysates may be quite disproportionate: a limited number of DUBs may be responsible for most of the cleavage activity in the lysates, and these DUBs may be dienone-insensitive. However, at a concentration of 20 μM, VLX1570 did not show general DUB inhibitory activity on a panel of recombinant DUBs.[48] It was recently reported that total DUB activity in cell lysates is inhibited by 10 and 50 μM b-AP15.[93] The same authors reported inhibition of the activity of different recombinant DUBs using 100 μM b-AP15. This concentration is in the order of >100 times the IC_{50} and difficult to interpret. In general, results obtained using biochemical *in vitro* assays of enzyme activity are unreliable. Thus, findings of no inhibition of DUB enzyme activity can be incorrect due to limited compound solubility (a common problem encountered with these compounds). Conversely, findings of DUB inhibition may be incorrect due to the PAINS nature of these compounds. Considering the reported ability of dienones to inhibit DUBs of both the USP and UCH families in exposed cells, it seems reasonable to conclude that dienones are very likely to be partially selective DUB inhibitors.[44]

Given that dienones are partially selective DUB inhibitors, the question arises with regard to the importance of inhibition of proteasome-associated DUBs, in relation to other DUBs, for the cytotoxicity of dienones. CRISPR deletion screening has demonstrated that the majority of DUB enzymes are nonessential for cell viability.[94,95] In contrast, co-transfection with siRNA to both USP14 and UCHL5 is known to result in decreased processing of proteasome substrates[84,87] and decreased cell viability.[85] We have, so far, been unable to generate USP14/UCHL5 double knock-out cells to test their sensitivity to b-AP15.

Binding to the Ubiquitin Receptor Rpn13. Anchoori et al.[55] reported that, similar to other dienones, RA-190 (Figure
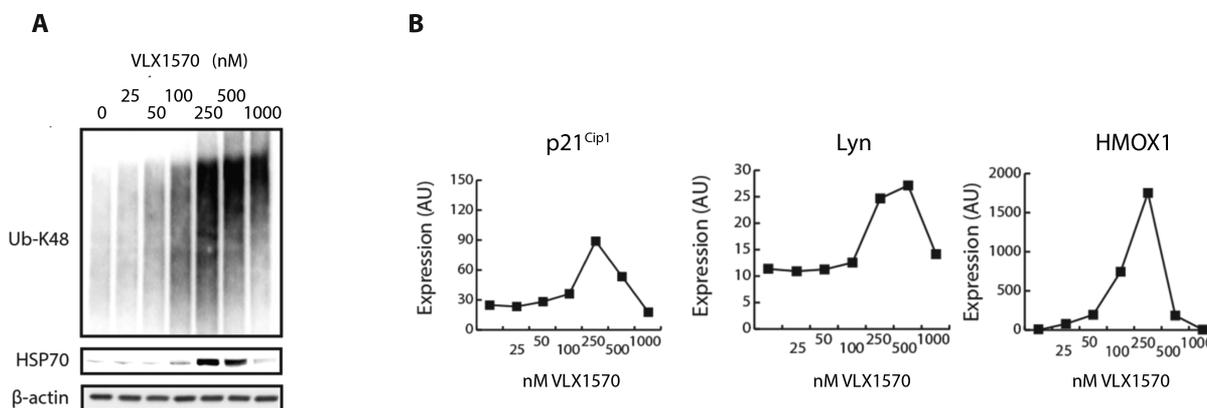
Figure 4. Concentration-dependent responses to VLX1570. (A) Induction of polyubiquitinated proteins in RS4;11 ALL cells. Note strong increases of polyubiquitin and induction of Hsp70 at 250 nM VLX1570. (B) Increases in p21[Cip1], LYN, and HMOX1. RS4;11 cells were exposed to VLX1570, and different markers were analyzed in the same extracts using a PCR-based multiplex method (OLINK, Uppsala, Sweden). Note the increases of HMOX1 at 50−100 nM VLX1570 and the decreases of all markers at high concentrations. Figure from ref 157, which licensed under CC BY 3.0.

1) induces accumulation of polyubiquitin in exposed cancer cells. These authors found that RA-190 binds to Cys88 of the proteasome−ubiquitin receptor Rpn13 and suggested this to be the mechanism of action of the compound. A similar mechanism of action, involving Rpn13, has been suggested for EF24.[96] Rpn13 is known to bind and to activate the DUB UCHL5, and it was subsequently shown that RA-190 does not affect the interaction of Rpn13 with UCHL5 but directly inactivates UCHL5.[90]

The studies of the role of Rpn13 and UCHL5 with regard to the mechanism of action of RA-190 are complicated by the fact that knock-out or knock-down of Rpn13 will lead to loss of UCHL5. In a recent study, Osei-Amponsa et al.[97] showed that a cell line expressing an Rpn13 receptor lacking the Pru domain, necessary for proteasome and ubiquitin binding, is less sensitive to RA-190 compared to parental cells. Although these cells were not completely resistant to RA-190, the result demonstrates the involvement of the proteasome in conferring sensitivity to RA-190.

Organelle Stress. The robust induction of cell death by dienones (i.e., apoptosis-induction in Apaf-1 negative cells, BCL2 overexpressing cells, and p53[null] cells; see further below) can be interpreted in terms of major disruptions of cellular homeostasis. Many studies have shown that dienone compounds induce ER stress.[98,23,99,39,57,81] This is expected considering the common finding of disturbancies of the ubiquitin−proteasome system observed by many investigators. Other types of organelle stress such as mitochondrial stress and lysosomal permeabilization have also been reported.

In their initial study of G5/F6, Brancolini and co-workers[44] showed that these compounds induce mitochondrial fragmentation prior to induction of membrane permeabilization and cytochrome c release. The 20S proteasome inhibitor MG-132 induced a similar effect.[44] Dienones have been suggested to induce direct mitochondrial membrane permeability transition by oxidation or cross-linking of membrane proteins.[100] Zhang et al. showed that polyubiquitinated proteins become associated with mitochondrial outer membranes in cells exposed to b-AP15.[52] The amounts of misfolded proteins on mitochondrial membranes correlated with decreases in oxidative phosphorylation.[52] Dienone compounds, including b-AP15, were identified in a screen for compounds that induce the lysosomal pathway of apoptosis.[47] Knock-down of the

lysosomal enzyme cathepsin-D reduced caspase-cleavage in cells exposed to b-AP15 and also to other inhibitors of the UPS.[47]

An association between proteotoxic stress and organelle stress would offer an explanation for the selective sensitivity of tumor cells to dienones (due to high protein synthesis and a high production of misfolded proteins) and the robust induction of cell death.

Stimulation of Steroid Receptor Coactivators. MCB-613 (Figure 1) was identified in a cell-based screen for compounds that stimulate the activity of SRC-1 and SRC-3 steroid receptor coactivators.[23] MCB-613 increases the interactions of SRC proteins with other coactivators and induces ER stress and generation of reactive oxygen species. MCB-613 was also found to induce cellular accumulation polyubiquitinated proteins but this was not considered to be the primary mechanism of action of the compound.[23] MCB-613 (CB826) was subsequently identified in a screen for compounds that inhibit the processing of a proteasome-degradable reporter and was found to increase the levels of polyubiquitinated proteins on proteasomes[57] (Figure 3).

Cross-Linking of Proteins. The dienone structure of 1,5-diaryl-3-oxo-1,4-pentadienes raises the possibility of protein cross-linking. Ward et al. recently raised the possibility of cross-linking being an important mechanism of action of b-AP15/VLX1570.[93] The distance between the carbonyls is approximately 5.1 Å apart in 3,5-bis(arylidene)-4-piperidones,[101] arguably a short distance for a chemical cross-linker. Ward et al. did show that b-AP15 can bind two molecules of GSH. The thiols of GSH would, however, be expected to be more sterically available than most cysteine thiols on larger protein structures. Ward et al. demonstrated the formation of protein dimers in cells exposed to VLX1570.[93] Of particular interest was the finding of dimers of anamorsin/CIAPIN1, an apoptotic regulator, raising the possibility that functional depletion of this protein is important for the mechanism of action of VLX1570. The authors did, however, not detect direct cross-linking of anamorsin/CIAPIN1 involving thiols and the mechanism of dimer formation is unclear.

Cross-linked protein material, detected as slowly migrating material using by SDS−PAGE and Coomassie Blue staining, was observed using 10 μM b-AP15/1570.[93] We also observe high MW protein complexes in AML cells exposed to 10 μM

b-AP15 (Selvaraju et al., unpublished data). It should be noted that 10 μM b-AP15 is a quite high concentration (10- to 100-fold the IC$_{50}$ in most cells) and that the cell death mode elicited by b-AP15 shifts from apoptosis to necrosis at concentrations of $\geq$5 μM.[46] High molecular weight material is generally not observed using SDS−PAGE using IC$_{50}$ concentrations of dienone compounds (e.g., RAMB1 (Figure 1), Figure 7A in Anchoori et al., 2011[54]), and the molecular weights of various cellular proteins are not altered on immunoblots. However, the molecular weights of polyubiquitinated proteins gradually increase at pharmacologically more relevant concentrations of VLX1570 (0.5−1 μM, see Figure 4A). We initially interpreted the increases in molecular weight in terms of DUB inhibition leading to longer polyubiquitin chains being attached to proteins.[22] An alternative interpretation is that cysteines in unfolded proteasome substrates will be more available to adduction by dienones. The formation of high molecular weight complexes and aggregation of proteins by Michael acceptors need to be further studied to evaluate mechanisms of aggregation and whether protein aggregation is coupled to cytotoxicity.

The cystic fibrosis transmembrane conductance regulator (CFTR) can be cross-linked into dimers after exposure of cells or microsomes to curcumin.[102] Cross-linking of the CFTR was observed also in the presence of high concentrations of reducing agents and using a cysteine-free CFTR construct, showing that oligomerization was not due to disulfide cross-linking. The authors suggested that cross-linking was associated with an oxidative reaction.[102] Protein cross-linking has been observed using also other compounds. Stanwell et al. tested the tyrosine kinase inhibitor erbstatin and related compounds for cross-linking of cellular proteins using an assay based on boiling cells for 10 min in 2% SDS/20 mM DTT and filtering through cellulose membranes.[103] Methyl 2,5-dihydroxycinnamate (Figure 1), containing a single Michael acceptor, was found to generate SDS/DTT insoluble protein material in treated cells. Cross-linking was observed at concentrations ~5-fold the IC$_{50}$ in cell proliferation assays, suggesting that it was not associated with inhibition of cell proliferation. Cross-linking was observed at both +37 °C and +4 °C, and a mechanism involving oxidation of compounds to reactive quinones that cross-link protein nucleophiles was suggested.[104] Pinto et al. reported that a benzophenazine photosensitizer, OR141, induced the formation of high molecular weight complexes.[105] Interestingly, OR141 was shown to induce polyubiquitinated proteins and to inhibit USP14 and UCHL5 activity *in vitro*. OR141 was speculated to induce disulfide bond formation, leading to formation of high molecular weight complexes.

The findings of cross-link formation by dienone compounds lead to the possibility that (i) specific proteins of importance for cellular homeostasis may be depleted and/or that (ii) cross-linked protein complexes are formed in amounts sufficient to elicit cytotoxicity. The results showing depletion of anamorsin/CIAPIN1 support the first notion (further discussed below).[93] We are, however, not aware of evidence supporting the notion that protein complexes formed at pharmacologically relevant concentrations of dienones lead to cytotoxicity.

Induction of Oxidative Stress. Induction of oxidative stress by dienone compounds has been reported in a number of studies.[23,29,32,36,39,81,98,106] Some of these studies use *N*-acetyl cysteine (NAC) as a tool to demonstrate that oxidative stress is essential for apoptosis induction. This is problematic

since NAC will bind directly to the compounds, leading to their inactivation.[107] Other scavengers have, however, also been used, and the results showed that oxidative stress induction is involved in triggering apoptosis (but not cell death).[98] Different mechanisms have been put forward to explain the induction of oxidative stress. The enzymatic activity of thioredoxin reductase (TrxR), an enzyme essential for cellular redox-homeostasis, is dependent on the motif Gly-Cys-Sec-Gly in which Sec is selenocysteine and particularly reactive to electrophiles.[108] Both b-AP15 and EF24 inhibit TrxR,[87,106] presumably by binding to the selenocysteine. An alternative mechanism for oxidative stress induction has been proposed involving mitochondria. Stimulation of oxidative stress by b-AP15 was diminished in Rho$^{\text{null}}$ cells depleted of mitochondrial DNA,[109] suggesting that the oxidative stress observed in cells exposed to b-AP15 is partly of mitochondrial origin.

Inhibition of Translational Elongation. Bortezomib treatment results in the induction of the unfolded protein response (UPR), including induction of phosphorylation of eIF2α.[110] Induction in eIF2α phosphorylation will decrease translation and the production of proteasome substrates, constituting a protective response.[110] We observed that the induction of various markers such as p21$^{\text{Cip1}}$ and LYN, proteins known to be proteasome substrates,[111,112] peak at concentrations of 250−500 nM VLX1570 and then decrease (Figure 4B). The lower level of induction at higher drug concentrations would be consistent with induction of the UPR, leading to decreased translation. Interestingly, however, the same pattern was observed for Hsp70 (Figure 4A). Hsp70 translation is cap-independent due to the presence of an internal ribosome binding site,[113] and translation should therefore not be affected by induction of the UPR. VLX1570 was indeed shown to inhibit translation by another mechanism, at the level of translational elongation, in a recent study.[114]

Inhibition of translation may be relevant to the mechanism of action of VLX1570. Tumor cells are sensitive to obstructions in protein homeostasis, and an inhibitor of translational elongation (omacetaxine mepesuccinate) has been approved for treatment of chronic myeloid leukemia.[115] However, it should be noted that translational inhibition is observed at slightly higher concentrations compared to proteasome inhibition.

Inhibition of Intracellular Signaling Systems. Various investigators have examined the effects of dienones on important cellular signaling molecules such as STAT transcription factors, MAP, AKT, and Src kinases. HO-3867, Tris DBA, and b-AP15 have been described to inhibit STAT3 phosphorylation.[36,41,66,86] Tris DBA and NC2213 (Figure 1) inhibit Src phosphorylation.[25,116] AKT has been demonstrated to be targeted by 2c using mass spectrometry,[83] and AKT phosphorylation is inhibited by b-AP15 in lymphoma cells,[86] by EF24 in colon cancer cells,[26] by the compounds RL90 and RL91 in breast cancer cells,[58] by E10 in prostate cancer cells,[63] and by Tris DBA in melanoma cells.[66] Furthermore, ERK phosphorylation is inhibited by b-AP15[49,86] and by DBA/Tris DBA[34,66] and E10[63] in different tumor cells. The basal levels of GTP-loaded RAS are decreased by b-AP15.[49] Whether the effects on these signaling systems are instrumental to the mechanism(s) of action of various compounds or are secondary consequences of severe stress is not well understood.

Methionine Aminopeptidase. Methionine aminopeptidases (MetAPs) remove methionine from the amino terminus
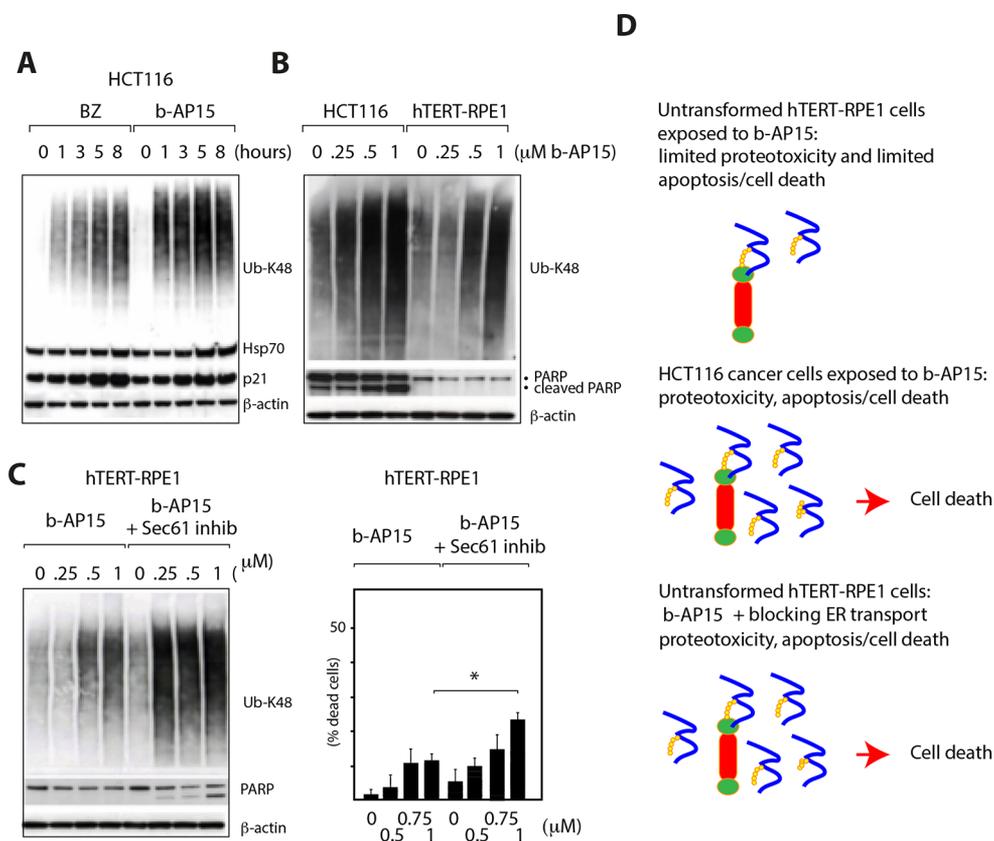
Figure 5. Association between polyubiquitin association and cell death. (A) HCT116 colon cancer cells were exposed to 1 μM b-AP15 or 100 nM bortezomib and harvested at the indicated time points. Lysates were subjected to immunoblotting for K48-linked polyubiquitin, HSP-70B', p21^{Cip1}, PARP, or β-actin (loading control). (B) HCT116 cells or hTERT-RPE1 cells (immortalized epithelial cells) were exposed to different concentrations of b-AP15 for 1 h, followed by washing and incubation for 16 h in drug-free medium. Lysates were subjected to immunoblotting for K48-linked polyubiquitin, PARP and β-actin (loading control). Note the stronger accumulation of polyubiquitinated proteins and the induction of PARP cleavage in the colon cancer cells. (C) CpdA is an inhibitor of the Sec61 complex of anterograde protein export into the ER.[130] Immortalized hTERT-RPE1 epithelial cells were exposed to increasing concentration of b-AP15 in the presence or absence of CpdA ("Sec61 inhib"). Note the increase in polyubiquitin accumulation, PARP cleavage, and cell death in the presence of CpdA (* $p < 0.05$). Parts A−C are reproduced from Sun et al.,[129] which is licensed under CC BY 4.0. (D) Graphic illustration of the results. Shown are proteasomes (red, 20S; green, 19S) and polyubiquitinated proteins.

of some newly synthesized proteins, essential for co-translational protein maturation processes such as myristoylation.[117] MetAP2 is essential for the growth of different types of cancer cells.[118] MetAP2 expression decreases in HT29 cancer cells after exposure to NC2213 (Figure 1).[116] Decreased levels of Src phosphorylation were observed in parallel to the decreases in MetAP2; Src is a myristoylated oncoprotein, and a causal relationship was suggested.[116]

Reactivation of Mutant p53. Much interest has been focused on the possibility of reactivating mutant p53 molecules to active forms.[119] It was reported that HO-3867 (Figure 1) covalently binds to mutant p53, resulting in restoration of a wild-type p53-like response.[38] This mechanism would explain the selective cytotoxicity toward cancer cells by this class of compounds. The result is, however, in apparent conflict with findings using b-AP15 where antiproliferative effects and/or apoptosis induction was found to be insensitive to p53 mutational status.[22,81] Furthermore, the IC$_{50}$ of p53^{wt} cell lines in the NCI$_{60}$ panel to b-AP15 is 0.18 μM, and the IC$_{50}$ of p53^{mut} cell lines is 0.15 μM ($p = 0.70$). The discrepancy could be explained by b-AP15 and HO-3867 having different mechanisms of action (HO-3867 has not been examined for possible inhibition of proteasome function).

Effects on DNA Repair Pathways. The ataxia telangiectasia mutated (ATM) gene product is activated in cells exposed to 2cPE (a pegylated derivative of 2c).[120] ATM is known to be activated by oxidative stress due to disulfide-cross-linked dimerization,[121] and the stimulation of ATM signaling may therefore be a consequence of oxidative stress. ATM inhibition was found to increase 2cPE-induced cell death.[120] A similar finding has been reported for EF24, showing that ATM-deficient cells are more sensitive to this compound.[122] EF24 was also found to inhibit the Fanconi anemia pathway, possibly due to inhibition of IκB kinase complex.[122]

ANALYSES OF GENE EXPRESSION PROFILES (CMap)

The connectivity map (CMap) is a freely available and quite useful resource for initial characterization of the cellular response to small molecules. The CMap method was originally described in 2006,[4] and an updated database was presented more recently.[123] In the original study by Lamb et al.[4] it was demonstrated that compounds sharing molecular targets induce similar molecular signatures (i.e., alterations in gene expression patterns, determined by the microarray method).
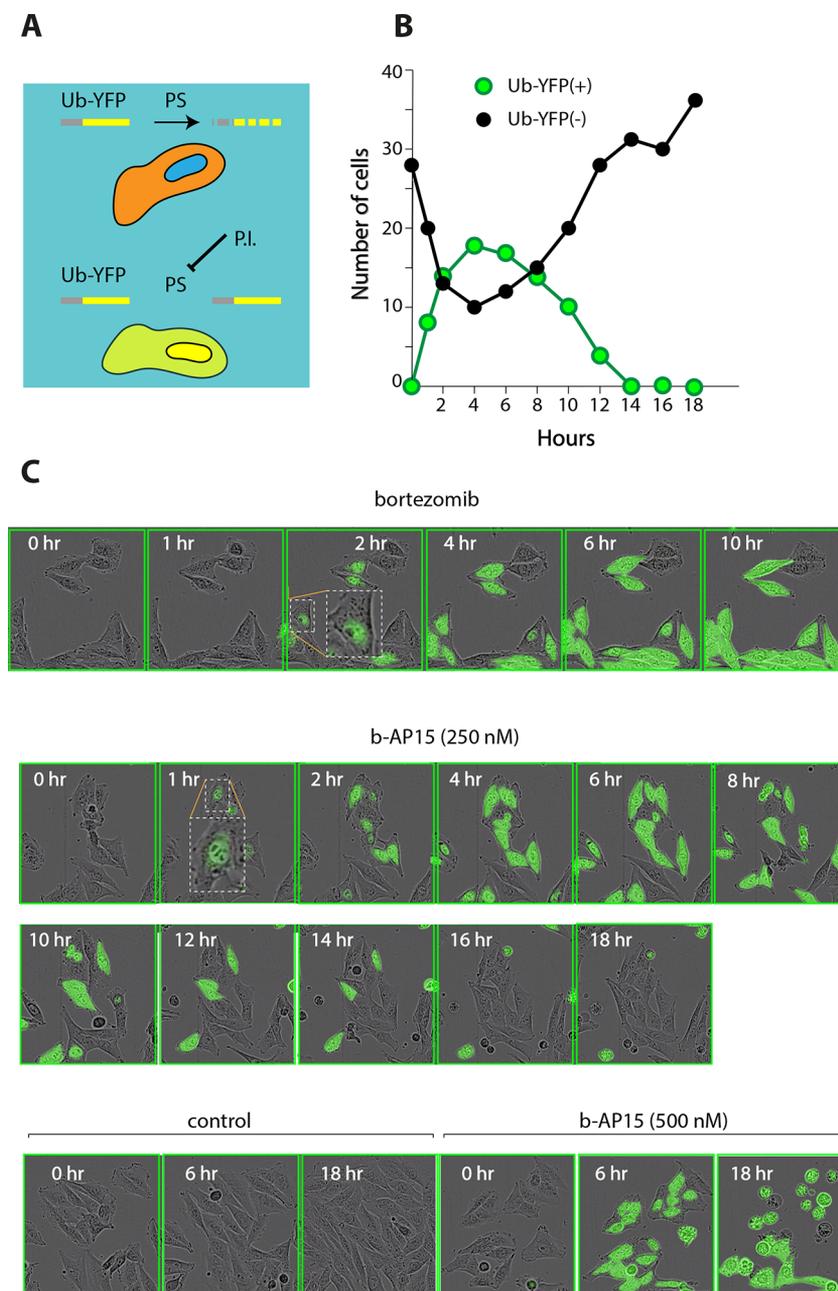
Figure 6. Proteasome inhibition induced by b-AP15 is associated with cell death. (A) A reporter protein (Ub^{G76V}-YFP) is degraded by the proteasome (PS) in human melanoma cells. Proteasome inhibition (PI) leads to accumulation of the reporter protein in the cells.[133] (B) Tracing individual cells expressing Ub^{G76V}-YFP following exposure to 250 nM b-AP15. Cells become Ub^{G76V}-YFP positive after 1−4 h, showing accumulation of the proteasome reporter. YFP-positive cells subsequently round up and the YFP leaks out from the cells after membrane disintegration. In contrast, cells that remain YFP-negative at 4 h of exposure survive and continue to divide. Results were obtained using an IncuCyte instrument (see refs 87 and 98). (C) Time-lapse recordings of Ub^{G76V}-YFP-expressing human melanoma cells after addition of compounds. Upper panel: Human melanoma cells expressing Ub^{G76V}-YFP were exposed to 100 nM bortezomib and traced for 10 h. Notice the appearance of YFP fluorescence in cell nuclei at 2 h and later appearance in the cytoplasm (one cell highlighted at 2 h). Middle panel: Cells were exposed to 250 nM b-AP15. Note the similar pattern of initial nuclear and later cytoplasmic fluorescence (one cell highlighted at 1 h), similar to the pattern observed using bortezomib. Also note that cells either become positive and die or remain completely negative and survive. Lower panel, left: control. Vehicle-treated control cells remain YFP-negative and proliferate. Lower panel, right: >90% of the cells exposed to 500 nM b-AP15 become YFP-positive after 6 h, and most of these cells have rounded up at 18 h, some have lost membrane integrity and the YFP content. Original data were published in refs 87 and 98.

Conditions were standardized with regard to the concentration of small molecules, time of exposure, and the cell line used (usually the MCF-7 breast cancer cells). The authors were able to show that CMap could be used to elucidate the mechanism of action of a natural product.[4]

The responses to dienones have been characterized using gene expression profiling (CMap).[21,22,48,120] These experiments show high correlation (connectivity) to known inhibitors of the 20S proteasome such as MG-132.[22] Induction of HSPA6 mRNA transcripts (an inducible form of the Hsp70

family of molecular chaperones) after 6 h of exposure to b-AP15 or P1 was at the level of ∼1000-fold, stronger than that observed using standard 20S proteasome inhibitors[21,98] (P1 is identical to EWS-W; Figure 1). Gene expression analyses have also been performed on cells exposed to 2c-PE and showed induction of expression of chaperones, particularly transcripts encoding Hsp70 proteins.[120] The response to the compound MCB-613/CB826 has also been studied.[23,57] Gene set enrichment analyses (GSEA)[124] showed an enrichment score of >2 for genes encoding proteins involved in protein folding.[57] The mechanism underlying the stronger induction of chaperone genes by dienones in comparison to bortezomib is unclear but may at least partly be explained by the limited formation of aggresomes.[81,125] The formation of aggresomes represents a mechanism for cells to escape cytotoxic effects of unfolded cellular proteins,[126] and ineffective formation of these structures may lead to increased proteotoxicity.

Compound F6 (Figure 1) is in the CMap database, and the connectivity score (tau-score) between F6 and loss of function of the proteasome pathway (deletion of proteasome subunits) is +99.55 (https://clue.io/query). Tau-scores of +90 or higher are considered as strong indicators of related mechanisms of action (https://clue.io/connectopedia/connectivity_scores). It should be noted, however, that compounds that affect protein homeostasis at other levels also show strong connectivity to loss of function of the proteasome pathway, including 17-DMAG (Hsp90 inhibitor, tau-score of +95.9) and puromycin (protein synthesis inhibitor, tau-score of +97.4). Furthermore, heat shock factor-1 (HSF1), essential for induction of chaperones, is activated by electrophiles[127] and electrophiles will therefore show connectivity to UPS inhibitors. The extent of this latter effect may, however, be limited since the dienone curcumin shows only weak connectivity to loss of function of the proteasome pathway (tau-score of +19.5). We conclude that CMap data are consistent with the notion that dienone compounds are proteasome inhibitors but do not provide positive proof.

■ ASSOCIATION BETWEEN INHIBITION OF PROTEASOME FUNCTION AND CELL DEATH

It is known that a considerable fraction of newly synthesized proteins is not folded correctly.[128] High levels of protein synthesis will therefore result in an increased production of proteasome substrates.[128] This would explain the therapeutic window of proteasome inhibitors, tumor cells being more active in protein synthesis, particularly the high sensitivity of myeloma cells that synthesize massive amounts of immuno-globulins. We previously reported that enhancing the levels of proteasome substrates by blocking anterograde transport of proteins through the ER increases the cytotoxic effect of b-AP15 (for details, see Figure 5).[129] Interestingly, elevation of proteasome substrate levels increases the sensitivity of immortalized hTERT-RPE1 epithelial cells and normal human fibroblasts to b-AP15-induced apoptosis and to cell death (Figure 5C)[129] and suggests that the difference in sensitivity of cancer cells and normal cells to b-AP15 is, at least in part, due to differences in the production of proteasome substrates (Figure 5D).

Evidence linking proteasome inhibition to cell death has been presented using cell lines expressing a proteasome-degradable reporter protein (Figure 6A).[87,98] Cells can be followed after initiation of treatment and the fate of individual cells determined. Using concentrations close to IC_{50}, it is

found that cells where the proteasome reporter accumulates subsequently die (where loss of membrane integrity results in leakage of YFP from the cells), whereas cells where proteasome inhibition is not observed survive and continue to proliferate[87,98] (Figure 6B). Bortezomib induces an increase in nuclear fluorescence after 2 h, followed by increases in cytoplasmic fluorescence (Figure 6C). This pattern is consistent with reports of predominant presence of 26S proteasomes in the nucleus.[131] Exposure to b-AP15 results in the same pattern of initial nuclear fluorescence (Figure 6D), first observed after 1 h. The difference in onset can be explained by the more rapid uptake of b-AP15 into cells compared to bortezomib.[87,132]

The accumulation of the Ub^{G76V}-YFP reporter in cells could be argued to be due to cross-linking and aggregation of the reporter protein and an inaccessibility of aggregated proteins to proteasomal degradation. We find this scenario unlikely for a number of reasons: (i) the same pattern of initial nuclear accumulation of Ub^{G76V}-YFP is observed in b-AP15- and bortezomib-treated cells; (ii) the phenomenon of "all-or-nothing" in terms of Ub^{G76V}-YFP positivity; it is difficult to understand how chemical cross-linking of Ub^{G76V}-YFP, leading to aggregation, will occur in some cells, whereas no (or undetectable) cross-linking and aggregation will occur in neighboring cells. This "all-or-nothing" phenomenon is identical in cells exposed to b-AP15 and bortezomib and is likely to reflect differences in the thresholds of proteasome degradation capacity and/or generation of proteasome substrates in different cells; (iii) we have not observed aggregation of Ub^{G76V}-YFP by immunoblotting in b-AP15-exposed cells using pharmacologically relevant concentrations;[129] (iv) cross-linking of cellular proteins[93] (see above) could be expected to result in general toxicity, not consistent with the tumor cell selectivity of dienones and the relative insensitivity of noncancerous cells to b-AP-15 and other dienones.

The notion that inhibition of the UPS is of importance for cytotoxicity of dienones is supported by experiments involving genetic manipulation: (i) deletion of Rpn13 and deletion of the Pru-domain of Rpn13 lead to a decreased (but not totally lost) sensitivity to RA-190;[97] (ii) overexpression of USP14 results in decreased sensitivity to b-AP15;[81] (iii) knock-out of *SRC-3*, the protein product of which has been suggested to be upstream of proteasome inhibition, leads to a decreased induction of DDIT3/CHOP and other stress-related markers.[23]

■ STUDIES OF BINDING AND DEGRADATION OF ELECTROPHILE-ADDUCTED PROTEINS

Some information is available with regard to the binding of dienones to the cellular proteome and the subsequent fate of adducted proteins. It was recently reported that 44 proteins were covalently adducted by VLX1570, including the anamorsin/CIAPIN1 apoptosis regulator.[93] Studies have been performed on 4-hydroxy-2-nonenal (HNE), an α,β-unsaturated aldehyde that shows reactivity to proteomic cysteines,[134] and the endogenous electrophile 15-deoxy-$\Delta^{12,14}$-prostaglandin-J2 (PG-J2, a cross-conjugated α,β-unsaturated dienone). HNE is known to be selective, and only a limited number of nucleophilic residues are carbonylated within each protein.[135] The reactivities of different residues appear to be affected by the protein environment.[135,136] PG-J2 has been demonstrated to bind ∼350 proteins, the largest

group related to metabolic processes involved in protein modification, protein folding, translation, and proteolysis.[137]

Thioredoxin reductase (TrxR) is expected to be a common target of cytotoxic electrophiles due to its nucleophilic active site Sec residue, present in an unstructured portion of the enzyme.[138] We recently evaluated 141 cytotoxic α,β-unsaturated compounds for their ability to inhibit proteasome function and their ability to inhibit TrxR. Surprisingly, whereas 28 compounds were found to inhibit proteasome function, only 20 could be shown to inhibit TrxR.[57] One of the compounds that was found not to inhibit TrxR was the dienone compound CB826/MCB-613. The finding was unexpected both with regard to the presence of the nucleophilic Sec residue in the TrxR enzyme and with regard to the PAINS properties of many of the compounds. Furthermore, the findings did not show a direct relationship between electrophilicity of the compounds and their cytotoxicity.[57]

The inhibition of proteasome DUB activity and of USP14 was reported to be reversible under *in vitro* conditions.[22,87] In surface plasmon resonance experiments (SPR), VLX1570 rapidly associates and subsequently dissociates from proteasomes.[49] We have subsequently found that extended incubation of 19S proteasomes with b-AP15 (20 min at 37 °C) results in reduced recovery of (~60%) of the DUB activity after dilution.[57] We interpret these results to suggest that b-AP15 recognizes proteasome-associated DUBs by noncovalent interactions, increasing the residence time to facilitate subsequent covalent binding.

Is the strong and rapid accumulation of polyubiquitinated proteins in cells exposed to dienones a secondary consequence of adduct formation and aggregation? An early study suggested that HNE-modified proteins were significantly ubiquitinated and showed that proteasome inhibitors suppressed the elimination of such proteins.[139] A later study showed that the elimination of HNE-modified proteins in cells was not stabilized by proteasome inhibitors, whereas the lysosome inhibitor chloroquine prevented their loss.[140] In a study of HNE-induced degeneration of neurons, Zhang et al. observed an abnormal accumulation of both K48- and K63-linked polyubiquitinated proteins.[141] The increases in K48-linked polyubiquitinated proteins were, however, quite modest, approximately 2-fold by 24 h. A particularly interesting study using mass spectrometry was published by Yang et al.[134] These authors showed that the alkylation of some cysteine residues by HNE was stable over time, whereas and interestingly the modification of other cysteines decreased rapidly after removal of HNE. These findings suggest the existence of a repair mechanism for HNE adducts in cells but is not consistent with alkylated proteins being removed by mechanisms of global protein degradation. Although the literature is conflicting with regard to mechanisms of degradation of HNE-adducted proteins, we are unaware of any study demonstrating accumulation of K48-linked polyubiquitinated proteins similar to what is observed using b-AP15 (Figures 4 and 5). Since both the kinetics and amplitude of the increases in polyubiquitin are similar to those observed using the 20S proteasome inhibitor bortezomib, our interpretation is that they are likely due to a direct inhibitory effect of proteasome function.

PHARMACOLOGY AND TOXICITY

Measurement of HO-3867 uptake in tissue cultured cells showed maximal uptake within 60 min followed by a decline of intracellular levels.[142] HO-3867 could still be detected after 72 h in cells exposed for 1 h followed by change of medium.[142] We have reported very similar results using b-AP15, demonstrating a rapid uptake from the culture medium and enrichment of the compound in exposed cells.[87] Intracellular enrichment of drugs is not uncommon and is also observed for the 20S proteasome inhibitor bortezomib.[132] The underlying mechanism for the rapid uptake and enrichment of dienone compounds is unclear, but we have observed that cellular uptake was impaired by 30 min of pretreatment of cells with a relatively low concentration of *N*-ethylmaleimide (10 μM). One interpretation of this result is that the uptake is driven by the availability of intracellular thiol groups. Alternatively, uptake is dependent on a *N*-ethylmaleimide-sensitive transporter.

The higher potency of b-AP15 and VLX1570 in intact cells compared to its potency using *in vitro* assays for USP14 and UCHL5 has been recently discussed.[93] Intracellular enrichment explains the findings by different investigators of USP14 activity being inhibited in cells exposed to relevant concentrations (0.1–1 μM) of b-AP15/VLX1570.[22,48–50,81,86,87]

The relevant drug concentrations that are to be used in *in vitro* studies have been an enigma in cancer drug research since compounds are often reactive and exhibit polypharmacology. Cisplatin can be used to illustrate this problem. Cisplatin is known to cross-link DNA, representing the major mechanism of action.[143] The cisplatin molecule will also react with proteins, in particular with thiols, and a number of other targets will become relevant using suprapharmacological concentrations of the drug. Cisplatin-induced acute apoptotic cell death, studied by many groups (PubMed search September 2020, cisplatin + apoptosis + mechanism: ~4000 hits), may be of limited importance for clinical drug sensitivity since concentrations of >10-fold the IC$_{50}$ are required to induce this phenomenon.[144]

A number of investigators have demonstrated that dienone compounds display considerable higher cytotoxicity to tumor cells compared to noncancerous cells.[18–26,38,42,53,59,60,129] Some of these studies have presented IC$_{50}$ values, and these are summarized in Figure 2 and Supplementary Table 1. These results, using *in vitro* cultured cells, are interesting from a mechanistic point of view but are difficult to extrapolate to toxicity in living organisms. It was recently reported that b-AP15/VLX1570 shows similar cytotoxicities to cancer cell lines and HUVECs (human vascular endothelial cells) and, based on this observation, it was suggested that these compounds are generally cytotoxic.[93] The sensitivity of HUVECs to b-AP15/VLX1570 is consistent with reports from other studies of dienones. In one study the IC$_{50}$ of EF24 toward HUVECs was reported to be 2.6 μM, lower in comparison to two different liver cancer cell lines (3.8 and 4.4 μM).[30] In another study, the IC$_{50}$ of EF24 toward HUVECs was reported to be 1.1 μM, compared to 0.2 μM for leukemia cells, 0.3 μM for colon cancer cells, 0.4 μM for prostate cancer cells, and 1.4 μM for melanoma cells.[15] MCB-613 showed IC$_{50}$ values of 3–6 μM in cancer cell lines, whereas no effect was observed on the proliferation of primary hepatocytes or fibroblasts at these concentrations.[23] HUVECs were, however, sensitive to high
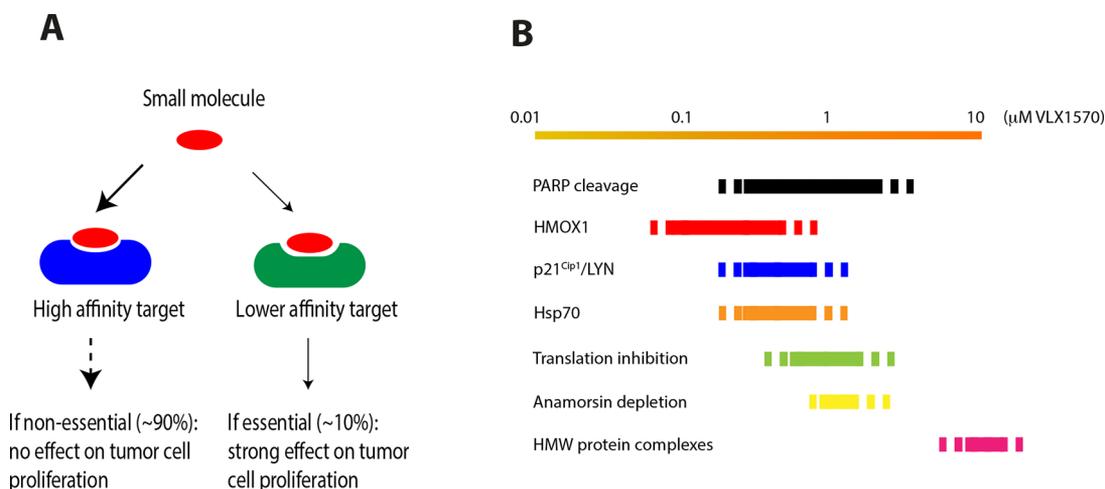
Figure 7. (A) Target affinity does not necessarily correlate to mechanism of action. A major fraction of cellular genes (∼90%) have been found to be nonessential for proliferation of tumor cells *in vitro*.[155] Small molecules that bind to the protein products of these genes are therefore unlikely to affect tumor cell proliferation. In contrast, low affinity binding to essential proteins may result in strong inhibition of proliferation. An example is microtubule dynamics, known to be sensitive to low concentrations of small molecules[156] and for which off-target effects may occur for various compounds when used in screens at 5−10 μM. (B) Illustration of responses to VLX1570 reported in different studies.[93,114,157] HMOX1 is induced at lower concentration of b-AP15/VLX1570 than other markers (see Figure 4). Nrf-2/KEAP is targeted by electrophiles, and the Nrf-2 system is likely to be particularly sensitive to electrophiles.[158] Increases of polyubiquitinated proteins, Hsp70 chaperones, and proteasome substrates such as p21^{Cip1} are observed at somewhat higher concentrations of VLX1570; increased levels of Hsp70 are also observed at these concentrations (Figure 4).[157] Inhibition of translation is observed at increasing concentrations of VLX1570,[114,157] leading to a blunted response of Hsp70, HMOX1, p21^{Cip1}, and LYN expression (Figure 4). Depletion of anamorsin/CIAPIN1 was reported to occur at concentrations of 0.5−1 μM[93] and the formation of higher molecular weight protein complexes at 10 μM[93] (using HeLa cells).

doses of MCB-613.[23] On the basis of the sensitivity of endothelial cells, dienones have been advocated as angiogenesis inhibitors.[145] Whether studies of the *in vitro* sensitivity of HUVECs predict the antiangiogenic potential of small molecules or whether HUVEC sensitivity can be used to argue that dienones are generally cytotoxic can be debated.

Multiple reports that demonstrated preclinical antitumor activity of dienones did not report animal toxicity (see above). In the specific case of VLX1570, preclinical use requires oil/detergents for formulation, leading to complications in the evaluation of toxicology due to species differences in the tolerance to such formulations.[146] A phase 1 study has been performed using VLX1570 in a formulation based on polyethylene glycol, polyoxyethylated castor oil, and polysorbate 80 (PCT) formulation.[147] The study used an accelerated protocol, and pulmonary toxicity was encountered at 1.2 mg/kg.[147] Pulmonary toxicity has also been observed with bortezomib,[148] and it was found that the PCT formulation enhances this type of adverse event by bortezomib and VLX1570.[147] These problems will hopefully be possible to resolve using prodrugs that do not require oil/detergent for administration.

CELL DEATH MODE INDUCED BY DIENONES

A number of different cell death modes have been described,[149] many of which may be induced by anticancer drugs. Describing the cell death mode being elicited by a particular compound is of importance both for understanding mechanisms of action and for understanding potential mechanisms of resistance.

The death mode induced by G5 has been studied in some detail.[150−152] G5-induced apoptosis requires the Bcl-2 family proteins Bax and Bak.[152] However, and in distinction to bortezomib, G5 is able to kill apoptosis-resistant cells deficient in Bax and Bak by activating a necrotic response.[152] In fact, the kinetics of cell death observed in G5-exposed cultures is indistinguishable using wild-type cells and Bax/Bak defective cells.[152] The morphology of cells exposed to G5 is characterized by massive cytoplasmic vacuolization.[152] Interestingly, a similar phenomenon of vacuolization was reported in cells exposed to MCB-613[23] and has also been demonstrated for b-AP15-treated cells.[52] The death mode induced by MCB-613 was interpreted as paroptosis,[23] a nonapoptotic form of cell death characterized by massive cytoplasmic vacuolization due to dilation of the ER and mitochondria,[153] and it is possible that all dienone compounds induce this cell death mode.

b-AP15 induces various stress responses, including ER stress and oxidative stress.[98] Inhibition of these stress responses impairs apoptosis but does not prevent cell death.[98] It was recently demonstrated that VLX1570 induces dimerization of the apoptosis inhibitor anamorsin/CIAPIN1, leading to a depletion of the active monomeric form.[93] This finding raises the possibility that inactivation of anamorsin contributes to apoptosis induction by VLX1570. However, the findings that dienones are able to kill apoptosis-resistant cells, possibly by paroptosis, lead to questions with regard to the importance of anamorsin depletion for cell death by VLX1570 and other dienones.

CONCLUSIONS

Dienones such as b-AP15/VLX1570 have obvious chemical liabilities leading to predictions of engagement of multiple targets and of general toxicity. Dienones have indeed been demonstrated to engage a number of target proteins in cells. These different targets will not be equivalent in terms of the pharmacological response that will be elicited and will not be engaged at the same drug concentrations (Figure 7A,B).

Spliceosome, ribosome, and proteasome functions represent major vulnerabilities of tumor cells;[154] evidence reviewed here suggests that effects on the UPS can be linked to the cytotoxicity of dienones. The exact mechanism(s) by which dienones elicit these effects, by inhibition of proteasome-associated DUBs, by effects on Rpn13, and/or by additional components, is (are) not clear at present.

Despite the chemical liabilities of the class of compounds reviewed here, a substantial number of studies have shown that these compounds show preferential toxicity to tumor cells and show activity in *in vivo* tumor models. The ability of dienones to kill apoptosis-resistant cells and the limited development of resistance have stimulated considerable interest in these molecules. These compounds are unsuitable as, and should not be used as, biological probes. They could possibly be developed into anticancer drugs, however, if soluble prodrugs such as 2c-PE will be found to be safe and effective.

■ ASSOCIATED CONTENT

ⓈⒾ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00812.

Table 1 listing selective antiproliferative activity to tumor cells, as verified by other laboratories (PDF)

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Notes

The authors declare no competing financial interest.

Biographies

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■ ABBREVIATIONS USED

AKT, protein kinase B; ATM, ataxia telangiectasia mutated; CETSA, cellular thermal shift assay; CFTR, cystic fibrosis transmembrane conductance regulator; CIAPIN1, cytokine-induced inhibitor of apoptosis; CMap, connectivity map; DBA, dibenzylidene-acetone; DDIT3/CHOP, DNA damage-inducible transcript 3/C/EBP homologous protein; DUB, deubiquitinase; DTT, dithiotreitol; ERK, extracellular signal-regulated; HMOX1, heme oxygenase 1; HNE, 4-hydroxy-2-nonenal; HPV, human papilloma virus; Hsp, heat shock protein; hTERT, human telomerase reverse transcriptase; HUVEC, human vascular endothelial cell; JNK, c-Jun N-terminal kinase; mRNA, messenger RNA; LYN, Src family tyrosine kinase LYN; MALDI, matrix-assisted laser desorption/ionization; MAP kinase, mitogen activated protein kinase; NF-κB, nuclear factor κB; PAINS, pan-assay interference compounds; PARP, poly ADP-ribose polymerase; PG-J2, prostaglandin-J2; Rpn, regulatory particle of non-ATPase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sec61, human secretory translocase; Src, sarcoma oncogene; SRC, steroid receptor coactivator; STAT, signal transducers and activators of transcription; TNF, tumor necrosis factor; TrxR, thioredoxin reductase; UPR, unfolded protein response; USP, ubiquitin specific peptidase; UCH, ubiquitin C-terminal hydrolase; UPS, ubiquitin proteasome system; YFP, yellow fluorescent protein

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