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Tuning isoform selectivity and bortezomib sensitivity with a new class of alkenyl indene PDI inhibitor

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Abstract

Protein disulfide isomerase (PDI, PDIA1) is an emerging therapeutic target in oncology. PDI inhibitors have demonstrated a unique propensity to selectively induce apoptosis in cancer cells and overcome resistance to existing therapies, although drug candidates have not yet progressed to the stage of clinical development. We recently reported the discovery of lead indene compound E64FC26 as a potent pan-PDI inhibitor that enhances the cytotoxic effects of proteasome inhibitors in panels of Multiple Myeloma (MM) cells and MM mouse models. An extensive medicinal chemistry program has led to the generation of a diverse library of indene-containing molecules with varying degrees of proteasome inhibitor potentiating activity. These compounds were generated by a novel nucleophilic aromatic ring cyclization and dehydration reaction from the precursor ketones. The results provide detailed structure activity relationships (SAR) around this indene pharmacophore and show a high degree of correlation between potency of PDI inhibition and bortezomib (Btz) potentiation in MM cells. Inhibition of PDI leads to ER and oxidative stress characterized by the accumulation of misfolded poly-ubiquitinated proteins and the induction of UPR biomarkers ATF4, CHOP, and Nrf2. This work characterizes the synthesis and SAR of a new chemical class and further validates PDI as a therapeutic target in MM as a single agent and in combination with proteasome inhibitors.

Graphical Abstract

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Declaration of competing interest

R.M.R. and N.G.D. are inventors on patents. N.G.D. has an ownership interest in Leukogene Therapeutics, Inc. The other authors declare that they have no conflict of interest.

Supplementary Data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.xxx.xxx>.

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Keywords

Protein disulfide isomerase (PDI); Multiple Myeloma; Proteasome Inhibitor; Drug Resistance; ER Stress; UPR

1. Introduction

Protein disulfide isomerase (PDI) is a resident protein of the endoplasmic reticulum (ER) that catalyzes the formation and isomerization of disulfide bonds in nascent polypeptides [1, 2]. This process is critical for proper protein folding and the maintenance of general protein homeostasis in cells [3]. Malignant cells are exquisitely sensitive to disruptions in protein homeostasis due to their heightened proliferative and metabolic demands, thus making PDI an attractive therapeutic target in oncology [4, 5]. In support of this hypothesis, studies have shown that PDI is overexpressed in a wide range of cancer types and imparts resistance to cancer therapy [6–8]. Experimental PDI inhibitors have been reported in the literature demonstrating preclinical anti-cancer activity [9–14]. However, despite the discovery of these molecules and the clear rationale for targeting PDI in oncology, there are currently no approved or developmental PDI inhibitors in cancer clinical trials. This is the product of several challenges in PDI inhibitor programs that have limited the discovery of suitable clinical candidates. These include limitations in the *in vitro* PDI activity assays used for high throughput screening [15], challenges in the druggability of the PDI enzyme [16], which includes multiple reactive cysteines that rely on redox chemistry to oxidize and reduce protein thiols [17], and a lack of clarity on precisely which PDI isoforms, in a family of >20 members, are most salient for the treatment of cancer [18].

A recent report by our group characterized a novel structural class of alkenyl indene PDI inhibitor (lead E64FC26) with nanomolar potency, favorable ADME-PK, and anti-cancer activity *in vitro* and *in vivo* [19]. A further differentiating characteristic of this indene structural class was its pan-style of inhibition, showing high potency against multiple PDI isoforms, including PDIA1 (PDI, P4HB), PDIA3 (ERp57), PDIA4 (ERp72), PDIA6 (ERp5), and TXNDC5 (ERp46). Multiple myeloma (MM), a plasma cell malignancy, was the disease

focus of this previous work. PDI inhibition by E64FC26 induced apoptosis in panels of genetically diverse MM cells as a single agent, and, notably, synergistically enhanced the activity of FDA-approved proteasome inhibitors (bortezomib/VELCADE®, carfilzomib/KYPROLIS®, and ixazomib/NINLARO®) in sensitive as well as resistant MM cells. E64FC26 induced a cascade of signaling events in MM cells including robust ER stress and oxidative stress responses. This was characterized by the accumulation of misfolded poly-ubiquitinated proteins and the induction of canonical ER stress and oxidative stress pathway effectors like ATF4, CHOP and Nrf2. Proteotoxic stress is exacerbated in MM given the high rate of immunoglobulin production and protein load in MM plasma cells. However, PDI is a promising drug target in a variety of malignancies, including ovarian cancer and glioblastoma [10, 13, 14], as well as in other disease states such as neurodegeneration and thrombosis [20–27].

In the current study we explored structure activity relationships (SAR) of various derivatives of the original E64FC26 indene scaffold. Herein, we describe novel aspects of the synthetic approaches used to generate these derivatives as well as their activity in biochemical PDI assays *in vitro* and in MM cellular models utilizing synergy with the proteasome inhibitor bortezomib (Btz) as a phenotypic screening tool. This enabled the development of a detailed SAR and the discovery of analogs with increased selectivity for PDI over other isoforms in the family, thus positioning this new structural class for development in MM and other cancer indications.

2. Results

2.1. Chemistry

Compound **1** was condensed with the corresponding ketone and LDA to give ketone **2** (Fig. 1). Upon treatment with pTsOH, a ring cyclized alkenyl indene was obtained (**3** and **4**) instead of the expected acyclic enone. Dihydroxy-containing compounds were obtained by treatment of dimethoxy-containing precursors at -78°C (Schemes S1–S3). Detailed synthesis for each compound is given in Schemes S1–S7 as general condensation to form the indene moiety is covered in Fig. 1. The conversion of **2** to **3** and **4** under acidic conditions is facilitated by the highly electron rich aromatic ring and the presence of the electron-withdrawing substituent (CF_3 , amide, carboxylic acid, or ester) on the β -carbon (more detailed chemistry given in Supplemental Information). This nucleophilic aromatic ring cyclization reaction is relatively uncharacterized, with previous work showing the acid-catalyzed cyclization of arylacetates and a β - CF_3 , β -phenyl enone [28, 29]. The alkenyl indene functionality is found in the drug sulindac, which is marketed as an NSAID, is also being investigated for its potential use for the treatment of cancer due to the inhibition of Ras activation caused by the major metabolite sulindac sulfide and also binding to retinoid X receptor- α which leads to activation of the death receptor-mediated apoptotic pathway [30, 31]. Derivatives of sulindac, sulindac benzylamine and sulindac sulfide amide are also being investigated for their use in lung, colon, and breast cancers through inhibition of cyclic guanosine 3',5'-monophosphate phosphodiesterase and suppression of Akt/mTOR and TGF β /miR-21 signaling, respectively [32–34]. Using this synthetic method, a diverse variety

of analogs with varying substitutions on the aryl ring of the indene, the β -carbon, and varying pendant substitutions on the alkenyl substituent were prepared.

After condensation of the acyclic ketone **2** by pTsOH, the resulting mixture of stereoisomers generated by protonation, cyclization, and dehydration were separated by normal phase chromatography. The geometric *E/Z* stereochemistry of the major and minor products was determined by the synthesis of deuterated substrates in which assignments could be made in an unambiguous manner. This resulted in the nuclear Overhauser effect (n.O.e) not being observed due to the proximity of adjacent hydrogens where protons were replaced with deuterium. 1,1,1,3,3-Pentadeuterio-2-heptanone was condensed with **1** to give **5** and **6** (Fig. 2). The n.O.e. was blocked with **5** between the alkenyl deuterium and the proton on the aryl ring (Supplemental NMR Spectra). This allowed correlation with the alkenyl proton that was associated with the geometric isomer in the undeuterated form. Therefore, the alkenyl proton of **5** without deuterium labeling corresponded to a chemical shift of 6.8 ppm and the alkenyl proton in **6** without deuterium labeling had a shift of 6.5 ppm, so the *E* isomer alkenyl proton was upfield of the *Z* isomer alkenyl proton. Using this relative positioning of the alkenyl protons, we were able to assign relative stereochemistry after crystallization in homologous analogs in the series. Using these synthesis techniques and characterization methods, a diverse series of alkenyl indenenes were synthesized (Fig. 3, Supplemental NMR Spectra, Tables S1–S2).

2.2 Btz Potentiation and PDI Inhibition

2PDI inhibition *in vitro* and Btz potentiation studies in Btz resistant (BzR) MM.1S cells were conducted for all analogs using methods and synergy analysis described in previous studies [19, 35, 36]. A summary of the SAR is shown in the scheme in Fig. 4. The trifluoromethyl-containing indenenes were the most potent PDI inhibitors *in vitro*, and we determined that this group was critical for synergistically enhancing the cytotoxicity of Btz (Figs. 5A and S1–S3). By comparison, carboxylic acid (**52**), ester (**54**), and amide (**62**) derivatives were weak PDI inhibitors and showed either additive effects with Btz or no effect. Substitution of the vicinal dihydroxy groups with dimethoxy (**49** and **50**) or methylenedioxy (**65**) groups led to the most Btz potentiation while monomethoxy (**61**) or ethylenedioxy (**67**) substitution abrogated PDI inhibition and Btz potentiation. While the vicinal oxygens appeared to be necessary for activity, they were not sufficient, as derivatives **52**, **54**, **62**, **67**, **82**, **83**, and **84** were all weak/inactive PDI inhibitors and potentiators of Btz despite the presence of these groups. Changes to length (**42** and **43**), cyclization (**79** and **80**), and stereochemistry of the alkane tail did not have a significant effect on compound activity indicating that the molecule is flexible to alkyl isomerization. Stereochemistry only had a significant effect on activity when a benzyloxy group was added to the terminus of the alkane tail. Interestingly, only the dimethoxy- containing (*Z*) stereoisomer (**81**) potentiated Btz while its stereoisomer (**84**) and methylene-containing compounds with identical tails (**82** and **83**) possessed no activity. This indicates that stereochemistry is only a main driver in activity when there are added functional groups that could have an enzyme specific interaction. Potentiation of Btz in MM cells by indene analogs tightly correlated with their ability to inhibit PDI activity in biochemical assays (Figs. 5B–D and S5–S6). In addition to using MM.1S BzR cells, a diverse panel of mantle cell lymphoma, acute myeloid leukemia,

pancreatic cancer, ovarian cancer, and glioblastoma all showed varying degrees of Btz potentiation by compound **26** (Fig. S4) suggesting this indene class of PDI inhibitor could potentially be used in different cancers in combination with Btz to bring out previously undetected clinical responses. There was a high degree of statistical significance between PDI inhibition *in vitro* and in cellular assays measuring Btz potentiation in MM (Fig. 5D). Potentiation of Btz in MM.1S BzR cells showed a bimodal correlation as only compounds with a calculated IC₅₀ value <2 μ M potentiated Btz in MM.1S BzR cells at 0.5 μ M while less active compounds against PDI (*i.e.*, IC₅₀ >2 μ M) showed no Btz potentiation in MM cells at this concentration (p=0.0009, N=10 and 8 respectively). This indicates that there is potentially an on-target threshold by PDI inhibitors that needs to be overcome in order to potentiate Btz. For instance, high micromolar inhibitors of PDI fail to potentiate the cytotoxic effects of Btz while mid-nanomolar inhibitors show a combination effect with the proteasome inhibitor. The indene-containing sulindac, by comparison, showed minimal effects on the activity of Btz (Fig. S7). At a concentration of 50 μ M, a 100-fold higher concentration than what was used to evaluate the other indenenes, sulindac increased sensitivity to Btz by only 1.6-fold (Fig. S7A). Compared to E64FC26, a very high concentration of sulindac was required to induce MM.1S BzR cell death (EC₅₀ >100 μ M; data not shown). Furthermore, sulindac was a weak PDI inhibitor *in vitro* (IC₅₀ > 50 μ M; Fig. S7B), thus demonstrating that the novel alkenyl indene PDI inhibitors that we describe here are distinct from existing indene-containing compounds both in structure and in anti-cancer mechanism of action. Taken together, these findings demonstrate that the potency of indene analogs in PDI biochemical assays closely mirrors the phenotypic synergy with Btz in cellular models, and these results establish clear SAR for this new class of alkenyl indene PDI inhibitor.

2.3. Induction of ER and Oxidative Stress Pathways as Biomarkers of Indene Activity

Previously, we showed that induction of ER stress and oxidative stress pathway effectors were biomarkers of response to E64FC26 treatment in MM cells [19]. Therefore, we evaluated the effect of synthesized indene analogs on induction of these stress pathways in MM.1S BzR cells. Specifically, we measured the accumulation of poly-ubiquitinated proteins, an indicator of misfolded proteins that have been flagged for degradation by the proteasome, and the induction of downstream ER and oxidative stress pathway effectors, including ATF4, CHOP, and Nrf2 for each analog (Fig. 6A). The ability of indene analogs to induce these stress markers strongly correlated with their abilities to potentiate Btz (defined as > 2-fold) in MM.1S BzR cells and inhibit PDI *in vitro* (Figs. 6B–I). The threshold for Btz potentiation of >2-fold was used as the cutoff in this study as the synthesized compounds here either sensitized cells above this threshold, or had no effect on Btz. For example, band densitometry analysis revealed that the most potent PDI inhibitors *in vitro* and Btz sensitizers in cells also induced the highest levels of ubiquitinated protein accumulation (Fig. 6B, F), ATF4 induction (Fig. 6C, G), CHOP induction (Fig. 6D, H), and Nrf2 stabilization in the nucleus (Figs. 6E, I and S8). GRP78 (BiP) levels were unaffected with treatment of any of the synthesized analogs while splicing of XBP1 was strongest with the Btz sensitizers (Fig. S9). These findings further support the SAR of alkenyl indene PDI inhibitors and demonstrate the complex downstream cellular stress response induced by this class of molecule.

2.4. Indene Analogs with Increased PDI Selectivity

Compound **26** (E64FC26) was previously characterized as a pan-inhibitor of the PDI family (PDIA1, PDIA3, PDIA4, TXNDC5, and PDIA6), although it exhibited the highest potency against PDI (PDIA1) [19]. We identified the catechol moiety of E64FC26 as a potential liability given that it is a documented pan assay interference (PAIN) group that can perform redox-cycling and metal chelation [37]. We were confident that this group was not responsible for false positivity in our biochemical assays, as there were multiple catechol-containing compounds that were inactive against PDI and failed to potentiate Btz. If this series of compounds were acting as PAINS, we would expect only minor differences between derivatives, which is not the case. There is a large range in activities between each compound with high statistical significance between PDI inhibition, Btz potentiation, and cellular signaling events. Furthermore, the high potency of analogs **49** and **65** demonstrated that we could modulate the catechol “head” of the indene pharmacophore with dimethoxy or methylenedioxy groups while maintaining its Btz potentiation phenotype and PDI inhibition. Protection of the catechol group in these compounds would also remove any non-specific PAIN effects, if present, and lead to negative hits. This is further evidence that the catechol-containing compounds produced do not fall into the category of PAINs. Interestingly, we found that protection of the catechol group shifted the selectivity of **49** and **65** towards PDI with no inhibition observed with thioredoxin and significantly higher IC₅₀ values in PDIA3, PDIA4, TXNDC5, and PDIA6 (Table 1; Fig. S10). It is also noteworthy that **26** (E64FC26) was originally tested against PDI isoforms at 25°C (*i.e.*, room temperature), which led to complete enzyme inhibition at the top end of the dose response curve [19]. By comparison, analogs **49** and **65**, when incubated at 25°C for 1 hour, only produced partial inhibition of PDI (Fig. S11). However, pre-incubation of **26**, **49**, and **65** at 37°C produced nearly identical dose response curves and IC₅₀ values for PDI (Table 1 and Fig. S10), with complete inhibition observed at low micromolar concentrations of each compound. These data shows that protection of the catechol moiety of **26** does not compromise on- target activity and leads to increased isoform selectivity for PDIA1. Compounds **26** and **65** also did not inhibit CYP3A4, an abundant cytochrome P450 in the ER, indicating the specificity towards PDI for these indene-containing analogs (Fig. S12). Furthermore, these findings suggest that, with regards to the major PDI family members present in the ER, high-affinity selective inhibition of PDI alone may be sufficient for re-sensitizing resistant MM cells to Btz.

3. Discussion

The indene chemical scaffold has been used in the development of pharmacological agents for the treatment of various diseases. Sulindac, a non-steroidal anti-inflammatory drug (NSAID) used to reduce pain and inflammation, is the only FDA approved indene-containing small molecule. In recent years, however, this drug and its derivatives have gained traction as potential candidates for the treatment of cancer, including prostate, colon, lung, and breast cancers [32, 33, 38–40]. Inhibition of COX-1 and COX-2 is the primary mechanism of sulindac-class molecules, although other mechanisms, including inhibition of the Ras oncoprotein, have been described and attributed to its anti-cancer activity [30, 38]. In this study we characterize the SAR of a new class of indene with a unique structure and mechanism that is distinct from sulindac and other indene-containing molecules. These

molecules, exemplified by lead E64FC26, are potent inhibitors of the PDI family of ER oxidoreductases and have the unique ability to sensitize MM cells to FDA-approved proteasome inhibitors like Btz and others [19]. We report here the synthetic approach used to generate these alkenyl indenenes through a novel nucleophilic aromatic ring cyclization reaction, and the ability to tune the selectivity of derivatives toward the PDIA1 (PDI) isoform.

Treatment for MM has improved dramatically over the past decade, although the disease remains incurable for most patients. The introduction of proteasome inhibitors like Btz revolutionized the clinical management of MM and has contributed to improvements in survival rates [41–43]. Proteasome inhibitors are now standards of care in the frontline and in relapsed/refractory MM, although most patients eventually develop resistance, rendering the drugs ineffective. With this clinical obstacle in mind, we engaged in a screening program to find Btz sensitizing compounds, which we reasoned could prolong the duration of treatment response in MM patients. This led to the discovery of E64FC26, which enhanced the activity of proteasome inhibitors in a diverse panel of resistant and naïve MM cells [19]. E64FC26 induced these anti-MM effects *in vitro* and *in vivo* via inhibition of PDI family members, leading to the accumulation of toxic misfolded proteins in cells. This triggers ER and oxidative stress pathway induction that is exacerbated by proteasome inhibition, and thus drives a synergistic apoptotic response in MM cells. In support of this connection between potency at the molecular target and the resulting signaling and cellular outcomes, we observe a strong correlation between PDI inhibition, Btz potentiation, and ER and oxidative stress pathway induction (Figs. 5 and 6) for the analogs we tested in this study. These findings demonstrate clear SAR and may be used to guide decision-making and selection of a clinical candidate to be evaluated in early stage trials in humans.

The catechol group of E64FC26 was one of the structural moieties we targeted for modification as this group can interact with proteins in non-drug like ways by covalently binding to cysteine residues after oxidation into the *o*-quinone form [44]. Our SAR clearly shows that the vicinal oxygens here are required for activity, but do not guarantee activity as compounds **52**, **54**, **62**, **67**, **82**, **83**, and **84** were all non-functional in our cellular and biochemical assays. This strongly indicates that inhibition by this series of compounds is due to a specific interaction between the indene-containing compounds and PDI. When we substituted dimethoxy (**49**) and methylene (**65**) groups, respectively, for the dihydroxy (**26**) motif we significantly reduced potency for PDI isoforms PDIA3, PDIA4, TXNDC5, and PDIA6 while maintaining the potency against PDI (Table 1 and Fig. S10). Furthermore, we completely eliminated activity against the non-PDI family member thioredoxin, demonstrating the ability to direct the selectivity of these alkenyl indenenes to PDI through modification of the catechol. Protection of the catechol removes two hydrogen bond donors from the molecule, thus reducing the possible number of interactions within the binding site of the protein target. This appears to influence binding to PDIA3, PDIA4, TXNDC5, PDIA6, and thioredoxin, but not PDI, the inhibition of which remains unchanged. However, modification of the catechol group to an ethylenedioxy group in **67** resulted in activity being abolished completely (Fig. 5). The ethylenedioxy group is very similar chemically to the dimethoxy group in **49** / **50** and is further indication that 3,4 vicinal oxygens on this indene

scaffold do not function as indiscriminate PDI inhibitors. Interestingly, we noted that while compounds **49** and **65** were essentially equivalent to **26** with regards to Btz potentiation in cell models and as PDI inhibitors *in vitro*, they only displayed partial inhibition of PDI when the compound:protein incubation temperature was reduced from 37°C to 25°C. By comparison, **26** exhibited full inhibition independent of temperature. This observation is of trivial translational/therapeutic significance, but may suggest a distinct mode of binding by these two analogs to PDI when compared to **26**. PDI is known to have conformational flexibility, an attribute that is necessary given the diverse set of client proteins for which it catalyzes disulfide bond formation [45]. We propose that a temperature-dependent conformational change may be required for optimal binding of **49** and **65**, which could explain why complete inhibition of the enzyme population is only achieved at 37°C. Co-crystallization studies are required to address this hypothesis and resolve the precise mode of drug:target binding, and these structural studies are currently in progress.

The majority of reported PDI inhibitors, including 16F16, PACMA 31, KSC-34, E61, and E64FC26, are covalent in nature [10, 19, 21, 46]. PDI contains 4 redox active cysteine residues in the active site that facilitate disulfide bond formation [17]. Electrophilic compounds can be used to take advantage of the nucleophilic nature of the active site cysteines in PDI, as it provides a natural site of attachment to the protein. Our SAR indicates that the trifluoro-containing molecules are not indiscriminate electrophiles of PDI as several compounds with that group at R₂ (**67**, **82**, **83**, and **84**) were ineffective as PDI inhibitors and stabilizers of Nrf2 (Fig. 6A). However, there are also reversible inhibitors such as bepristat 2a, rutin, LOC14, and estradiol that all bind to and inhibit PDI [47–51]. However, these inhibitors are much less potent than those that bind irreversibly, suggesting that covalent inhibition of PDI could result in the most potent inhibitors with the highest probability of success in the clinic [19]. In fact, the emergence of covalent inhibitors has been an increasing trend in oncology and drug discovery as a whole [52–54]. The inhibitors we developed in this series appear to be among the most potent PDI inhibitors available and possess the unique characteristic of potentiating proteasome inhibitor therapy in MM. The treatment landscape for MM has expanded in recent years with the approval of new immunomodulatory agents and monoclonal antibodies [55–60]. Moreover, with CAR-T therapy showing great promise in early clinical trials [61], there is optimism that the rate and durability of treatment responses in MM will continue to improve. Proteasome inhibitors like Btz will likely remain cornerstone agents in an increasingly complex treatment paradigm that incorporates multi-drug combinations in cocktail regimens. Our data support the exploration of adding alkenyl indene PDI inhibitors to such regimens to overcome or ward off resistance and enhance proteasome inhibitor efficacy. PDI inhibitors have potential in cancers other than MM, and our group is currently screening for additional cancer types that would benefit most from alkenyl indene PDI inhibitors as well as other classes of FDA-approved agents that are enhanced by this new class of small molecule.

4. Experimental Section

4.1. Chemistry

All reagents and solvents were purchased from commercial sources and used without additional purification. Normal phase column chromatography was performed using silica columns with a Teledyne ISCO CombiFlash chromatography system. Reverse phase chromatography was performed on a Gilson Preparative HPLC System. ^1H NMR spectra were recorded at 25°C on a Varian 300 MHz NMR located at the Fox Chase Chemical Diversity Center located in Doylestown, PA. Mass spectra were obtained from the Mass Spectrometry and Proteomics Facility at Notre Dame University. The purity of the compounds synthesized was determined to be >95% as determined by HPLC using a Waters Alliance HPLC model e2695.

4.4.1. General procedure for the synthesis of (E)-2-deutero-1-(1-deutero-pentylidene)-3- (trifluoromethyl)-1H-indene-5,6-diol (5)—To a solution of 2-heptanone-1,1,1,3,3,3-d₅ (238 mg, 2.0 mmol) in THF (5 mL) at –78°C, LDA (2 M solution in THF, 1.05 mL, 2.1 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4- dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (61 mg, 0.35 mmol), and heated at 125°C for 2 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes to give the title compound. Yield 99 mg, 0.315 mmol (31.5%). LC/MS: R_f = 6.72 min. Purity > 95%. ^1H NMR (300 MHz, CH_2Cl_2) δ 7.12–7.24 (m, 1H), 7.19 (s, 1H), 7.05 (d, J =1.17 Hz, 1H), 6.97 (s, 1H), 6.70–6.88 (m, 1H), 3.95 (d, J =3.51 Hz, 6H), 3.91 (d, J =1.76 Hz, 1H), 2.60 (d, J =6.44 Hz, 1H), 2.51–2.65 (m, 1H), 1.57 (d, J =7.62 Hz, 1H), 1.43 (d, J =7.03 Hz, 1H), 1.18–1.74 (m, 3H), 0.96 (t, J =7.32 Hz, 3H), 1.01 (br. s., 1H). ESI-MS(+): m/z 315.59 [$M + H$]⁺.

4.1.2. General procedure for the synthesis of (E)-1-nonylidene-3-(trifluoromethyl)-1H-indene-5,6-diol (26)—To a solution of undecan-2-one (306 mg, 1.8 mmol) in THF (8 mL) at –78°C, LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4- dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (48 mg, 0.25 mmol), and heated at 125°C for 1 hour. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–10% ethyl acetate/hexanes. The residue was then re-suspended in CH_2Cl_2 (5 mL) and mixed with BBr_3 (1 M solution in

CH₂Cl₂, 1.3 mL, 1.3 mmol) at –78°C. The mixture was stirred at –78°C for 1 hour. The mixture was then warmed to –20°C and stirred for 3 hours. Then, 20 mL of saturated NH₄Cl and 40 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 100 mg, 0.294 mmol (29.4%). LC/MS: R_f = 6.93 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*): δ 7.16 (s, *J*=3.71, 3.71 Hz, 1H), 7.03 (s, 1H), 6.97 (s, *J*=3.44 Hz, 1H), 6.65–6.73 (m, 1H), 5.37 (d, *J*=18.16 Hz, 2H), 2.54 (q, *J*=7.62 Hz, 2H), 1.45–1.63 (m, 2H), 1.20–1.41 (m, 10H), 0.84–0.96 (m, 3H). ESI- MS(+): *m/z* 341.1723 [M + H]⁺.

4.1.3. General procedure for the synthesis of (Z)-1-nonylidene-3-

(trifluoromethyl)-1H- indene-5,6-diol (40)—To a solution of undecan-2-one (306 mg, 1.8 mmol) in THF (8 mL) at –78°C, LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4- dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 3 hours. 40 mL of saturated NH₄Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (48 mg, 0.25 mmol), and heated at 125°C for 1 hour. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–10% ethyl acetate/hexanes. The residue was then re-suspended in CH₂Cl₂ (2 mL) and mixed with BBr₃ (1 M solution in CH₂Cl₂, 0.33 mL, 0.33 mmol) at –78°C. The mixture was stirred at –78°C for 1 hour. The mixture was then warmed to –20°C and stirred for 3 hours. Then, 10 mL of saturated NH₄Cl and 20 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 25 mg, 0.073 mmol (7.3%). LC/MS: R_f = 6.93 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*): δ 7.36 (br. s., 1H), 7.00 (br. s., 1H), 6.69 (br. s., 1H), 6.43–6.56 (m, 1H), 5.34–5.69 (m, 2H), 2.73 (q, *J*=7.03 Hz, 2H), 1.51–1.77 (m, 3H), 1.18–1.51 (m, 11H), 0.79–1.11 (m, 3H). ESI-MS(+): *m/z* 341.1723 [M + H]⁺.

4.1.4. General procedure for the synthesis of (E)-1-heptylidene-3-

(trifluoromethyl)-1H- indene-5,6-diol (42)—To a solution of nonan-2-one (255 mg, 1.8 mmol) in THF (8 mL) at –78°C, LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4- dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 3 hours. 40 mL of saturated NH₄Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (40 mg, 0.21 mmol), and heated at 100°C for 18 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes. The residue was then re-suspended in CH₂Cl₂ (3 mL) and mixed with BBr₃ (1 M solution in CH₂Cl₂, 0.6 mL, 0.6 mmol) at –78°C. The mixture was stirred at –78°C for 1 hour. The

mixture was then warmed to -20°C and stirred for 3 hours. Then, 10 mL of saturated NH_4Cl and 20 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 44 mg, 0.141 mmol (14.1%). LC/MS: $R_f = 6.29$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM-}d$): δ 7.28 (s, 1H), 7.17 (s, 2H), 7.04 (s, 1H), 6.98 (s, 2H), 6.69 (t, $J=7.91$ Hz, 1H), 5.52 (br. s., 3H), 2.56 (q, $J=7.62$ Hz, 3H), 1.43–1.67 (m, 4H), 1.22–1.43 (m, 10H), 0.76–1.07 (m, 5H). ESI-MS(+): m/z 313.1410 $[\text{M} + \text{H}]^+$.

4.1.5. General procedure for the synthesis of (Z)-1-heptylidene-3-

(trifluoromethyl)-1H-indene-5,6-diol (43)—To a solution of nonan-2-one (255 mg, 1.8 mmol) in THF (8 mL) at -78°C , LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4-dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (40 mg, 0.21 mmol), and heated at 100°C for 18 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes. The residue was then re-suspended in CH_2Cl_2 (1 mL) and mixed with BBr_3 (1 M solution in CH_2Cl_2 , 0.2 mL, 0.2 mmol) at -78°C . The mixture was stirred at -78°C for 1 hour. The mixture was then warmed to -20°C and stirred for 3 hours. Then, 10 mL of saturated NH_4Cl and 20 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 10 mg, 0.032 mmol (3.2%). LC/MS: $R_f = 6.38$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM-}d$): δ 7.35 (s, $J=3.36$ Hz, 1H), 7.27 (s, 1H), 6.98 (s, 1H), 6.68 (s, $J=3.60$, 3.60 Hz, 1H), 6.46 (t, $J=7.62$ Hz, 1H), 5.36 (d, $J=13.47$ Hz, 2H), 2.73 (q, $J=7.62$ Hz, 2H), 1.74 (br. s., 1H), 1.53–1.70 (m, 2H), 1.25–1.49 (m, 7H), 0.85–0.98 (m, 3H). ESI-MS(+): m/z 313.1410 $[\text{M} + \text{H}]^+$.

4.1.6. General procedure for the synthesis of (Z)-5,6-dimethoxy-1-

nonylidene-3-(trifluoromethyl)-1H-indene (49)—To a solution of undecan-2-one (306 mg, 1.8 mmol) in THF (8 mL) at -78°C , LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4-dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (48 mg, 0.25 mmol), and heated at 125°C for 1 hour. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–10% ethyl acetate/hexanes to give the title compound. Yield 35 mg, 0.095 mmol (9.5%). LC/MS: $R_f = 8.42$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM-}d$): δ 7.32 (s, $J=3.51$ Hz, 1H), 7.26 (s, $J=3.04$

Hz, 1H), 6.98 (s, $J=3.30$ Hz, 1H), 6.66–6.72 (m, 1H), 6.50 (s, 1H), 3.94 (d, $J=2.93$ Hz, 6H), 2.77 (d, $J=7.62$ Hz, 2H), 1.65 (d, $J=7.62$ Hz, 3H), 1.23–1.47 (m, 11H), 0.83–0.93 (m, 3H), 0.00 (s, 1H). m/z 391.1855 $[M + Na]^+$.

4.1.7. General procedure for the synthesis of (E)-5,6-dimethoxy-1-nonylidene-3- (trifluoromethyl)-1H-indene (50)—

To a solution of undecan-2-one (306 mg, 1.8 mmol) in THF (8 mL) at -78°C , LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4-dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (48 mg, 0.25 mmol), and heated at 125°C for 1 hour. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–10% ethyl acetate/hexanes to give the title compound. Yield 138 mg, 0.375 mmol (37.5%). LC/MS: $R_f = 8.27$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM-}d$) δ 7.26 (s, 1H), 7.17 (s, $J=3.63$, 3.63 Hz, 1H), 7.03 (br. s., 1H), 6.95 (br. s., 1H), 6.78 (br. s., 1H), 3.85–4.01 (m, 7H), 2.58 (d, $J=7.62$ Hz, 2H), 1.45–1.66 (m, 4H), 1.18–1.44 (m, 12H), 0.88 (br. s., 3H), 0.00 (s, 1H). m/z 391.1855 $[M + Na]^+$.

4.1.8. General procedure for the synthesis of 1-heptylidene-5,6-dihydroxy-1H-indene-3- carboxylic acid (52)—

To a solution of nonan-2-one (511 mg, 3.6 mmol) in THF (10 mL) at -78°C , LDA (2 M solution in THF, 2.0 mL, 4.0 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of ethyl 2-(3,4-dimethoxyphenyl)-2-oxoacetate (476 mg, 2.0 mmol) in THF (2 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 60 mL of saturated NH_4Cl was added and the mixture was extracted from 40 mL of diethyl ether twice. The ether layers were combined and washed with 40 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (12 mL), mixed with pTsOH (130 mg, 0.75 mmol), and heated at 125°C for 2 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes. The residue was then re-suspended in CH_2Cl_2 (2 mL) and mixed with BBr_3 (1 M solution in CH_2Cl_2 , 0.6 mL, 0.6 mmol) at -78°C . The mixture was stirred at -78°C for 1 hour. The mixture was then warmed to -20°C and stirred for 3 hours. Then, 3 mL of saturated NH_4Cl and 10 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 45 mg, 0.156 mmol (7.8%). LC/MS: $R_f = 6.07$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM-}d$) δ 7.55 (br. s., 1H), 7.35–7.50 (m, 1H), 7.16–7.33 (m, 2H), 6.73–6.94 (m, 1H), 5.17–5.33 (m, 1H), 2.71 (br. s., 1H), 2.36 (q, $J=7.42$ Hz, 2H), 1.43 (br. s., 3H), 1.26 (br. s., 6H), 0.84 (br. s., 3H). ESI-MS(+): m/z 289.1434 $[M + H]^+$.

4.1.9. General procedure for the synthesis of ethyl 1-heptylidene-5,6-dihydroxy-1H-indene-3-carboxylate (54)—To a solution of nonan-2-one (511 mg, 3.6 mmol) in THF (10 mL) at -78°C , LDA (2 M solution in THF, 2.0 mL, 4.0 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of ethyl 2-(3,4-dimethoxyphenyl)-2-oxoacetate (476 mg, 2.0 mmol) in THF (2 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 60 mL of saturated NH_4Cl was added and the mixture was extracted from 40 mL of diethyl ether twice. The ether layers were combined and washed with 40 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (12 mL), mixed with pTsOH (130 mg, 0.75 mmol), and heated at 125°C for 2 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes. The residue was then re-suspended in CH_2Cl_2 (1 mL) and mixed with BBr_3 (1 M solution in CH_2Cl_2 , 0.21 mL, 0.21 mmol) at -78°C . The mixture was stirred at -78°C for 1 hour. The mixture was then warmed to -20°C and stirred for 3 hours. Then, 3 mL of saturated NH_4Cl and 10 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 15 mg, 0.047 mmol (2.4%). LC/MS: $R_f = 6.01$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM}-d$) δ 7.41–7.67 (m, 2H), 7.29–7.34 (m, 1H), 7.26 (s, 6H), 7.14 (s, 1H), 6.63–6.81 (m, 1H), 6.47–6.57 (m, 1H), 5.57–5.67 (m, 1H), 5.22–5.36 (m, 1H), 4.37 (d, $J=7.03$ Hz, 2H), 2.47–2.83 (m, 2H), 1.56 (s, 7H), 1.40 (d, $J=7.03$ Hz, 16H), 0.81–1.00 (m, 5H). ESI-MS(+): m/z 317.1747 $[\text{M} + \text{H}]^+$.

4.1.10. General procedure for the synthesis of (E)-1-heptylidene-5-methoxy-3-(trifluoromethyl)-1H-indene (61)—To a solution of nonan-2-one (255 mg, 1.8 mmol) in THF (8 mL) at -78°C , LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3-methoxyphenyl)ethan-1-one (204 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (43 mg, 0.25 mmol), and heated at 100°C for 5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes to give the title compound. Yield 41 mg, 0.132 mmol (13.2%). LC/MS: $R_f = 7.78$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM}-d$) δ 7.52 (d, $J=8.79$ Hz, 1H), 7.18 (s, $J=3.54$ Hz, 1H), 7.00 (br. s., 1H), 6.84 (d, $J=8.20$ Hz, 2H), 3.84–3.88 (m, 3H), 2.58 (d, $J=7.62$ Hz, 2H), 1.50–1.63 (m, 2H), 1.26–1.44 (m, 6H), 0.84–0.97 (m, 3H). ESI-MS(+): m/z 311.1617 $[\text{M} + \text{H}]^+$.

4.1.11. General procedure for the synthesis of 1-heptylidene-5,6-dihydroxy-N-methyl-1H-indene-3-carboxamide (62)—To a solution of nonan-2-one (511 mg, 3.6 mmol) in THF (10 mL) at -78°C , LDA (2 M solution in THF, 2.0 mL, 4.0 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of ethyl 2-(3,4-dimethoxyphenyl)-2-oxoacetate (476 mg, 2.0 mmol) in THF (2 mL) was added

slowly. The solution was stirred at -78°C for 3 hours. 60 mL of saturated NH_4Cl was added and the mixture was extracted from 40 mL of diethyl ether twice. The ether layers were combined and washed with 40 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (12 mL), mixed with pTsOH (130 mg, 0.75 mmol), and heated at 125°C for 2 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–20% ethyl acetate/hexanes. The residue was re-suspended in THF (1.5 mL) and mixed with MeNH_2 (2 M solution, 0.4 mL, 0.8 mmol) and HATU (90 mg, 0.24 mmol). The solution was stirred at 50°C for 24 hours. The residue was then re-suspended in CH_2Cl_2 (1 mL) and mixed with BBr_3 (1 M solution in CH_2Cl_2 , 0.15 mL, 0.15 mmol) at -78°C . The mixture was stirred at -78°C for 1 hour. The mixture was then warmed to -20°C and stirred for 3 hours. Then, 3 mL of saturated NH_4Cl and 10 mL of ether were added. The organic layer was separated, dried, and concentrated. The residue was purified by normal phase chromatography to give the title compound. Yield 4.9 mg, 0.016 mmol (0.8%). LC/MS: $R_f = 5.43$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM}-d$) δ 8.19 (d, $J=2.31$ Hz, 1H), 7.27 (s, $J=4.18$ Hz, 1H), 7.15 (s, $J=7.91$ Hz, 1H), 6.91 (d, $J=8.14$ Hz, 1H), 3.22 (s, 3H), 2.38 (s, 2H), 1.23–1.55 (m, 9H), 0.90 (s, 3H). ESI-MS(+): m/z 302.1751 $[\text{M} + \text{H}]^+$.

4.1.12. General procedure for the synthesis of (E)-5-heptylidene-7-

(trifluoromethyl)-5H- indeno[5,6-d][1,3]dioxole (65)—To a solution of nonan-2-one (983 mg, 6.9 mmol) in THF (50 mL) at -78°C , LDA (2 M solution in THF, 4.0 mL, 8.0 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 1-(benzo[d][1,3]dioxol-6-yl)-2,2,2- trifluoroethan-1-one (1.0 g, 4.6 mmol) in THF (5 mL) was added slowly. The solution was stirred at -78°C for 4 hours. 100 mL of saturated NH_4Cl was added and the mixture was extracted from 100 mL of diethyl ether twice. The ether layers were combined and washed with 50 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (25 mL), mixed with pTsOH (1.62 g, 4.5 mmol), and heated at 125°C for 1.5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica with 0–10% ethyl acetate/hexanes. Then, further purified by C18 with 75–100% $\text{MeCN}/\text{H}_2\text{O}$ to give the title compound. Yield 288 mg, 0.889 mmol (19.3%). LC/MS: $R_f = 7.94$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM}-d$) δ 7.09 (s, $J=9.09$, 9.09 Hz, 1H), 5.99 (s, 1H), 2.57 (d, $J=7.62$ Hz, 1H), 1.55 (s, 1H), 1.20–1.44 (m, 4H), 0.90 (s, 2H). ESI-MS(+): m/z 325.1410 $[\text{M} + \text{H}]^+$.

4.1.13. General procedure for the synthesis of (E)-6-heptylidene-8-

(trifluoromethyl)-2,3- dihydro-6H-indeno[5,6-b][1,4]dioxine (67)—To a solution of nonan-2-one (255 mg, 1.8 mmol) in THF (8 mL) at -78°C , LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 1-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-2,2,2- trifluoroethan-1-one (232 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over

Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes. The residue was re-suspended in toluene (4 mL), mixed with pTsOH (46 mg, 0.25 mmol), and heated at 100°C for 3 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to give the title compound. Yield 14 mg, 0.041 mmol (4.1%). LC/MS: R_f = 7.86 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*) δ 7.09 (s, *J*=9.09, 9.09 Hz, 1H), 5.99 (s, 1H), 2.57 (d, *J*=7.62 Hz, 1H), 1.55 (s, 1H), 1.20–1.44 (m, 4H), 0.90 (s, 2H). ESI-MS(+): *m/z* 325.1410 [M + H]⁺.

4.1.14. General procedure for the synthesis of (E)-5-(2-cyclopentylethylidene)-7- (trifluoromethyl)-5H-indeno[5,6-*d*][1,3]dioxole (79)—

To a solution of 4-cyclopentylbutan-2-one (252 mg, 1.8 mmol) in THF (8 mL) at –78°C, LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 1-(benzo[*d*][1,3]dioxol-6-yl)- 2,2,2-trifluoroethan-1-one (218 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 4 hours. 40 mL of saturated NH₄Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (3 mL), mixed with pTsOH (42 mg, 0.25 mmol), and heated at 90°C for 5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to give the title compound. Yield 15.2 mg, 0.047 mmol (4.7%). LC/MS: R_f = 7.60 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*) δ 6.98–7.13 (m, 2H), 6.91 (s, 1H), 6.76 (s, 1H), 5.98 (s, 2H) 2.57 (t, *J*=7.62 Hz, 2H), 1.79 (br. s., 4H), 1.65 (br. s., 2H), 1.58 (br. s., 3H). ESI-MS(+): *m/z* 323.1253 [M + H]⁺.

4.1.15. General procedure for the synthesis of (E)-5-(2-cyclohexylethylidene)-7- (trifluoromethyl)-5H-indeno[5,6-*d*][1,3]dioxole (80)—

To a solution of 4-cyclohexylbutan-2-one (278 mg, 1.8 mmol) in THF (8 mL) at –78°C, LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 1-(benzo[*d*][1,3]dioxol-6-yl)- 2,2,2-trifluoroethan-1-one (218 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 4 hours. 40 mL of saturated NH₄Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (5 mL), mixed with pTsOH (50 mg, 0.3 mmol), and heated at 90°C for 7 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to give the title compound. Yield 15 mg, 0.045 mmol (4.5%). LC/MS: R_f = 7.26 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*) δ 7.26 (s, 2H), 7.09 (s, 1H), 7.00–7.03 (m, 1H), 6.91 (s, *J*=2.52 Hz, 1H), 6.77 (t, *J*=8.15 Hz, 1H), 5.98 (s, 2H), 2.46 (t, *J*=7.62 Hz, 2H) 1.62–1.79 (m, 6H), 1.54 (s, 3H), 1.21 (d, *J*=9.37 Hz, 4H), 1.02 (br. s., 2H). ESI- MS(+): *m/z* 337.1410 [M + H]⁺.

4.1.16. General procedure for the synthesis of (Z)-1-(7-

(benzyloxy)heptylidene)-5,6- dimethoxy-3-(trifluoromethyl)-1H-indene (81)—To a solution of 9-hydroxynonan-2-one (791 mg, 5 mmol) benzyl bromide (1.11 g, 6.5 mmol) was added in 50% aqueous sodium hydroxide (4 mL) and benzene (4 mL) in the presence of tetrabutylammonium hydrogen sulfate (353 mg, 1 mmol) and allowed to stir at room temperature for 5 hours. The mixture was neutralized with 5 N HCl, extracted with ethyl acetate, dried, and concentrated. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes to yield 9-(benzyloxy)nonan-2-one. The 9-(benzyloxy)nonan-2-one was resuspended in THF (8 mL) at –78°C, and LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4-dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 3 hours. 40 mL of saturated NH₄Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (3 mL), mixed with pTsOH (28 mg, 0.16 mmol), and heated at 90°C for 5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to give the title compound. Yield 10 mg, 0.022 mmol (2.2%). LC/MS: R_f = 7.60 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*) δ 7.25–7.37 (m, 7H), 6.97 (s, *J*=2.55 Hz, 1H), 6.68 (t, *J*=3.08 Hz, 1H), 6.48 (t, *J*=7.50 Hz, 1H), 4.49 (s, 2H), 3.87–3.99 (m, 7H), 3.47 (t, *J*=6.44 Hz, 2H), 2.76 (q, *J*=7.23 Hz, 2H), 2.15 (d, *J*=12.89 Hz, 1H), 1.55–1.70 (m, 5H), 1.46 (td, *J*=3.51, 7.03 Hz, 4H), 1.25 (s, 1H), 0.00 (s, 1H). ESI-MS(+): *m/z* 447.73 [M + H]⁺.

4.1.17. General procedure for the synthesis of (Z)-5-(7-

(benzyloxy)heptylidene)-7- (trifluoromethyl)-5H-indeno[5,6-*d*][1,3]dioxole (82)

—To a solution of 9-hydroxynonan-2-one (791 mg, 5 mmol) benzyl bromide (1.11 g, 6.5 mmol) was added in 50% aqueous sodium hydroxide (4 mL) and benzene (4 mL) in the presence of tetrabutylammonium hydrogen sulfate (353 mg, 1 mmol) and allowed to stir at room temperature for 5 hours. The mixture was neutralized with 5 N HCl, extracted with ethyl acetate, dried, and concentrated. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes to yield 9-(benzyloxy)nonan-2-one. The 9-(benzyloxy)nonan-2-one was resuspended in THF (8 mL) at –78°C, and LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at –78°C for 45 minutes. Next, a solution of 1-(benzo[*d*][1,3]dioxol-6-yl)-2,2,2-trifluoroethan-1-one (218 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at –78°C for 4 hours. 40 mL of saturated NH₄Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na₂SO₄ and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (3 mL), mixed with pTsOH (28 mg, 0.16 mmol), and heated at 90°C for 5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to give the title compound. Yield 1.5 mg, 0.0035 mmol (0.35%). LC/MS: R_f = 8.07 min. Purity > 95%. ¹H NMR (300 MHz, CHLOROFORM-*d*) δ 7.23–7.40 (m, 6H), 6.98–7.12 (m, 1H), 5.96–6.02 (m, 2H), 4.49 (s, 2H), 3.38–3.54 (m, 2H),

2.56 (d, $J=7.62$ Hz, 2H), 1.52–1.85 (m, 6H), 1.33–1.52 (m, 4H), 1.25 (s, 2H). ESI-MS(+): m/z 431.1829 [M + H]⁺.

4.1.18. General procedure for the synthesis of (E)-5-(7-(benzyloxy)heptylidene)-7- (trifluoromethyl)-5H-indeno[5,6-d][1,3]dioxole (83)

—To a solution of 9-hydroxynonan-2-one (791 mg, 5 mmol) benzyl bromide (1.11 g, 6.5 mmol) was added in 50% aqueous sodium hydroxide (4 mL) and benzene (4 mL) in the presence of tetrabutylammonium hydrogen sulfate (353 mg, 1 mmol) and allowed to stir at room temperature for 5 hours. The mixture was neutralized with 5 N HCl, extracted with ethyl acetate, dried, and concentrated. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes to yield 9-(benzyloxy)nonan-2-one. The 9-(benzyloxy)nonan-2-one was resuspended in THF (8 mL) at -78°C , and LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 1-(benzo[d][1,3]dioxol-6-yl)-2,2,2-trifluoroethan-1-one (218 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 4 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (3 mL), mixed with pTsOH (28 mg, 0.16 mmol), and heated at 90°C for 5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to give the title compound. Yield 10 mg, 0.023 mmol (2.3%). LC/MS: $R_f = 7.78$ min. Purity > 95%. ^1H NMR (300 MHz, $\text{CHLOROFORM-}d$) δ 7.29–7.38 (m, 6H), 7.25 (s, 2H), 7.00–7.11 (m, 3H), 6.89–6.94 (m, 1H) 6.73 (t, $J=8.13$ Hz, 1H), 5.98 (s, 3H), 4.50 (s, 3H), 3.46 (t, $J=6.44$ Hz, 3H), 2.56 (d, $J=7.03$ Hz, 3H), 1.49–1.68 (m, 6H), 1.33–1.48 (m, 6H), 1.25 (s, 2H), 0.00 (s, 1H). ESI- MS(+): m/z 453.1648 [M + Na]⁺.

4.1.19. General procedure for the synthesis of (E)-1-(7-(benzyloxy)heptylidene)-5,6- dimethoxy-3-(trifluoromethyl)-1H-indene (84)—To a solution of 9-hydroxynonan-2-one (791 mg, 5 mmol) benzyl bromide (1.11 g, 6.5 mmol) was added in 50% aqueous sodium hydroxide (4 mL) and benzene (4 mL) in the presence of tetrabutylammonium hydrogen sulfate (353 mg, 1 mmol) and allowed to stir at room temperature for 5 hours. The mixture was neutralized with 5 N HCl, extracted with ethyl acetate, dried, and concentrated. The residue was purified by silica chromatography with 0–20% ethyl acetate/hexanes to yield 9-(benzyloxy)nonan-2-one. The 9-(benzyloxy)nonan-2-one was resuspended in THF (8 mL) at -78°C , and LDA (2 M solution in THF, 1.1 mL, 2.2 mmol) was added slowly. The mixture was stirred at -78°C for 45 minutes. Next, a solution of 2,2,2-trifluoro-1-(3,4-dimethoxyphenyl)ethanone (234 mg, 1.0 mmol) in THF (0.5 mL) was added slowly. The solution was stirred at -78°C for 3 hours. 40 mL of saturated NH_4Cl was added and the mixture was extracted from 20 mL of diethyl ether twice. The ether layers were combined and washed with 20 mL of brine, dried over Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by silica chromatography with 0–10% ethyl acetate/hexanes. The residue was re-suspended in toluene (3 mL), mixed with pTsOH (28 mg, 0.16 mmol), and heated at 90°C for 5 hours. After completion, the mixture was concentrated by rotary evaporation and purified by silica then, further purified by C18 to

give the title compound. Yield 41 mg, 0.092 mmol (9.2%). LC/MS: R_f = 7.68 min. Purity > 95%. ^1H NMR (300 MHz, CHCl_3) δ 7.29–7.39 (m, 5H), 7.25 (s, J =3.86, 3.86 Hz, 2H), 7.16 (s, 1H), 6.95 (s, 2H), 4.50 (s, 2H), 3.93 (d, J =2.93 Hz 7H), 3.46 (t, J =6.44 Hz, 2H), 2.57 (d, J =7.03 Hz, 2H), 1.56 (s, 6H), 1.22–1.49 (m, 6H), –0.01 (s, 1H). ESI-MS(+): m/z 447.2142 $[\text{M} + \text{H}]^+$.

4.2 Cell Lines and Reagents

MM.1S BzR cells were a generous gift from Dr. Brian Van Ness at the University of Minnesota. They were selected for Btz resistance and cultured as previously described [62]. T-cells were derived from human peripheral blood mononuclear cells by activating with CD3/CD28/CD2 trivalent antibody (STEMCELL Technologies) according to the manufacturer's specifications and 10 ng/mL hIL-2. T-cells were allowed to expand for 2 weeks and were cultured in ImmunoCult™-XF T Cell Expansion Medium (STEMCELL Technologies).

4.3 Cell Viability Assay

Cell viability was measured as previously described using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega) on a SpectraMax L Microplate Reader (Molecular Devices) [19].

4.4 Protein Expression and Purification

2All PDIs were expressed in *E. coli* BL21(DE3) in pET15TEV_NESG and purified by immobilized metal affinity chromatography as previously described [19].

4.5 Insulin Reduction Assay

Enzymatic activity for the PDI family members were measured by the insulin reduction assay as previously described [19]. All compounds were incubated with the designated PDI for 1 hour at 25°C or 37°C (as specified).

4.6 CYP3A4 Inhibition Assay

CYP3A4 inhibition was measured using midazolam in 0.2 mg/mL of mix-gender, pooled human liver microsomes in 100 mM potassium phosphate pH 7.0 and 2 mM NADPH. Reactions were performed with varying **26**, **65**, or 100 μM ketoconazole as a positive control at 37°C for 30 minutes. Reactions were quenched with two volumes of acetonitrile, centrifuged to remove precipitated protein, and analyzed by LC-MS at each of the test compound concentrations. A decrease in the formation of hydroxylated midazolam compared to vehicle control was used to calculate percent inhibition at each concentration of compound.

4.7 Western Blotting

Western Blots were generated as previously described [19]. Densitometry analysis was performed in ImageJ. Band densities for each biomarker were normalized to actin densities from the corresponding samples. Then, those ratios were normalized to the signal compound **26** produced in each of the blots, as it was used for a comparison between membranes as not

all samples could be fit on one blot. Thus, all the signals for each of the analyses for compound **26** are set to an arbitrary value of 1.

4.8 Statistical Analysis

Welch's t-tests were performed in GraphPad Prism (v. 7). Compounds were grouped by their Btz potentiation characteristics using a threshold of 2-fold. Compounds **26**, **40**, **42**, **43**, **49**, **50**, **65**, **79**, **80**, and **81** (N=10) were in the Btz potentiation group and compounds **52**, **54**, **61**, **62**, **67**, **82**, **83**, and **84** (N=8) were in the Btz non-potentiation group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADME-PK	absorption distribution metabolism and excretion-pharmacokinetics
Akt	protein kinase B
ATF4	activating transcription factor 4
Btz	bortezomib
BzR	bortezomib resistant
CAR-T	chimeric antigen receptor T cells
CF₃	trifluoromethyl
CHOP	C/EBP homologous protein
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CYP3A4	cytochrome P450 3A4
EC₅₀	half maximal effective concentration
ER	endoplasmic reticulum
FDA	United States Food and Drug Administration

HPLC	high-performance liquid chromatography
IC₅₀	half maximal inhibitory concentration
LDA	lithium diisopropylamide
miR-21	microRNA 21
MM	multiple myeloma
mTOR	mammalian target of rapamycin
nOe	nuclear Overhauser effect
NMR	nuclear magnetic resonance
Nrf2	nuclear factor-like 2
NSAID	nonsteroidal anti-inflammatory drug
PAIN	pan-assay interference compound
PDI	protein disulfide isomerase
PDIA1	protein disulfide isomerase A1
PDIA3	protein disulfide isomerase A3
PDIA4	protein disulfide isomerase A4
PDIA6	protein disulfide isomerase A6
PI	proteasome inhibitor
pTsOH	para-toluenesulfonic acid
TGFβ	transforming growth factor-beta
TXNDC5	thioredoxin domain containing 5
UPR	unfolded protein response

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Highlights

- Indene PDI inhibitors are a new class of therapeutic for the treatment of cancer
- Indene derivatives exhibit structure activity relationships in PDI activity assays
- Indene SAR in vitro correlates with bortezomib sensitization in myeloma cell models
- ER stress pathway biomarkers are key indicators of indene activity in myeloma cells
- PDI isoform selectivity is tunable with modification to the indene scaffold

Fig. 1.
General synthesis of compounds containing the indene pharmacophore.

Fig. 2.
Deuterium-labeling of indene compounds to assign conformation.

Fig. 3.
Molecular structures of synthesized indene-containing compounds.

Fig. 4.

Summary of indene-containing SAR. Modification of the catechol group at R₁ to methylene dioxy or dimethoxy groups leads to an increase in PDIA1 selectivity. Activity of the compounds correlates to electrophilicity of the R₂ group. The tail at position R₃ is flexible to modification as well as stereochemical orientation.

Fig. 5.

Btz potentiation in MM.1S BzR cells by indene-containing analogs of **26** strongly correlates with their ability to inhibit PDI. A) MM.1S BzR cells were treated for 24 hours with 0 – 200 nM Btz (+/-) 0.5 μM of the indicated molecule. Cell death induced by each molecule was accounted for by normalizing viability to the absence of Btz. Potentiation is defined as the fold decrease in EC₅₀ value for Btz in the presence of a compound compared to DMSO. Thus, a compound with a potentiation value of 1 has no effect on the efficacy of Btz and compounds with values higher than 1 are Btz potentiators. B) PDI IC₅₀ values of each of the synthesized compounds. C) PDI IC₅₀ values vs. their Btz potentiation effects in MM.1S BzR cells. D) Welch's t-test of PDI IC₅₀ values in C between compounds with Btz potentiation values >2 (black) and compounds with Btz potentiation values <2 (white).

Fig. 6.

Indene-containing compounds that potentiate Btz induce ER and oxidative stress in MM.1S BzR cells. Cells were treated with 0.5 μM of each compound for 8 hours. A) Western blots of poly-ubiquitinated protein accumulation, ATF4 and CHOP induction, and Nrf2 stabilization. B) PDI IC_{50} values vs. densitometry analysis of poly-ubiquitinated protein accumulation. C) PDI IC_{50} values vs. densitometry analysis of ATF4 induction. D) PDI IC_{50} values vs. densitometry analysis of CHOP induction. E) PDI IC_{50} values vs. densitometry analysis of Nrf2 stabilization. F) Welch's t-test of accumulation of poly-ubiquitinated proteins densitometry in B between compounds with Btz potentiation values >2 (black bar) and compounds with Btz potentiation values <2 (white bar). G) Welch's t-test of ATF4 induction densitometry in C between compounds with Btz potentiation values >2 (black) and compounds with Btz potentiation values <2 (white). H) Welch's t-test of CHOP induction densitometry in D between compounds with Btz potentiation values >2 (black) and compounds with Btz potentiation values <2 (white). I) Welch's t-test of Nrf2 stabilization densitometry in E between compounds with Btz potentiation values >2 (black) and compounds with Btz potentiation values <2 (white).

Table 1.

IC₅₀ values of **26**, **49**, and **65** in PDI family members. Compounds were pre- incubated with the specified protein for 1 hour at 37°C prior to testing enzymatic activity. All values are reported in μ M.

Compound	PDI	PDIA3	PDIA4	TXNDC5	PDIA6	Thioredoxin
26	0.60 $\pm$ 0.04	4.8 $\pm$ 0.4	33.1 $\pm$ 9.2	12.1 $\pm$ 1.6	5.7 $\pm$ 0.3	30.3 $\pm$ 1.5
49	1.12 $\pm$ 0.05	20.8 $\pm$ 5.5	61.6 $\pm$ 9.5	>100	16.3 $\pm$ 2.2	N.I.
65	0.79 $\pm$ 0.06	16.5 $\pm$ 5.2	>100	>100	12.0 $\pm$ 1.7	N.I.