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Journal:	*Journal of Medicinal Chemistry*
Manuscript ID	jm-2020-01088h.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Meng, Xiaoli; University of Liverpool, Molecular and Clinical Pharmacology Waddington, James; University of Liverpool, Department of Molecular and Clinical Pharmacology Tailor, Arun; University of Liverpool, Department of Molecular and Clinical Pharmacology Lister, Adam; University of Liverpool, Molecular and Clinical Pharmacology Hamlett, Jane; University of Liverpool, Molecular and Clinical Pharmacology Berry, Neil; University of Liverpool, Chemistry Park, Kevin; University of Liverpool, Institute of Translational Medicine Sporn, Michael; Dartmouth College Geisel School of Medicine, molecular and systems biology

CDDO-Imidazolide Targets Multiple Amino Acid Residues on the Nrf2 Adaptor, Keap1

Xiaoli Meng[*1], James C Waddington[1], Arun Tailor[1], Adam Lister[1], Jane Hamlett[1], Neil Berry[2], B Kevin Park[1], Michael B. Sporn[3]*

1 MRC Centre for Drug Safety Science, Department of Molecular & Clinical Pharmacology, University of Liverpool, Liverpool, L69 3GE, UK.

2 Department of Chemistry, University of Liverpool, Liverpool, L69 7ZD, UK.

3 Molecular and Systems Biology, Dartmouth Medical School, Lebanon, NH, 03756, USA.

*Corresponding Author

Dr Xiaoli Meng

MRC Centre for Drug Safety Science, Department of Pharmacology, University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, England

Telephone: 0044 151 7948368; e-mail: xlmeng@liverpool.ac.uk

Abstract

Synthetic triterpenoids including CDDO, its methyl ester (CDDO-Me, bardoxolone methyl), and its imidazolide (CDDO-Im) enhance Nrf2-mediated anti-oxidant and anti-inflammatory activity in many diseases by reacting with thiols on the adaptor protein, Keap1. Unlike monofunctional CDDO-Me, the bifunctional analog, CDDO-Im, has a second reactive site (imidazolide) and can covalently bind to amino acids other than cysteine on target proteins such as glutathione S-transferase pi (GSTP), serum albumin, or Keap1. Here we show for the first time that bifunctional CDDO-Im (in contrast to CDDO-Me), as low as 50 nM, can covalently transacylate arginine and serine residues in GSTP and cross link them to adjacent cysteine residues. Moreover, we show that CDDO-Im binds covalently to Keap1, by forming permanent Michael adducts with 8 different cysteines, and acyl adducts with lysine and several tyrosine residues. Modeling studies suggest the Tyr 85 adduct stabilizes the Keap1-Cul3 complex, thereby enhancing the potency of CDDO-Im.

Introduction

Synthetic oleanane triterpenoids such as 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) derivatives are multifunctional electrophilic agents that have been used to prevent and treat many chronic diseases in both *in vitro* and *in vivo* models[1-4]. The methyl ester derivative (CDDO-Me) has undergone clinical trials for the treatment of cancer, pulmonary disease, and chronic kidney disease[5]. The α,β unsaturated ketones in the A and C rings of CDDO derivatives are considered to be essential for their pharmacological action. It has been hypothesized that covalent binding to key cysteine residues on proteins through Michael addition disrupts protein-protein interaction, leading to a pharmacological response[1, 2, 6, 7]. Both CDDO-Me and CDDO-Imidazolide (CDDO-Im) can form covalent adducts at C1 of the A ring with cysteine residues in proteins through reversible Michael addition thio-alkylation (Figure 1 and Scheme 1)[6, 8]. Many cellular signalling proteins such as I-kappa-B-kinase (IKK), estrogen receptors (ER), insulin signalling proteins, and janus kinase/signal transducers and activators of transcription (JAK/STAT) have been identified as targets for CDDO-Im in cell cultures[1, 9].

One of the primary targets of triterpenoids is the Nrf2-Keap1 pathway, one of the most important cellular protective mechanisms against chemical/oxidative stresses[2]. Keap1, a cysteine-rich adaptor protein, contains an *N*-terminal Broad complex, Tram- track, and Bric-a-brac (BTB) domain, a central intervening region (IVR), and a Kelch domain close to the C-terminus (Figure 2A). Keap1 interacts with Nrf2 through binding of its C-terminal Kelch domain to two distinct degron motifs (DLG and ETGE degron) present in the Neh2 domain of Nrf2 (Figure 2B)[10, 11]. Under normal physiological conditions, Keap1 maintains low levels of Nrf2 by promoting its Cul3-Rbx1-mediated ubiquitination and subsequent proteasomal degradation. Under conditions of cellular stress, reactive species modify sensor cysteine

residues within Keap1 and block the degradation of Nrf2, leading to cellular accumulation of Nrf2, which subsequently promotes cytoprotective genes (Figure 2C). Several potential models have been proposed to explain how covalent modification of cysteine residues in Keap1 activates the Nrf2 pathway[12]. In the "hinge and latch" model (Figure 2D), covalent modification of key cysteine residues such as Cys151, Cys273, and Cys288 in the BTB and IVR domains of Keap1 lead to conformational changes, thus disrupting the interaction between Nrf2 and Kelch[13]. Alternatively, in the Cul3-dissociation model (Figure 2E), covalent modification of cysteine residues in the BTB domain decreases the binding of Cul3 to Keap1, leading to the loss of Nrf2 ubiquitination. In particular, modification of Cys151 that is in close proximity to the Cul3 binding interface, is consistent with this Cul3-dissociation model[14].

The triterpenoid CDDO-Im was designed to be a bifunctional drug, in contrast to the monofunctional molecule, CDDO-Me, in which only the A ring of oleanolic acid has been activated. Thus, the imidazolide moiety attached to carbonyl C-28 of CDDO-Im confers much greater potential for irreversible acylation of nucleophilic amino acid residues on proteins, compared to the less reactive methyl ester moiety of CDDO-Me (Scheme 1). Indeed, CDDO-Im has been found to have several biological activities not shared with CDDO-Me [1, 15], and in some instances, though not all [6, 16], CDDO-Im is significantly more potent than CDDO-Me. In almost every case examined, CDDO-Im is also much more potent than CDDO-free acid. The greater potency of CDDO-Im is particularly striking in the suppression of macrophage production of inducible nitric oxide synthase[17], which is an Nrf2-dependent gene[17, 18]. Thus, in primary macrophage cultures, CDDO-Im is more than 1,000-fold more potent than CDDO-Me or CDDO-free acid in such assays[7, 18]. This greater activity of CDDO-Im is also seen in assays for the induction of another Nrf2-dependent gene, heme oxygenase (HO-1), in which the imidazolide is markedly more potent than the methyl ester or

the free acid in enhancing both HO-1 gene and protein expression in several different cell types[15, 19]. Induction of HO-1 by CDDO-Im is almost completely abolished in Nrf2-knockout cells, compared to wild type[19]. Therefore, we hypothesized that additional molecular interactions between CDDO-Im and other amino acid residues in addition to cysteine could likely contribute to their potency and target selectivity. Thus, the aim of this study was to 1) investigate the binding of CDDO-Im to proteins using mass spectrometric methods and 2) explore its reactivity with Keap1 and its implication in the activation of Nrf2.

Results

Characterization of CDDO-Im Modified-GSTP In Vitro. To characterise the Michael adducts of CDDO-Im, human glutathione S-transferase pi (GSTP) was chosen as a model as it contains several reactive cysteine residues. His-GSTP captured on nickel beads was exposed to a range of concentrations of CDDO-Im (50 nM-10 μM). LC-MS/MS analysis of the tryptic digests of CDDO-Im treated GSTP revealed multiple types of adducts, including a simple Michael adduct formed by thio-alkylation with cysteine, a cross-linked adduct, and an acylation adduct formed with arginine residues (Table 1). Michael addition of cysteine in GSTP to CDDO-Im followed by the hydrolysis of the imidazolide amide bond resulted in adducts with a mass addition of 491.3 amu. Figure 3A shows a representative MS/MS spectrum for a doubly charged ion at m/z 785.986, corresponding to the tryptic peptide ^{45}ASCLYGQLPK^{54} with an additional mass of 491.3 amu. The peptide sequence was confirmed by partial singly charged y and b series ions. The modification site was confirmed by the presence of b3* (m/z 753.57), b4* (m/z 866.64), and b8*(m/z 1328.02), all with adduction of 491.3 amu. The presence of a fragment ion of m/z 464.45 and 447.42 that was derived from CDDO carboxylic acid provided further evidence of the modification. This adduct was only detected on Cys47; no other sites were identified.

Cross-linked adducts are anticipated due to the presence of the bifunctional groups on CDDO-Im. An abundant doubly charged ion at m/z 776.989 was detected, corresponding to the tryptic peptide ^{45}ASCLYGQLPK^{54} with an additional mass of 473.3 amu. The cross-linked adduct could be formed by reaction of CDDO-Im with Cys47 and the adjacent serine residue in the sequence ^{45}ASCLYGQLPK^{54}. A typical MS/MS spectrum representing the tryptic peptide ^{45}ASCLYGQLPK^{54} with a mass addition of 473.3 amu is shown in Figure 3B. The peptide sequence was confirmed by a series of y product ions, and the cross-linked was evidenced by the presence of b2*(m/z 632.56, Ser46) and y8* (m/z 1395.29, Cys47), both with a mass

increment of 473.3 amu. The cross-linked adducts were also detected on multiple sites including Arg13-Cys14 and Arg100-Cys101 (Table 1). Similar to the modifications observed with S46, a stable adduct derived from an arginine residue was also detected (Figure S1, Arg186, ^{183}LSARPK^{188}) when a high concentration of CDDO-Im was used (500 μM).

Covalent binding of CDDO-Im to GSTP was concentration-dependent (Figure 3C and 3D) with adducts being detectable at the lowest concentration of CDDO-Im (50 nM). CDDO-Me did not form adducts with GSTP, except at very high concentrations (500 μM). The cross-linked adducts appeared to be the major adducts formed between CDDO-Im and GSTP, probably due to the adduct stabilization through acylation at C28.

Characterization of HSA Modified by CDDO-Im In Vitro. Human serum albumin (HSA) has long been known to exhibit great affinity for many ligands and act as an endogenous target and/or quencher for numerous electrophilic compounds such as penicillins, α,β-unsaturated aldehydes, and acyl glucuronides [20-23]. Adducts could be formed in HSA with various nucleophilic amino acid residues including lysine, cysteine, and histidine. To probe the chemical basis of the putative Michael and acylation adducts of CDDO-Im with HSA, HSA was incubated with CDDO-Im at various concentrations and time points. LC-MS/MS analysis of the tryptic digests revealed 23 CDDO-Im modified peptides, including peptides containing lysine (n=10), arginine (n=2), serine (n=7) or tyrosine (n=5) residues after incubation at the highest concentration of CDDO-Im (1 mM) (Supplementary Table 1). At lower concentrations, CDDO-Im appeared to bind selectively to lysine and tyrosine residues and some adducts could be detected at concentrations as low as 10 nM (Table 2).

A typical MS/MS spectrum representing the tryptic peptide ^{411}YTK*K^{414} with a mass addition of 473.3 amu is shown in Figure 4A. A missed cleavage at the proposed site of covalent binding and the presence of y2* (m/z 748.4) and y3* (m/z 849.69) ions provided firm evidence of

acylation at Lys413. Similar to the modifications observed with lysine, stable adducts derived from tyrosine (Figure 4B) were also detected on multiple sites (Table 2). A semi-quantitative analysis of modification at each site revealed a concentration- and time-dependent increase for each modified peptide (Figure 4C & 4D).

Characterization of CDDO-Im Modified Keap1 In Vitro. LC-MS/MS analysis of the tryptic digests of CDDO-Im-treated recombinant Keap1 protein also revealed multiple types of stable adducts, including a Michael adduct formed with cysteine and acylation adducts formed with tyrosine and lysine (Table 3). A representative MS/MS spectrum shows the peptide ^{85}YQDAPAAQFMAHK^{96} modified by CDDO-Im at Tyr85 (Figure 5A). The presence of adducted a1 ion (m/z 609.37) provided firm evidence of acylation at Tyr85. Multiple cysteine residues in Keap1 including Cys13, Cys14, Cys38, Cys257, Cys288, Cys489, Cys513, and Cys613, were modified by CDDO-Im, all with a mass addition of 473.3 amu. The mass addition of 473.3 amu could correspond to either a thioester adduct or a Michael adduct followed by acylation of the N-terminal amino group of isolated peptides. The absolute structure of these adducts requires further investigation.

A concentration dependent increase of modification was observed at most sites except for Tyr85 (Figure 5B). Tyr85 appeared to be the major modification site when incubated with CDDO-Im at low concentration (100 nM); more sites were bound when the concentrations of CDDO-Im increased (Figure 5B and 5C). Both Cys151 and Tyr85 are located in a groove formed by the side chains of His 154, His 129 (Figure S2A), it is therefore not surprising that Tyr85 is readily modified by CDDO-Im. Covalent docking of CDDO-Im with Tyr85 demonstrated that the ring system positioned away from Cys151, with the E ring occupying a small cavity near the side chain of His154 (Figure 5D). Two hydrogen bonds are formed, one is between the backbone amide nitrogen of Met161 and the nitrile nitrogen of CDDO-Im

($r_{N...N}$ = 2.96Å); another is formed between the backbone oxygen of Gly128 and the enolic oxygen of the C-ring ($r_{O...O}$ =3Å) (Figure 5E). Surprisingly, modification of Cys151 by CDDO-Im was not detected in this study. However, Cleasby et al. have shown that when CDDO-free acid covalently bound to Cys151, the ring systems positioned in the groove, and the C-4 gem-dimethyl group occupying a small cavity near the side chain of Val 155 and the hydrophobic portion of Lys 131 (Figure S2B)[14]. The close proximity of Tyr85 to the carbonyl carbon of CDDO-Im (5.7Å) and its flexible location makes it theoretically possible to form a cross-linked adduct (Figure S2B). The difficulty to identify such a cross-linked adduct by searching algorithm may explain why Cys151 modification was not detected.

Notably, we could not detect any adducts of CDDO-Me with Keap1, even when incubated at concentrations as high as 10 μM. This result again emphasizes the difference between CDDO-Im and CDDO-Me and the importance of the imidazolide moiety for irreversible covalent binding.

Modification of Keap1 by CDDO-Im stabilizes the Keap1-Cul3 complex. To explore the impact of CDDO-Im modified Tyr85 on the Keap1-Cul3 complex formation, covalent docking of CDDO-Im with Tyr85 was performed using the crystal structure of the Keap1-Cul3 complex (PDB code: 5NLB). As shown in the crystal structure of the Keap1-Clu3 complex, Tyr85 was identified as a key amino acid interacting with the Cul3 N terminal domain (Figure 6A). Covalent binding of CDDO-Im to Tyr85 positions the ring system towards Cul3, forming a bridge between the Keap1 and Cul3 (Figure 6B and C). Three hydrogen bonds could be potentially formed between the nitrile nitrogen of CDDO and the guanidinium nitrogen of Arg188 in Cul3 ($r_{N...N}$ = 1.9Å, 3.2Å, and 3.6Å) (Figure 6D).

Discussion

Although many electrophiles have been reported to enhance transcriptional activity of Nrf2 through covalent modifications of cysteine thiols of Keap1, the detailed mechanisms by which these species affect the activity of Keap1 remain controversial[12]. Multiple mechanisms have been proposed including Keap1 conformation changes ("hinge and latch" model and Cul3-dissociation model) and increased Keap1 ubiquitination (ubiquitination switching model) owing to covalent modifications of multiple cysteine residues, all leading to disruption of Keap1-Nrf2 interaction and subsequent Nrf2 degradation[12, 13, 24, 25]. So far, cysteine residues in Keap1 have been identified as the only targets. Here we have demonstrated CDDO-Im, in contrast to CDDO-Me, covalently binds to amino acid residues other than cysteine through multiple chemical mechanisms. In particular, selective binding to Tyr85 in the BTB domain of human Keap1 protein may contribute to the greater potency of CDDO-Im to activate Nrf2 in certain contexts.

As a bifunctional triterpenoid, CDDO-Im has the potential to form not only thio-alkyl adducts with cysteine residues in proteins as initial studies demonstrated[6-8], but also acylation adducts with other nucleophilic amino acid residues such as lysine, arginine, serine, and tyrosine due to the presence of its reactive imidazolide group. More importantly, intra and inter cross-linked adducts can also be formed (Scheme 1). Among the 27 cysteine residues in human Keap1, 8 cysteine residues were modified by CDDO-Im, among which five (Cys257, Cys288, Cys489, Cys513, and Cys613) were the most readily modified by other electrophilic reagents[26, 27 28]. Cys273 and Cys288 are essential for Keap1 to control Nrf2 under both basal and stress conditions, whereas Cys151, located in a positively charged pocket, is identified as a primary target for many electrophiles under conditions of stress[14, 25, 26, 29]. Surprisingly, we did not detect CDDO-Im modified Cys151, although different forms of unmodified Cys151 containing peptide (CVLHVMNGAVMYQIDSVVR) were detected, in agreement with other

observations [30](Figure S1B and C). Failure to detect Cys151 modification could be due to the hydrophobicity of the peptide or potential cross-linked adducts that cannot be identified by software.

Modification of cysteine residues within the BTB and IVR domain (Cys151, Cys273 and Cys288) is postulated to alter the conformation of Keap1, which can either directly disrupt the interaction between Nrf2 and Kelch ("hinge and latch model) or block the ubiquitination of Nrf2 (Cul3-dissociation model), leading to Nrf2 nuclear accumulation[31]. However, the direct disruption of Keap1-Nrf2 interaction caused by a cysteine modification has been controversial. For example, Cys151 is remote from the Nrf2 substrate-binding domain, hence the conformation changes caused by modification of Cys151 may be unlikely to disrupt the Keap-Nrf2 complex [31]. Even though Cys489 and Cys513 are located within the Kelch domain that directly binds to Nrf2, covalent binding of CDDO-Im to either cysteine residue seems unlikely to cause any disruptions as shown in the computational modelling studies (Figure S3). On the other hand, Cys151 modification was also postulated to cause alteration of the interface between BTB and Cul3[14], leading to down regulation of Nrf2 ubiquitination and subsequent proteasomal degradation. However, studies have shown that cysteine modifications by many Nrf2-inducing chemicals do not dissociate Keap1 from Cul3[32-34]. Two possible hypotheses have been proposed to explain the down regulation of Nrf2 ubiquitination caused by modification of critical cysteine residues in Keap1. One suggested that cysteine residues within BTB and IVR domains could form thioester conjugates with ubiquitin to accomplish ubiquitin transfer from Cul3 to the lysine residues in Nrf2; modification of critical cysteine residues which are selective to different electrophiles ("cysteine code") thereby inhibits Keap1-dependent ubiquitination[12, 24, 35]. Alternatively, cysteine modifications might cause the switch of ubiquitination from Nrf2 to Keap1, leading to upregulation of Keap1 ubiquitination as well as Nrf2 nuclear accumulation[31].

Most importantly, we demonstrated for the first time that CDDO-Im selectively targets Tyr85 in Keap1. Located at the entrance of a groove formed by the side chains of His154, His 129, and Cys151[14], Tyr85 may adjust its position in response to interaction with particular ligands and this flexibility may play an important role in the formation of Keap1-Cul3 complex. Computational docking demonstrated that covalent binding of CDDO-Im to Tyr85 positions the CDDO ring system towards the Keap1-Cul3 binding interface. Three hydrogen bonds were formed between the CDDO nitrile nitrogen and Cul3 Arg188, which stabilise the Keap1-Cul3 complex rather than weaken or abrogate the complex formation as proposed previously[34]. The strong interaction between Keap1 and Cul3 was proposed to prevent Nrf2 binding to the complex for ubiquitination[36, 37]. This is in contrast with the observation that only CDDO-Im exceptionally reduces the interaction between Keap1 and Cul3, although this only occurred at high concentrations of CDDO-Im (6 or 18 μM of CDDO-Im)[29, 34]. It is important to note that CDDO-Im does not selectively bind to Tyr85 at these concentrations, indicating multiple mechanisms may contribute to the disruption of Keap1-Cul3 complex formation. In particular, cross-linked adducts formed at high concentrations of CDDO-Im may likely cause its unique function of disrupting the Keap1-Cul3 interaction. Thus, depending on the concentration, CDDO-Im may have significantly different functions[1, 19]. Selective modification of Tyr85 by CDDO-Im at low concentrations may have a pronounced effect on the stability of Keap-Cul3 complex, which may cause the switch of ubiquitination from Nrf2 to Keap1, leading to disruption of Keap1-Nrf2 interaction and increased self-ubiquitination of Keap1. In contrast, modification of multiple cysteine residues and the formation of cross-linked adducts by CDDO-Im at high concentrations may cause large conformational changes in Keap1, leading to the dissociation of Nrf2 from Keap1.

It is important to emphasize that even within the same class of compounds, CDDO-Im and CDDO-Me can have different, sometimes opposite, effects on cellular functions. It has been

shown that there are major differences in the sets of genes, both Nrf2-dependent and Nrf23-independent, upregulated or downregulated by either CDDO-Im or CDDO-Me[15]. It should also be noted that Nrf2 is not the only molecular target to which Keap1 binds. Significant direct interactions of Keap1 with the regulatory protein, p62, and the anti-oxidative protein, iASPP, have been reported [38, 39]. In all of these situations, the context, especially the dose, of CDDO-Im will be a key determinant of any final biological response. A low dose of CDDO-Im can give a certain response, and a higher dose an opposite effect[1, 19, 40]. The dose-dependent covalent binding of CDDO-Im to many amino acid residues, which we have shown here, may account for some of these contextual variations.

In all situations, context is of critical importance. Thus it has been published that the action of CDDO-Im as an inducer of Nrf2 is "independent of the presence of Cys 151" in Keap1 (Fig. 7 in Tayaka et al., 2012)[41], while the same group, using lower concentrations of CDDO-Im, later showed data indicating that this drug is "a Cys 151-preferring inducer" (Fig. 10 in Saito et al., 2016)[42]. Studies of interaction of triterpenoid molecules with Cys 151 have been performed both in silico and in cell cultures, with many different agents, including CDDO, CDDO-Me, an epoxide of CDDO-Me, CDDO-Im, new analogs of CDDO-Im, and even tricyclic ene-one analogs which are not true triterpenoids[43]. Different concentrations of drug have been used in different studies, and even the context of Keap1 has sometimes been different. In some experiments Keap1 has been an intact whole molecule, but in others only the BTB domain of Keap1 has been used[14]. For all future studies it will be important to perform a complete dose-response evaluation.

Conclusion

In summary, we have found that the bifunctional triterpenoid, CDDO-Im forms covalent adducts with many nucleophilic amino acids on important target proteins at concentrations as low as 10-50 nM, and can even form cross-links. The contrast with monofunctional CDDO-Me is striking, since we could not detect adduct formation by CDDO-Me at concentrations less than 500 μM. Our data are directly relevant to known differences between CDDO-Im and CDDO-Me, although clearly these two molecules also share many common mechanisms of action. Altogether our results highlight novel targets for bifunctional triterpenoids that may contribute to their important potency and activity. They provide new insights into the chemical mechanisms of action and pave the way for exploring potential novel cellular signaling pathways for bifunctional triterpenoids, including even newer pyridyl imidazolide derivatives[44] that also form covalent acylation adducts, which will be described in a future publication. In all future work, it will be essential to consider context.

Experimental Section

Chemicals. CDDO-Im was provided by Triterpenoid Therapeutics, Inc (USA). CDDO-Me and HSA (97-99% pure) were purchased from Sigma-Aldrich, trypsin from Promega (Madison, WI), liquid chromatography-mass spectrometry (LC-MS) grade solvents from Fisher Scientific UK Ltd (Loughborough, Leicestershire), and all other standard reagents from Sigma-Aldrich.

Concentration-Dependent Modification of HSA by CDDO-Im. CDDO-Im freshly dissolved in DMSO, followed by dilutions in phosphate buffer (10 mM, pH 7.4), was incubated with HSA (0.6 mM, 50 µL) in phosphate buffer in sealed Eppendorf tubes at 37 ^{o}C for 16 h. The molar ratios of drug to protein were 0.00001:1, 0.0001:1, 0.001:1, 0.01:1, 0.1:1, and 1:1. Protein was precipitated twice with 9 volumes of ice-cold methanol to remove free drug, and processed for LC-MS/MS analysis using previous methods[45].

Time-Dependent Modification of HSA by CDDO-Im. 10 µM CDDO-Im was incubated with HSA (0.6 mM, 300 µL) at 37 ^{o}C. Aliquots of 50 µL were removed after 10, 30, 60, and 180 minutes and processed for LC-MS/MS analysis.

Concentration-Dependent Modification of His-GSTP by triterpenoids. His-GSTP was expressed in E.coli as described previously[22]. Purified His-GSTP captured on nickel beads was incubated with a range of concentrations of CDDO-Im (50 nM-10µM) in phosphate buffer, pH 7.4 for 16 h. CDDO-Me (0.5 mM) was used as a positive control. The beads were then washed 5 times with 1000 µL phosphate buffer. The protein was subjected to on-bead tryptic digestion. In brief, a suspension of beads in 30 µL of 50 mM ammonium bicarbonate buffer was incubated with 20 ng of trypsin for 16 h at 37 °C and the digests were analyzed by LC-MS/MS.

Modification of Keap1 by CDDO-Im. Solutions of CDDO-Im, as described above, were incubated (0.1-10µM) with recombinant Keap1 protein[46] at 37 ^{o}C for 16 h in phosphate

buffer (10 mM, pH 7.4). The mixture was then purified by 1D-gel electrophoresis using established protocol, followed by in gel-digestion[45]. The digests were further purified by C18-ziptiping and analyzed by LC-MS/MS.

LC-MS/MS analysis of CDDO-Im protein adducts. The tryptic peptides were analyzed by a Triple TOF 5600 mass spectrometer (Sciex). Samples were reconstituted in 50 µL 0.1% formic acid and 2 µL of samples were delivered into the instrument using an Eksigent Nano-LC system mounted with a nanoACQUITY UPLC Symmetry C18 Trap Column and an analytical BEH C18 nanoACQUITY Column (Waters, MA, USA). A NanoSpray III source was fitted with a 10 µm inner diameter PicoTip emitter (New Objective). Samples were loaded in 0.1% formic acid onto the trap, which was then washed with 2% ACN/0.1% FA for 10 min at 2 µL/ min before switching in-line with the analytical column. A gradient of 2−50% (v/v) ACN/0.1% (v/v) FA over 90 min was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive ion mode using information-dependent acquisition, using mass ranges of 400−1600 amu in MS and 100−1400 amu in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approximately 10 Hz) using a threshold of 100 counts per s, with dynamic exclusion for 12 s and rolling collision energy.

Analysis of CDDO-Im modified proteins. LC-MS/MS data were searched against the reviewed human proteome (UniProt/SwissProt accessed October 2018), using ProteinPilot software, v4.0 (Sciex). Data were refined using default parameters and searches performed with the following parameters: enzymatic cleavage restriction for trypsin, fixed modification-carbamidomethylation of cysteine; variable modifications-methionine oxidation (+15.99), asparagine and glutamine deamidation (+0.98); CDDO-Im modification of lysine, serine, tyrosine, and arginine (+473.3); CDDO-Im modification of cysteine (+473.3 or 491.3).

Keap1 modelling. Crystal structures of BTB domain of human Keap1 (PDB code: 4CXI) and Keap1-Cul3 complex (PDB code: 5NBL) were used to generate models. GOLD 5.2 (CCDC

software) was used for covalent docking of CDDO-Im to Tyr85. To covalently link the CDDO-Im to Tyr85, the corresponding side chain was removed from the protein and the ligand modified to contain the side chain to allow flexibility. The site of covalent attachment was at the tyrosine Cα [30]. A generic algorithm with ChemPLP as the fitness function was used to generate 10 binding modes per ligand. Default settings were retained for the "ligand flexibility", "fitness and search options", and "GA" settings.

Author Information

Corresponding author

Dr Xiaoli Meng, MRC Centre for Drug Safety Science, Department of Pharmacology, The University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, England

Telephone: 0044 151 7948368; e-mail: xlmeng@liverpool.ac.uk

Funding

The project received funding from Triterpenoid Therapeutics, Inc. (USA) and the MRC Centre for Drug Safety Science (Grant Number MR/L006758/1).

Notes

M. B. Sporn is an employee and shareholder of Triterpenoid Therapeutics. The other authors declare no competing financial interest

Supporting Information

A supplementary table and additional figures (Figure S1-S3) show the formation of CDDO-Im protein adducts and the interaction of CDDO-Im with Keap1. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid; CDDO-Im, CDDO-Imidazolide; ER, estrogen receptor; JAK, janus kinase; STAT, signal transducers and activators of transcription.

References

1. Liby, K. T.; Sporn, M. B. Synthetic Oleanane Triterpenoids: Multifunctional Drugs with a Broad Range of Applications for Prevention and Treatment of Chronic Disease. *Pharmacol Rev* **2012,** 64, 972-1003.
2. Yamamoto, M.; Kensler, T. W.; Motohashi, H. The Keap1-Nrf2 System: A Thiol-Based Sensor-Effector Apparatus for Maintaining Redox Homeostasis. *Physiol Rev* **2018,** 98, 1169-1203.
3. Uruno, A.; Furusawa, Y.; Yagishita, Y.; Fukutomi, T.; Muramatsu, H.; Negishi, T.; Sugawara, A.; Kensler, T. W.; Yamamoto, M. The Keap1-Nrf2 System Prevents Onset of Diabetes Mellitus. *Mol Cell Biol* **2013,** 33, 2996-3010.
4. Johnson, N. M.; Egner, P. A.; Baxter, V. K.; Sporn, M. B.; Wible, R. S.; Sutter, T. R.; Groopman, J. D.; Kensler, T. W.; Roebuck, B. D. Complete Protection against Aflatoxin B(1)-Induced Liver Cancer with a Triterpenoid: DNA Adduct Dosimetry, Molecular Signature, and Genotoxicity Threshold. *Cancer Prev Res (Phila)* **2014,** 7, 658-665.
5. Chin, M. P.; Bakris, G. L.; Block, G. A.; Chertow, G. M.; Goldsberry, A.; Inker, L. A.; Heerspink, H. J. L.; O'Grady, M.; Pergola, P. E.; Wanner, C.; Warnock, D. G.; Meyer, C. J. Bardoxolone Methyl Improves Kidney Function in Patients with Chronic Kidney Disease Stage 4 and Type 2 Diabetes: Post-Hoc Analyses from Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes Study. *Am J Nephrol* **2018,** 47, 40-47.
6. Dinkova-Kostova, A. T.; Liby, K. T.; Stephenson, K. K.; Holtzclaw, W. D.; Gao, X.; Suh, N.; Williams, C.; Risingsong, R.; Honda, T.; Gribble, G. W.; Sporn, M. B.; Talalay, P. Extremely Potent Triterpenoid Inducers of the Phase 2 Response: Correlations of Protection against Oxidant and Inflammatory Stress. *Proc Natl Acad Sci U S A* **2005,** 102, 4584-4589.
7. Sporn, M. B.; Liby, K.; Yore, M. M.; Suh, N.; Albini, A.; Honda, T.; Sundararajan, C.; Gribble, G. W. Platforms and Networks in Triterpenoid Pharmacology. *Drug Develop Res* **2007,** 68, 174-182.
8. Sporn, M. B.; Liby, K. T.; Yore, M. M.; Fu, L.; Lopchuk, J. M.; Gribble, G. W. New Synthetic Triterpenoids: Potent Agents for Prevention and Treatment of Tissue Injury Caused by Inflammatory and Oxidative Stress. *J Nat Prod* **2011,** 74, 537-545.
9. Yore, M. M.; Kettenbach, A. N.; Sporn, M. B.; Gerber, S. A.; Liby, K. T. Proteomic Analysis Shows Synthetic Oleanane Triterpenoid Binds to Mtor. *PLoS One* **2011,** 6, e22862.
10. Lo, S. C.; Li, X.; Henzl, M. T.; Beamer, L. J.; Hannink, M. Structure of the Keap1:Nrf2 Interface Provides Mechanistic Insight into Nrf2 Signaling. *EMBO J* **2006,** 25, 3605-3617.
11. Tong, K. I.; Katoh, Y.; Kusunoki, H.; Itoh, K.; Tanaka, T.; Yamamoto, M. Keap1 Recruits Neh2 through Binding to Etge and Dlg Motifs: Characterization of the Two-Site Molecular Recognition Model. *Mol Cell Biol* **2006,** 26, 2887-2900.
12. Baird, L.; Yamamoto, M. The Molecular Mechanisms Regulating the Keap1-Nrf2 Pathway. *Mol Cell Biol* **2020,** 40, DOI:10.1128/MCB.00099-00020.
13. Kansanen, E.; Kuosmanen, S. M.; Leinonen, H.; Levonen, A. L. The Keap1-Nrf2 Pathway: Mechanisms of Activation and Dysregulation in Cancer. *Redox Biol* **2013,** 1, 45-49.
14. Cleasby, A.; Yon, J.; Day, P. J.; Richardson, C.; Tickle, I. J.; Williams, P. A.; Callahan, J. F.; Carr, R.; Concha, N.; Kerns, J. K.; Qi, H.; Sweitzer, T.; Ward, P.; Davies, T. G. Structure of the Btb Domain of Keap1 and Its Interaction with the Triterpenoid Antagonist Cddo. *PLoS One* **2014,** 9, e98896.
15. To, C.; Ringelberg, C. S.; Royce, D. B.; Williams, C. R.; Risingsong, R.; Sporn, M. B.; Liby, K. T. Dimethyl Fumarate and the Oleanane Triterpenoids, Cddo-Imidazolide and Cddo-Methyl Ester, Both Activate the Nrf2 Pathway but Have Opposite Effects in the a/J Model of Lung Carcinogenesis. *Carcinogenesis* **2015,** 36, 769-781.
16. Yates, M. S.; Tauchi, M.; Katsuoka, F.; Flanders, K. C.; Liby, K. T.; Honda, T.; Gribble, G. W.; Johnson, D. A.; Johnson, J. A.; Burton, N. C.; Guilarte, T. R.; Yamamoto, M.; Sporn, M. B.; Kensler, T. W. Pharmacodynamic Characterization of Chemopreventive Triterpenoids as Exceptionally Potent Inducers of Nrf2-Regulated Genes. *Mol Cancer Ther* **2007,** 6, 154-162.

17. Ahmed, S. M.; Luo, L.; Namani, A.; Wang, X. J.; Tang, X. Nrf2 Signaling Pathway: Pivotal Roles in Inflammation. *Biochim Biophys Acta Mol Basis Dis* **2017,** 1863, 585-597.
18. Kobayashi, E. H.; Suzuki, T.; Funayama, R.; Nagashima, T.; Hayashi, M.; Sekine, H.; Tanaka, N.; Moriguchi, T.; Motohashi, H.; Nakayama, K.; Yamamoto, M. Nrf2 Suppresses Macrophage Inflammatory Response by Blocking Proinflammatory Cytokine Transcription. *Nat Commun* **2016,** 7, 11624.
19. Liby, K.; Hock, T.; Yore, M. M.; Suh, N.; Place, A. E.; Risingsong, R.; Williams, C. R.; Royce, D. B.; Honda, T.; Honda, Y.; Gribble, G. W.; Hill-Kapturczak, N.; Agarwal, A.; Sporn, M. B. The Synthetic Triterpenoids, Cddo and Cddo-Imidazolide, Are Potent Inducers of Heme Oxygenase-1 and Nrf2/Are Signaling. *Cancer Res* **2005,** 65, 4789-4798.
20. Carter, D. C.; Ho, J. X. Structure of Serum Albumin. *Adv Protein Chem* **1994,** 45, 153-203.
21. Meng, X.; Jenkins, R. E.; Berry, N. G.; Maggs, J. L.; Farrell, J.; Lane, C. S.; Stachulski, A. V.; French, N. S.; Naisbitt, D. J.; Pirmohamed, M.; Park, B. K. Direct Evidence for the Formation of Diastereoisomeric Benzylpenicilloyl Haptens from Benzylpenicillin and Benzylpenicillenic Acid in Patients. *J Pharmacol Exp Ther* **2011,** 338, 841-849.
22. Meng, X.; Lawrenson, A. S.; Berry, N. G.; Maggs, J. L.; French, N. S.; Back, D. J.; Khoo, S. H.; Naisbitt, D. J.; Park, B. K. Abacavir Forms Novel Cross-Linking Abacavir Protein Adducts in Patients. *Chem Res Toxicol* **2014,** 27, 524-535.
23. Hammond, T. G.; Meng, X.; Jenkins, R. E.; Maggs, J. L.; Castelazo, A. S.; Regan, S. L.; Bennett, S. N.; Earnshaw, C. J.; Aithal, G. P.; Pande, I.; Kenna, J. G.; Stachulski, A. V.; Park, B. K.; Williams, D. P. Mass Spectrometric Characterization of Circulating Covalent Protein Adducts Derived from a Drug Acyl Glucuronide Metabolite: Multiple Albumin Adductions in Diclofenac Patients. *J Pharmacol Exp Ther* **2014,** 350, 387-402.
24. Zhang, D. D.; Hannink, M. Distinct Cysteine Residues in Keap1 Are Required for Keap1-Dependent Ubiquitination of Nrf2 and for Stabilization of Nrf2 by Chemopreventive Agents and Oxidative Stress. *Mol Cell Biol* **2003,** 23, 8137-8151.
25. Eggler, A. L.; Small, E.; Hannink, M.; Mesecar, A. D. Cul3-Mediated Nrf2 Ubiquitination and Antioxidant Response Element (Are) Activation Are Dependent on the Partial Molar Volume at Position 151 of Keap1. *Biochem J* **2009,** 422, 171-180.
26. Bollong, M. J.; Lee, G.; Coukos, J. S.; Yun, H.; Zambaldo, C.; Chang, J. W.; Chin, E. N.; Ahmad, I.; Chatterjee, A. K.; Lairson, L. L.; Schultz, P. G.; Moellering, R. E. A Metabolite-Derived Protein Modification Integrates Glycolysis with Keap1-Nrf2 Signalling. *Nature* **2018,** 562, 600-604.
27. Mills, E. L.; Ryan, D. G.; Prag, H. A.; Dikovskaya, D.; Menon, D.; Zaslona, Z.; Jedrychowski, M. P.; Costa, A. S. H.; Higgins, M.; Hams, E.; Szpyt, J.; Runtsch, M. C.; King, M. S.; McGouran, J. F.; Fischer, R.; Kessler, B. M.; McGettrick, A. F.; Hughes, M. M.; Carroll, R. G.; Booty, L. M.; Knatko, E. V.; Meakin, P. J.; Ashford, M. L. J.; Modis, L. K.; Brunori, G.; Sevin, D. C.; Fallon, P. G.; Caldwell, S. T.; Kunji, E. R. S.; Chouchani, E. T.; Frezza, C.; Dinkova-Kostova, A. T.; Hartley, R. C.; Murphy, M. P.; O'Neill, L. A. Itaconate Is an Anti-Inflammatory Metabolite That Activates Nrf2 Via Alkylation of Keap1. *Nature* **2018,** 556, 113-117.
28. Suzuki, T.; Yamamoto, M. Stress-Sensing Mechanisms and the Physiological Roles of the Keap1-Nrf2 System During Cellular Stress. *J Biol Chem* **2017,** 292, 16817-16824.
29. Rachakonda, G.; Xiong, Y.; Sekhar, K. R.; Stamer, S. L.; Liebler, D. C.; Freeman, M. L. Covalent Modification at Cys151 Dissociates the Electrophile Sensor Keap1 from the Ubiquitin Ligase Cul3. *Chem Res Toxicol* **2008,** 21, 705-710.
30. Wong, M. H.; Bryan, H. K.; Copple, I. M.; Jenkins, R. E.; Chiu, P. H.; Bibby, J.; Berry, N. G.; Kitteringham, N. R.; Goldring, C. E.; O'Neill, P. M.; Park, B. K. Design and Synthesis of Irreversible Analogues of Bardoxolone Methyl for the Identification of Pharmacologically Relevant Targets and Interaction Sites. *J Med Chem* **2016,** 59, 2396-2409.
31. Eggler, A. L.; Liu, G.; Pezzuto, J. M.; van Breemen, R. B.; Mesecar, A. D. Modifying Specific Cysteines of the Electrophile-Sensing Human Keap1 Protein Is Insufficient to Disrupt Binding to the Nrf2 Domain Neh2. *Proc Natl Acad Sci U S A* **2005,** 102, 10070-10075.

32. Baird, L.; Dinkova-Kostova, A. T. Diffusion Dynamics of the Keap1-Cullin3 Interaction in Single Live Cells. *Biochem Biophys Res Commun* **2013,** 433, 58-65.
33. Li, Y.; Paonessa, J. D.; Zhang, Y. Mechanism of Chemical Activation of Nrf2. *PLoS One* **2012,** 7, e35122.
34. Iso, T.; Suzuki, T.; Baird, L.; Yamamoto, M. Absolute Amounts and Status of the Nrf2-Keap1-Cul3 Complex within Cells. *Mol Cell Biol* **2016,** 36, 3100-3112.
35. Hur, W.; Gray, N. S. Small Molecule Modulators of Antioxidant Response Pathway. *Curr Opin Chem Biol* **2011,** 15, 162-173.
36. Zhang, D. D.; Lo, S. C.; Cross, J. V.; Templeton, D. J.; Hannink, M. Keap1 Is a Redox-Regulated Substrate Adaptor Protein for a Cul3-Dependent Ubiquitin Ligase Complex. *Mol Cell Biol* **2004,** 24, 10941-10953.
37. Wang, X. J.; Sun, Z.; Chen, W.; Li, Y.; Villeneuve, N. F.; Zhang, D. D. Activation of Nrf2 by Arsenite and Monomethylarsonous Acid Is Independent of Keap1-C151: Enhanced Keap1-Cul3 Interaction. *Toxicol Appl Pharmacol* **2008,** 230, 383-389.
38. Komatsu, M.; Kurokawa, H.; Waguri, S.; Taguchi, K.; Kobayashi, A.; Ichimura, Y.; Sou, Y. S.; Ueno, I.; Sakamoto, A.; Tong, K. I.; Kim, M.; Nishito, Y.; Iemura, S.; Natsume, T.; Ueno, T.; Kominami, E.; Motohashi, H.; Tanaka, K.; Yamamoto, M. The Selective Autophagy Substrate P62 Activates the Stress Responsive Transcription Factor Nrf2 through Inactivation of Keap1. *Nat Cell Biol* **2010,** 12, 213-223.
39. Ge, W.; Zhao, K.; Wang, X.; Li, H.; Yu, M.; He, M.; Xue, X.; Zhu, Y.; Zhang, C.; Cheng, Y.; Jiang, S.; Hu, Y. Iaspp Is an Antioxidative Factor and Drives Cancer Growth and Drug Resistance by Competing with Nrf2 for Keap1 Binding. *Cancer Cell* **2017,** 32, 561-573 e566.
40. Kensler, T. W.; Wakabayashi, N. Nrf2: Friend or Foe for Chemoprevention? *Carcinogenesis* **2010,** 31, 90-99.
41. Takaya, K.; Suzuki, T.; Motohashi, H.; Onodera, K.; Satomi, S.; Kensler, T. W.; Yamamoto, M. Validation of the Multiple Sensor Mechanism of the Keap1-Nrf2 System. *Free Radic Biol Med* **2012,** 53, 817-827.
42. Saito, R.; Suzuki, T.; Hiramoto, K.; Asami, S.; Naganuma, E.; Suda, H.; Iso, T.; Yamamoto, H.; Morita, M.; Baird, L.; Furusawa, Y.; Negishi, T.; Ichinose, M.; Yamamoto, M. Characterizations of Three Major Cysteine Sensors of Keap1 in Stress Response. *Mol Cell Biol* **2016,** 36, 271-284.
43. Naidu, S. D.; Muramatsu, A.; Saito, R.; Asami, S.; Honda, T.; Hosoya, T.; Itoh, K.; Yamamoto, M.; Suzuki, T.; Dinkova-Kostova, A. T. C151 in Keap1 Is the Main Cysteine Sensor for the Cyanoenone Class of Nrf2 Activators, Irrespective of Molecular Size or Shape. *Sci Rep-Uk* **2018,** 8, DOI:10.1038/s41598-41018-26269-41599.
44. Cao, M.; Onyango, E. O.; Williams, C. R.; Royce, D. B.; Gribble, G. W.; Sporn, M. B.; Liby, K. T. Novel Synthetic Pyridyl Analogues of Cddo-Imidazolide Are Useful New Tools in Cancer Prevention. *Pharmacol Res* **2015,** 100, 135-147.
45. Meng, X.; Earnshaw, C. J.; Tailor, A.; Jenkins, R. E.; Waddington, J. C.; Whitaker, P.; French, N. S.; Naisbitt, D. J.; Park, B. K. Amoxicillin and Clavulanate Form Chemically and Immunologically Distinct Multiple Haptenic Structures in Patients. *Chem Res Toxicol* **2016,** 29, 1762-1772.
46. Copple, I. M.; Goldring, C. E.; Jenkins, R. E.; Chia, A. J.; Randle, L. E.; Hayes, J. D.; Kitteringham, N. R.; Park, B. K. The Hepatotoxic Metabolite of Acetaminophen Directly Activates the Keap1-Nrf2 Cell Defense System. *Hepatology* **2008,** 48, 1292-1301.

Tables

Table 1. CDDO-Im modified GSTP peptides detected *in vitro*[1]

Entry	Amino acid	Peptide[2]	m/z	Δm
1	Cys14	GR*C*AALR	407.2529	473.3
2	Cys14	C*AALR	503.8094	473.3
3	Cys47	ASC*LYGQLPK	785.986	491.3
4	Cys47	AS*C*LYGQLPK	776.958	473.3
5	Cys101	DQQEAALVDMVNDGVEDLR*C*K	706.238	473.3
6	Arg186	LSAR*PK	572.9	473.3

1. CDDO-Im (50 nM-10 μM) was incubated with GSTP in phosphate buffer, pH 7.4 for 16 h.
2. The modified amino acid was labelled with *.

Table 2. CDDO-Im modified HSA peptides detected *in vitro*[1]

Entry	Amino acid	Peptide[2]	m/z	CDDO-Im concentration (μM)			
				0.01	1	10	100
1	K137	K*YLYEIAR	764.94	√	√	√	√
2	Y138	KY*LYEIAR(Me)	771.9877	--	--	√	√
3	Y161	RY*K	470.3134	--	--	√	√
4	K162	YK*AAFTECCQAADK	1010.501	--	--	--	√
5	K199	LK*CASLQK	710.9803	--	--	--	√
6	K351	LAK*TYETTLEK	885.5318	--	--	--	√
7	Y353	TY*ETTLEK	729.3912	--	--	--	√
8	Y411	Y*TK	442.7766	--	--	--	√
9	K413	YTK*K	506.8359	--	--	--	√
10	K524	QIK*K	495.3375	--	--	--	√
11	K525	K*QTALVELVK	801.593	--	--	√	√
12	K541	ATK*EQLK	645.9232	--	--	--	√

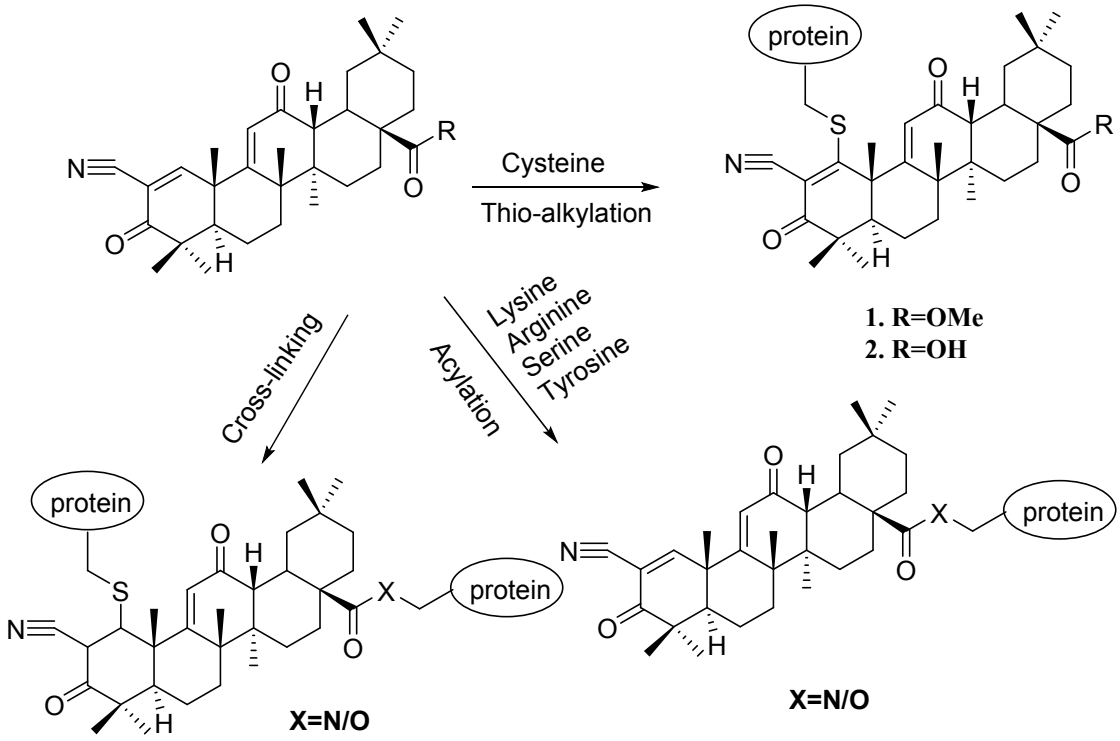
1. CDDO-Im (10 nM-100 μM) was incubated with GSTP in phosphate buffer, pH 7.4 for 16 h.
2. The modified amino acid was labelled with *.

Table 3. CDDO-Im modified Keap1 peptides detected *in vitro*[1]

Entry	Amino acid	Peptide sequence[2]	m/z	Reported binding sites
1	Cys13	PSGAGAC*C(iodo)R	676.3366	Y
2	Cys14	PRPSGAGACC*R	541.9338	Y
		PSGAGACC*R	685.8182	
3	Cys38	ASTEC*K	556.1458	N
4	Tyr85	Y*QDAPAAQFMAHK	651.9981	N
		Y*QDAPAAQFM(O)AHK	656.3312	
		(iodo)Y*QDAPAAQFM(O)AHK	675.3446	
5	Tyr208	EYIY*MHFGEVAK	654.0048	N
6	Cys257	YDC*EQR	643.801	Y
		YDC*EQ(Me)R	650.8192	
		(Iodo)YDC*EQR	672.3263	
7	Tyr263	FY*VQALLR	742.4025	N
8	Cys288	C*EILQSDSR	762.4541	Y
9	Lys323	APK*VGR	550.864	N
10	Tyr443	Y*EPER	583.812	N
11	Cys489	LNSAEC*YYPER	909.4423	Y
12	Cys513	SGAGVC*VLHN	715.3806	Y
13	Cys613	SGVGVAVTMEPC*R	897.9634	Y

1. CDDO-Im (10 μM) was incubated with Keap1 in phosphate buffer, pH 7.4 for 16 h.
2. The modified amino acid was labelled with *.

Scheme 1. Potential pathways for the formation of triterpenoid-protein adducts.



TOC

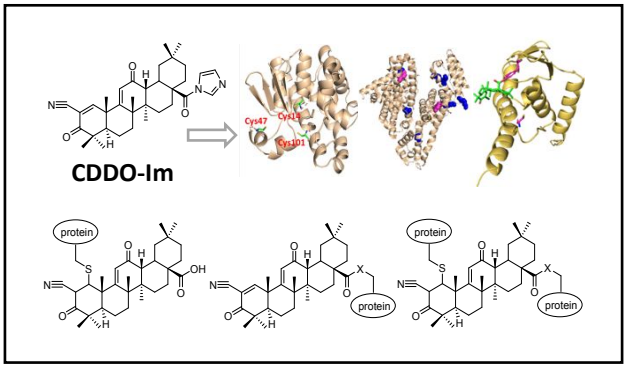

CDDO-Im
Cys47
Cys119
Cys101
protein
protein
protein
protein

Figure 1.

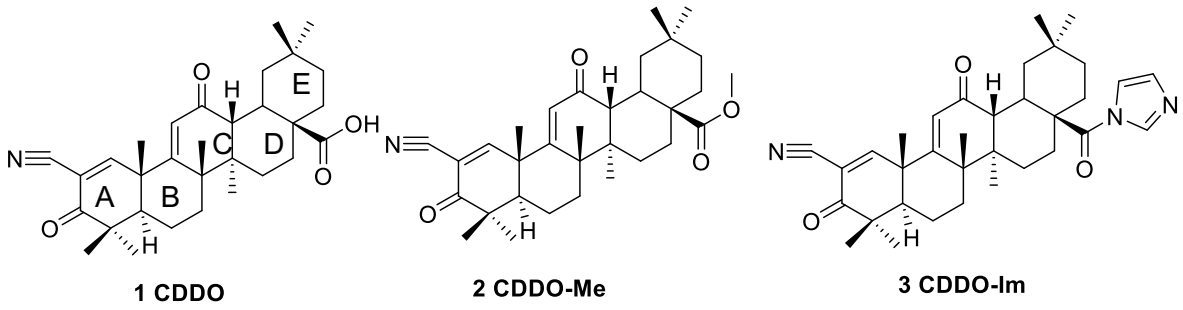


Figure 1.Chemical structures of triterpenoids

Figure 2.

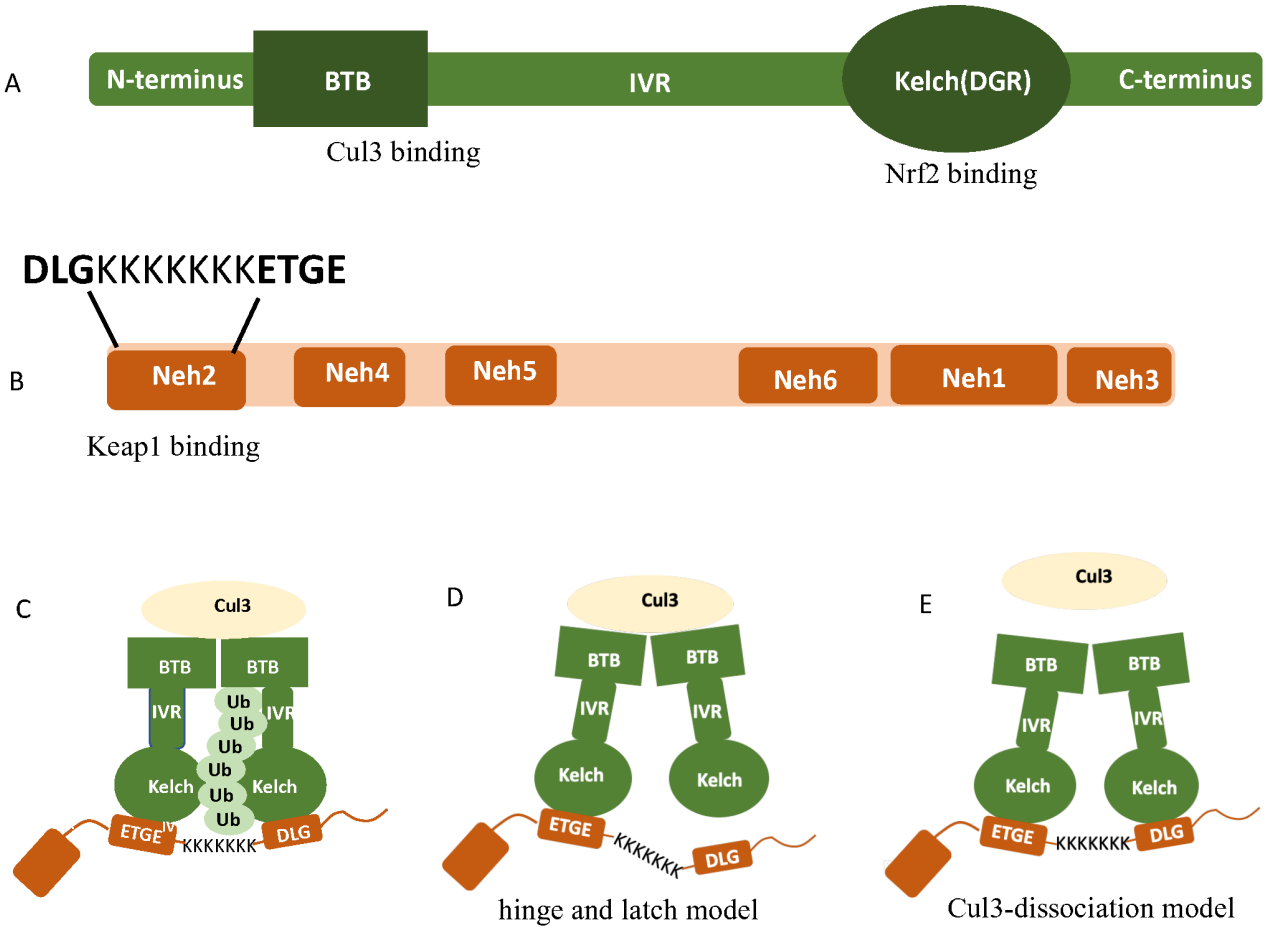


Figure 2. Current models of Keap1-Nrf2 interaction[10-14]. (A) Domain structures of human Keap1 and important binding partners are shown. (B) Domain structures of human Nrf2; DLG and ETGE binding motif in Neh2 are highlighted. (C) under basal conditions, Keap1 maintains low levels of Nrf2 by promoting its Cul3-Rbx1-mediated ubiquitination and subsequent proteasomal degradation. Under stressful conditions, covalent modification of key cysteine residues of Keap1causes conformational changes. This can either disrupt the interaction between Nrf2 and Kelch (D) or decrease the binding of Cul3 to Keap1 (E), leading to nuclear accumulation of Nrf2.

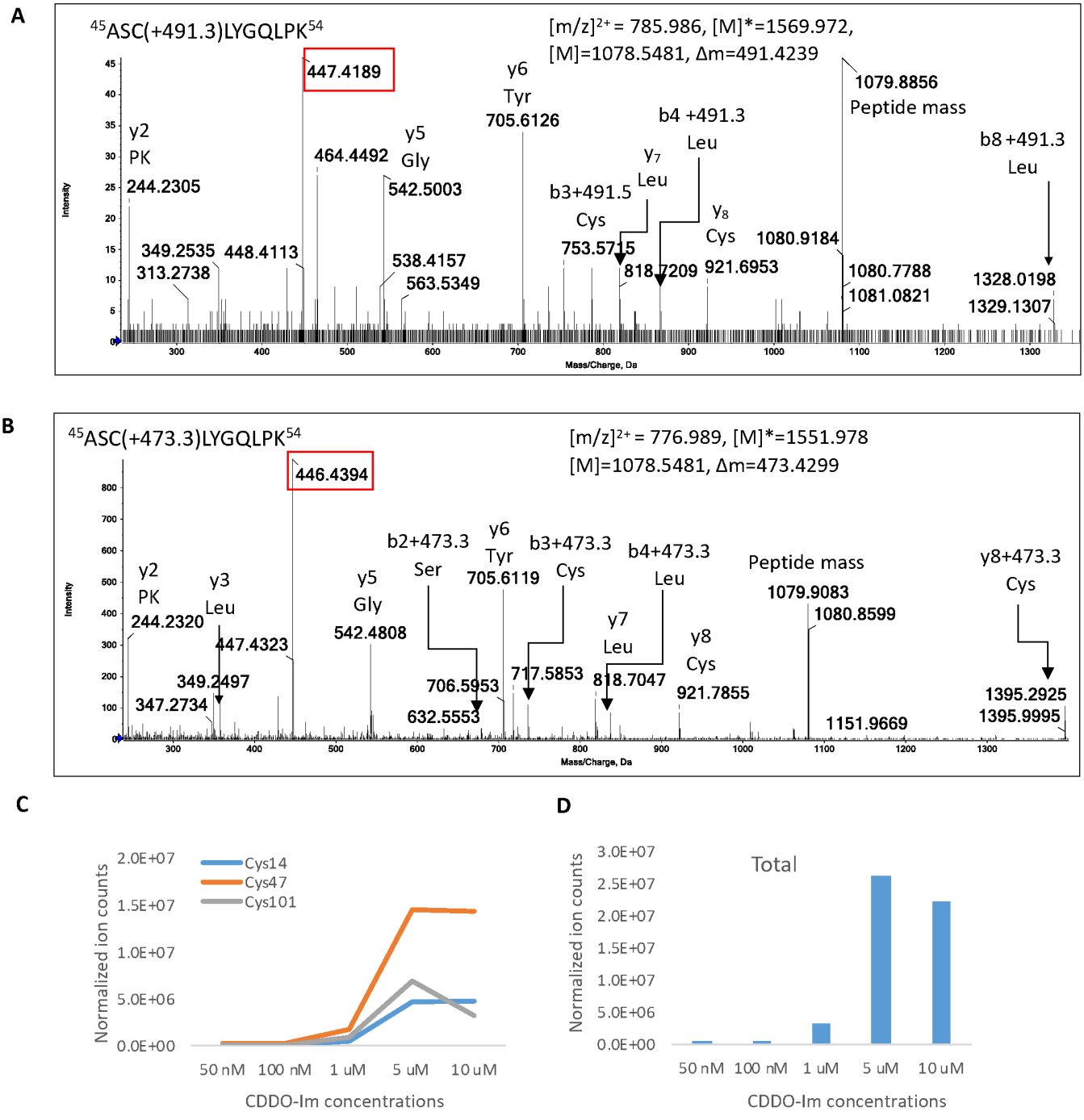


Figure 3. LC-MS/MS analysis of CDDO-Im modified GSTP peptides identified *in vitro*. MS/MS spectra show (A) peptide ^{45}ASCLYGQLPK^{54} was modified by CDDO carboxylate at Cys47 with a mass addition of 491.3 amu; (B) cross-linking adducts formed between Ser46 and Cys47 on peptide ^{45}ASCLYGQLPK^{54} with a mass addition of 473.3 amu; covalent binding to GSTP was concentration dependent as shown by individual sites (C) and total binding (D)

Figure 4

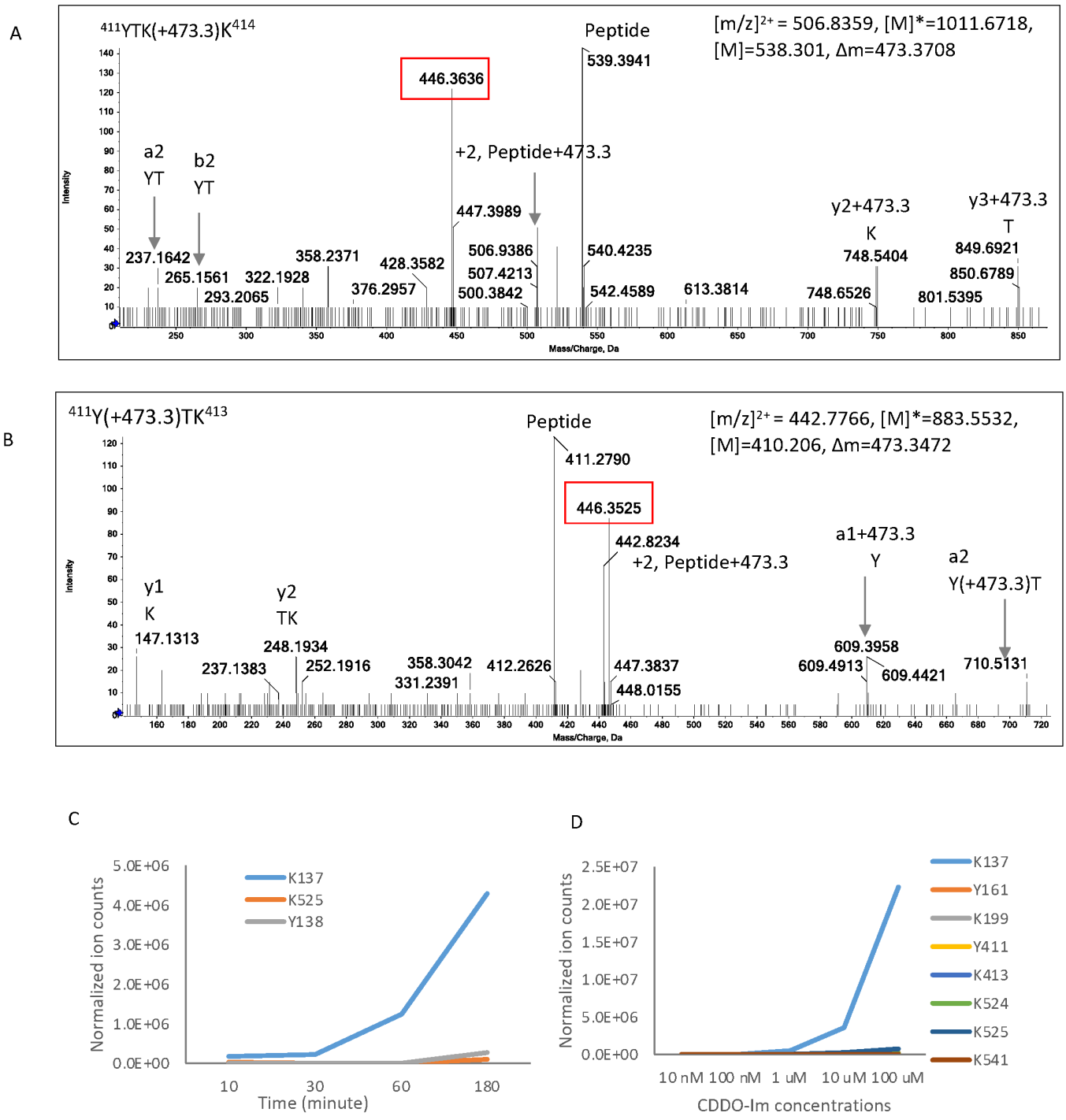


Figure 4. LC-MS/MS analysis of CDDO-Im modified HSA peptides identified *in vitro*. MS/MS spectra show (A) an acylation of Lys413 by CDDO-Im was detected on peptide ^{411}YTKK^{414} from HSA; (B) similar adducts were also detected on Tyr411 (^{411}YTK^{413}); covalent binding to HSA was time (5μM CDDO-Im) and concentration dependent (C and D)

Figure 5

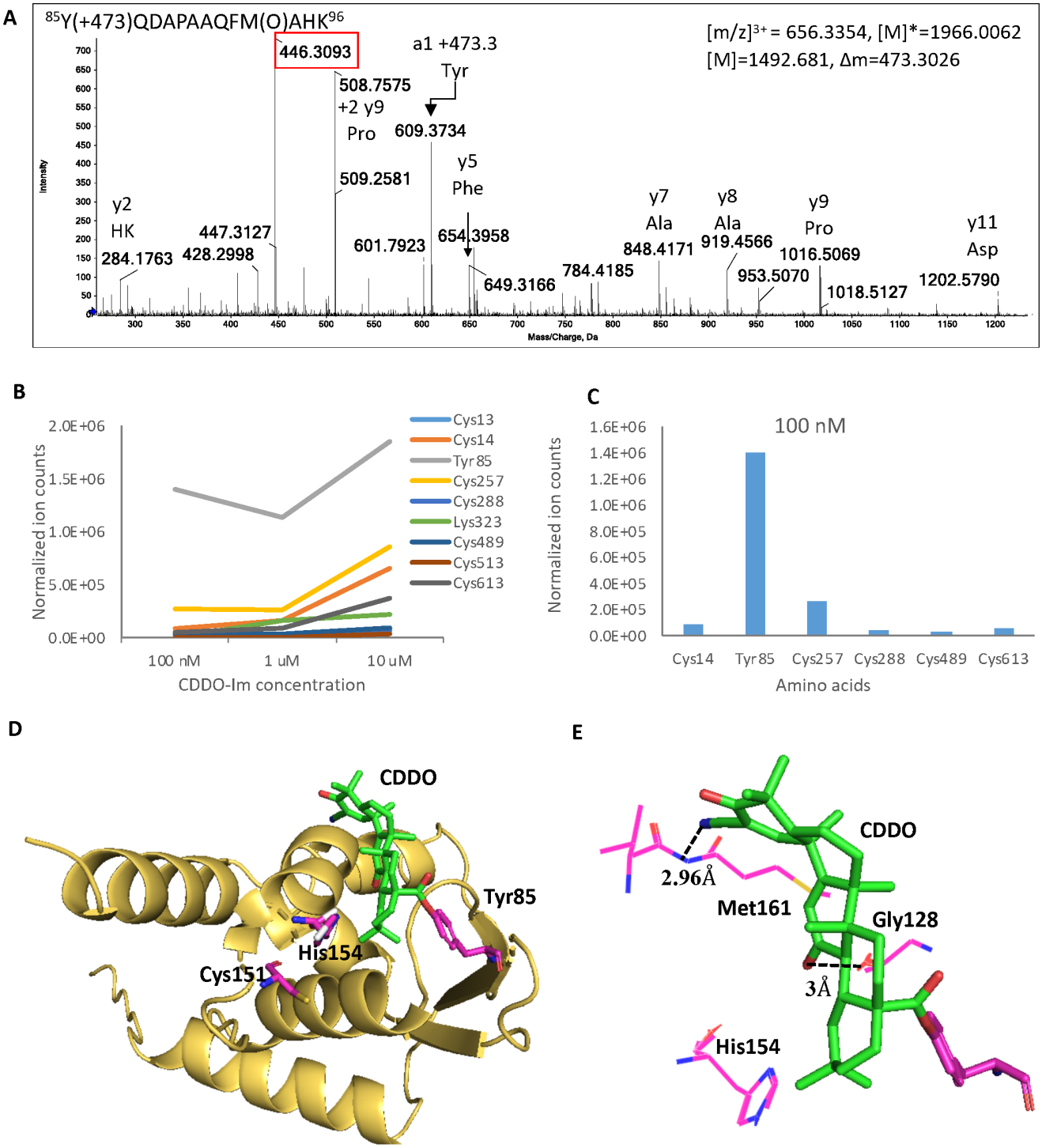


Figure 5. Covalent binding of CDDO-Im to Keap1 protein. (A) Representative MS/MS spectrum shows CDDO-Im forms an acylation adduct with Tyr85 in Keap1. (B) Covalent binding of CDDO-Im to Keap1 is concentration-dependent. (C) Relative quantification using normalized ion counts revealed that CDDO-Im (100 nM) selectively target Tyr85 when incubated with Keap1. (D) Covalent docking of CDDO-Im with Tyr85 into BTB domain of Keap1 (PDB code: 4CXI) demonstrated that the ring system positioned away from Cys151. (E) CDDO interacts with Met161 and Gly128 through hydrogen bonds that are denoted by dashed lines. Images are illustrated by PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.).

Figure 6

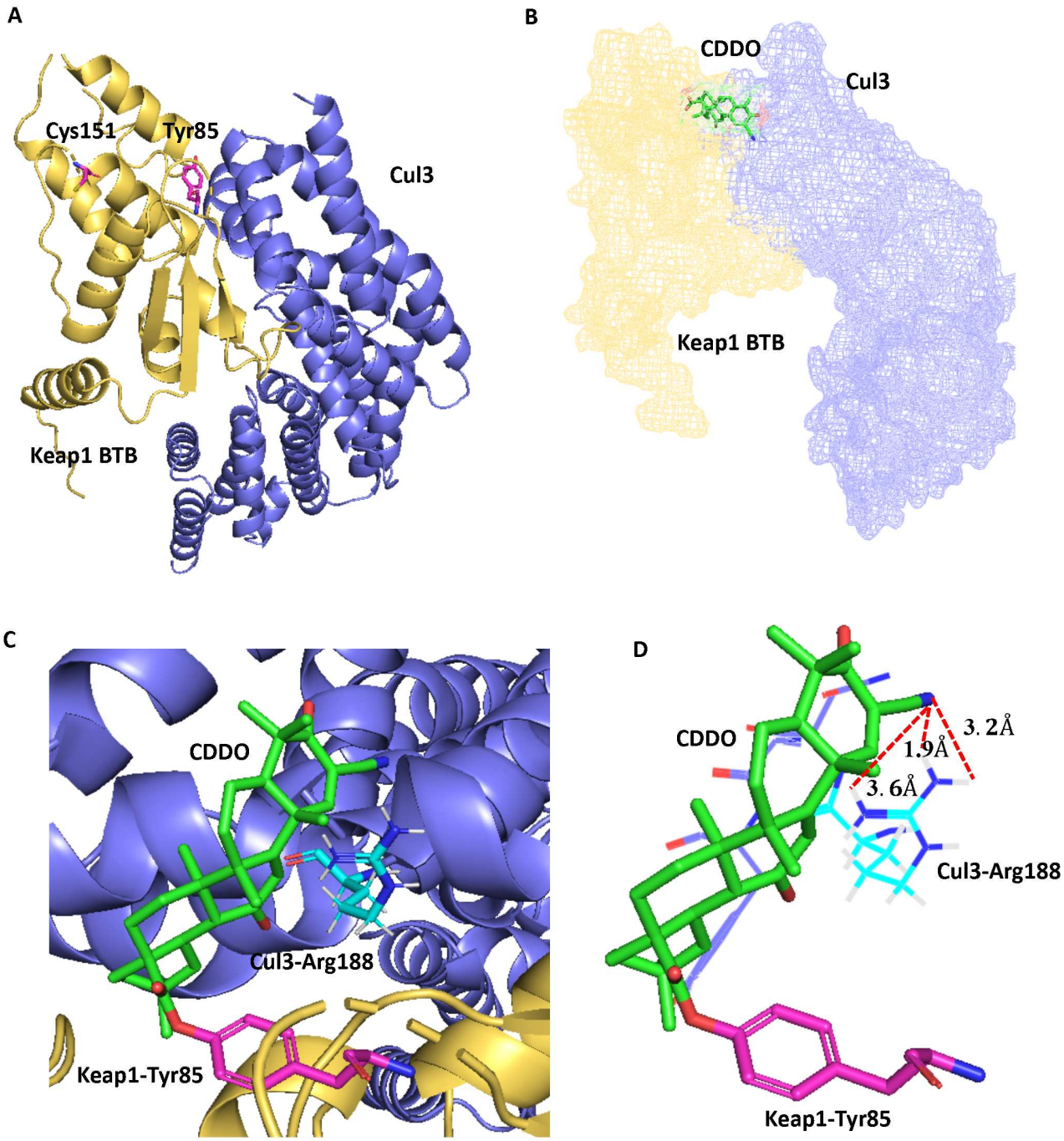


Figure 6. The effects of CDDO-Im on Keap1-Cul3 complex formation. (A) Crystal structure of Keap1-Cul3(PDB code: 5NBL) highlights the importance of Tyr85 for Cul3 binding. (B) Mesh representation of Keap1-CDDO-Cul3 complex showing CDDO can stabilize the Keap1-Cul3 complex. (C) Covalent binding of CDDO-Im to Tyr85 positions the ring system towards Cul3.(D) CDDO interacts with Arg188 in Cul3 through hydrogen bonds that are denoted by dashed lines. Images are illustrated by PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.).

Supporting information

CDDO-Imidazolide Targets Multiple Amino Acid Residues on the Nrf2 Adaptor, Keap1

Xiaoli Meng[*1], James C Waddington[1], Arun Tailor[1], Adam Lister[1], B Kevin Park[1], Neil Berry[2], Michael B Sporn[3]*

1 MRC Centre for Drug Safety Science, Department of Molecular & Clinical Pharmacology, University of Liverpool, Liverpool, UK.
2 Department of Chemistry, University of Liverpool, Liverpool, UK
3 Molecular and Systems Biology, Dartmouth Medical School, New Hampshire, USA.
*Corresponding Author

Dr Xiaoli Meng
MRC Centre for Drug Safety Science, Department of Pharmacology, University of Liverpool, Sherrington Building, Ashton Street, Liverpool L69 3GE, England
Telephone: 0044 151 7948368; e-mail: xlmeng@liverpool.ac.uk

A supplementary table and additional figures (Figure S1-S3) show the formation of CDDO-Im protein adducts and the interaction of CDDO-Im with Keap1. This material is available free of charge via the Internet at http://pubs.acs.org.

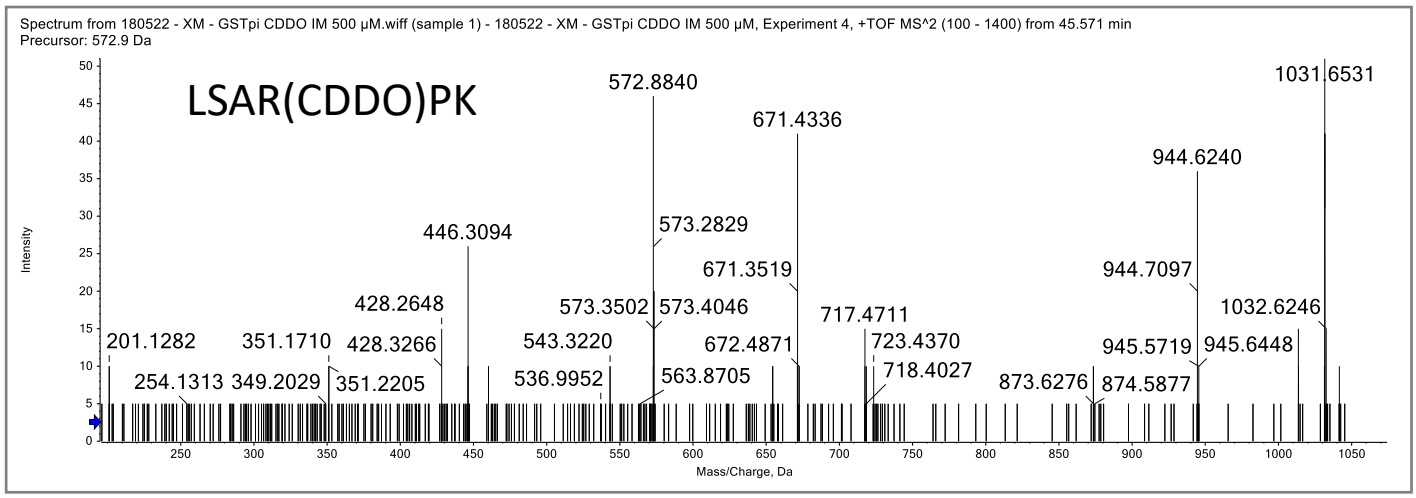
Table S1. CDDO-Im modified HSA peptides detected *in vitro*[1]

Amino acid	Peptide[2]	m/z	Retention time (minutes)
K137	K*YLYEIAR	764.9434	82.06
K195	ASSAK*QR	610.8541	68.57
K199	LK*CASLQK	710.9154	74.6
K413	YTK*K	506.8073	72.22
K432	NLGK*VGSK	638.3876	77.25
K436	VTK*CCTESLVNR	628.3306	69.94
K524	QIK*K	495.3224	72.87
K525	K*QTALVELVK	801.4988	82.61
K536	HK*PK	491.812	66.39
K541	ATK*EQLK	645.8875	73.81
R142	LDELR*DEGK	516.6239	67.25
R222	LSQR*FPK	674.7512	80.01
S193	ASS*AK	468.7725	81.83
S202	CAS*LQK	561.8137	75.9
S220	LS*QR	488.796	72.97
S232	AEFAEVS*K	677.3669	79.01
S435	VGS*K	432.2647	73.52
S470	TPVS*DR	574.3217	71.09
S489	RPCFS*ALEVDETYVPK	776.4546	81.03
Y140	YLY*EIAR	700.8973	94.19
Y161	RY*K	470.2868	76.7
Y319	NY*AEAK	584.815	83.05
Y353	TY*ETTLEK	729.3921	85.8
Y411	Y*TK	442.2242	72.97

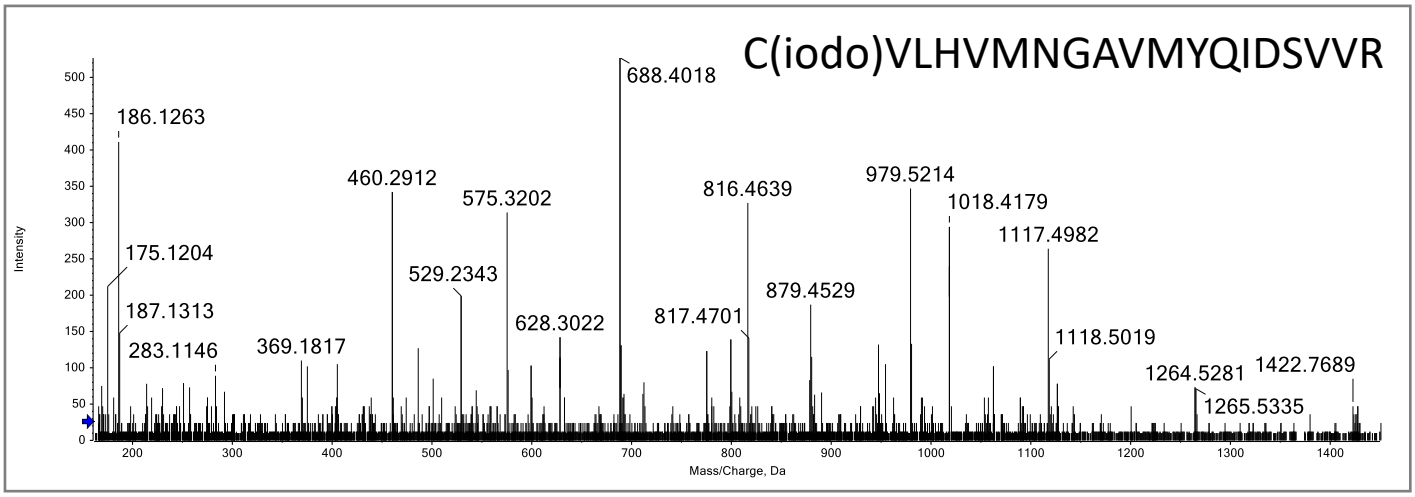
1. CDDO-Im (1mM) was incubated with HSA in phosphate buffer, pH 7.4 for 16 h.
2. The modified amino acid was labelled with *.

Figure S1

A



B



C

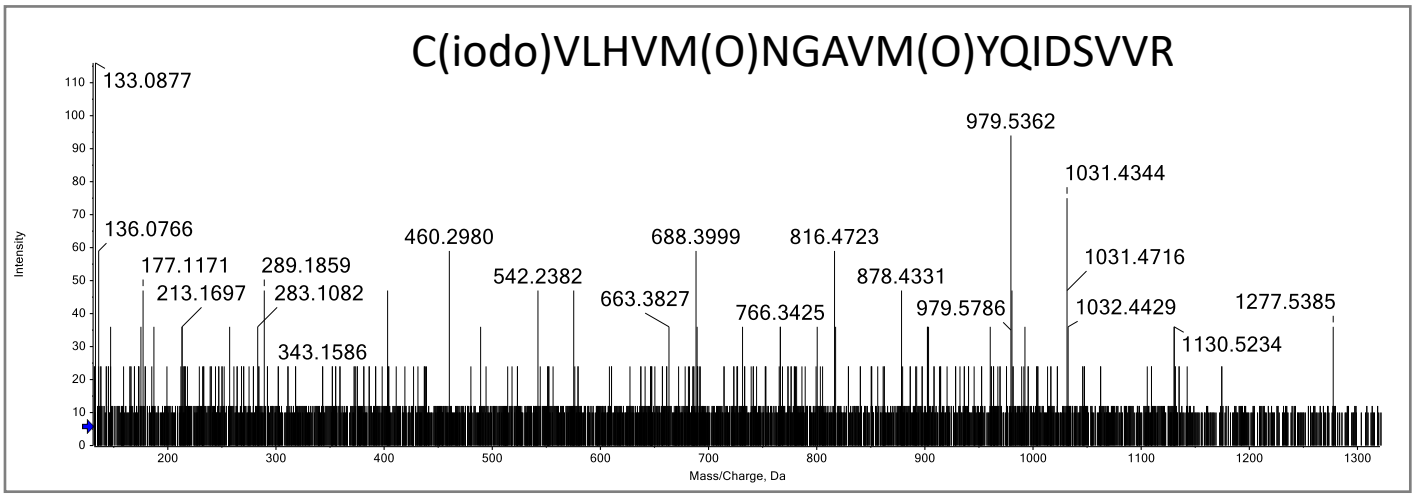


Figure S1. MS/MS spectra show (A) peptide LSARPK from GSTP was modified by CDDO-Im (500 µM) at Arg186 with a mass addition of 473.4 amu; GSTP was incubated with CDDO-Im (500μM) in phosphate buffer (10 mM, pH 7.4) for 16 h. (B& C) unmodified Cys151 containing peptide CVLHVMNGAVMYQIDSVVR from Keap1.

Figure S2

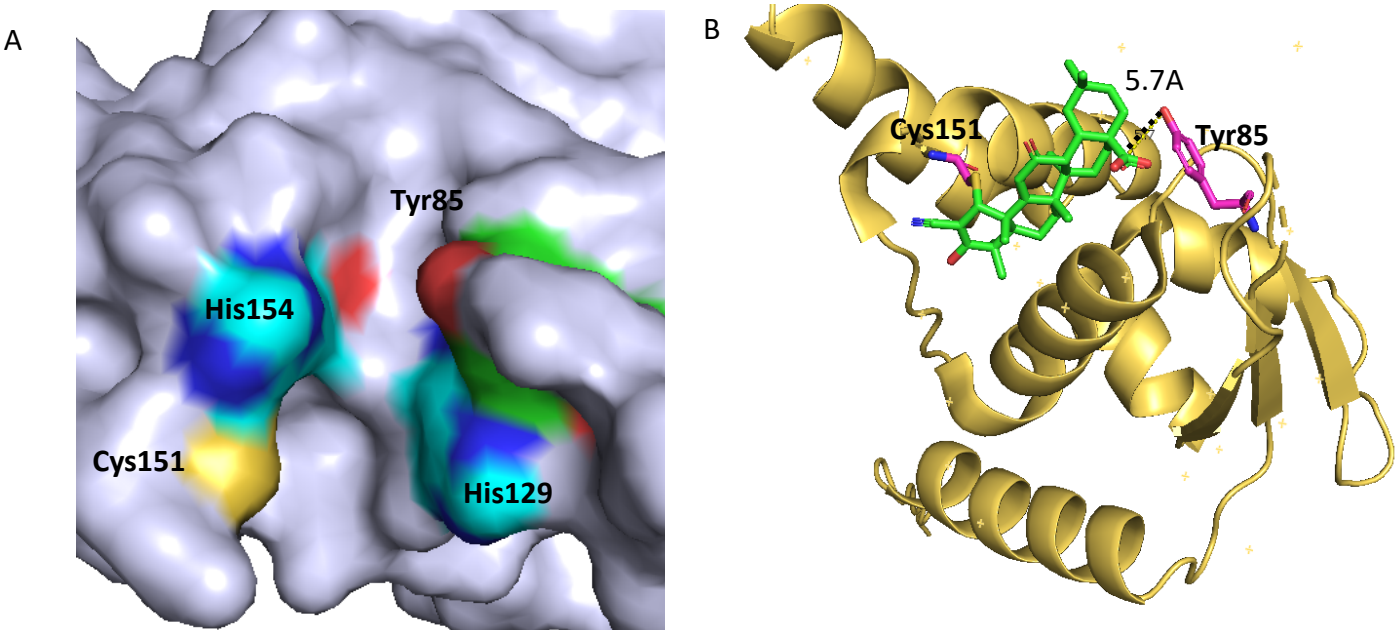


Figure S2. The structure of Keap1 BTB domain. (A) Surface representation of the Keap1 BTB domain highlighting the location of Cys151 and Tyr85 (PDB 4CXI). (B) Cleasby et al have shown that CDDO carboxylate is covalently bound to Cys151(PDB 4CXI)[1], the close proximity of carbonyl carbon to Tyr85 (5.7A) suggests that a cross-linking adduct could be formed between Cys151 and Tyr85 if CDDO-Im is bound to Cys151 in a similar conformation. Images are illustrated by PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.).

Reference

1. Cleasby, A.; Yon, J.; Day, P. J.; Richardson, C.; Tickle, I. J.; Williams, P. A.; Callahan, J. F.; Carr, R.; Concha, N.; Kerns, J. K.; Qi, H.; Sweitzer, T.; Ward, P.; Davies, T. G. Structure of the Btb Domain of Keap1 and Its Interaction with the Triterpenoid Antagonist Cddo. *PLoS One* **2014,** 9, e98896.

Figure S3

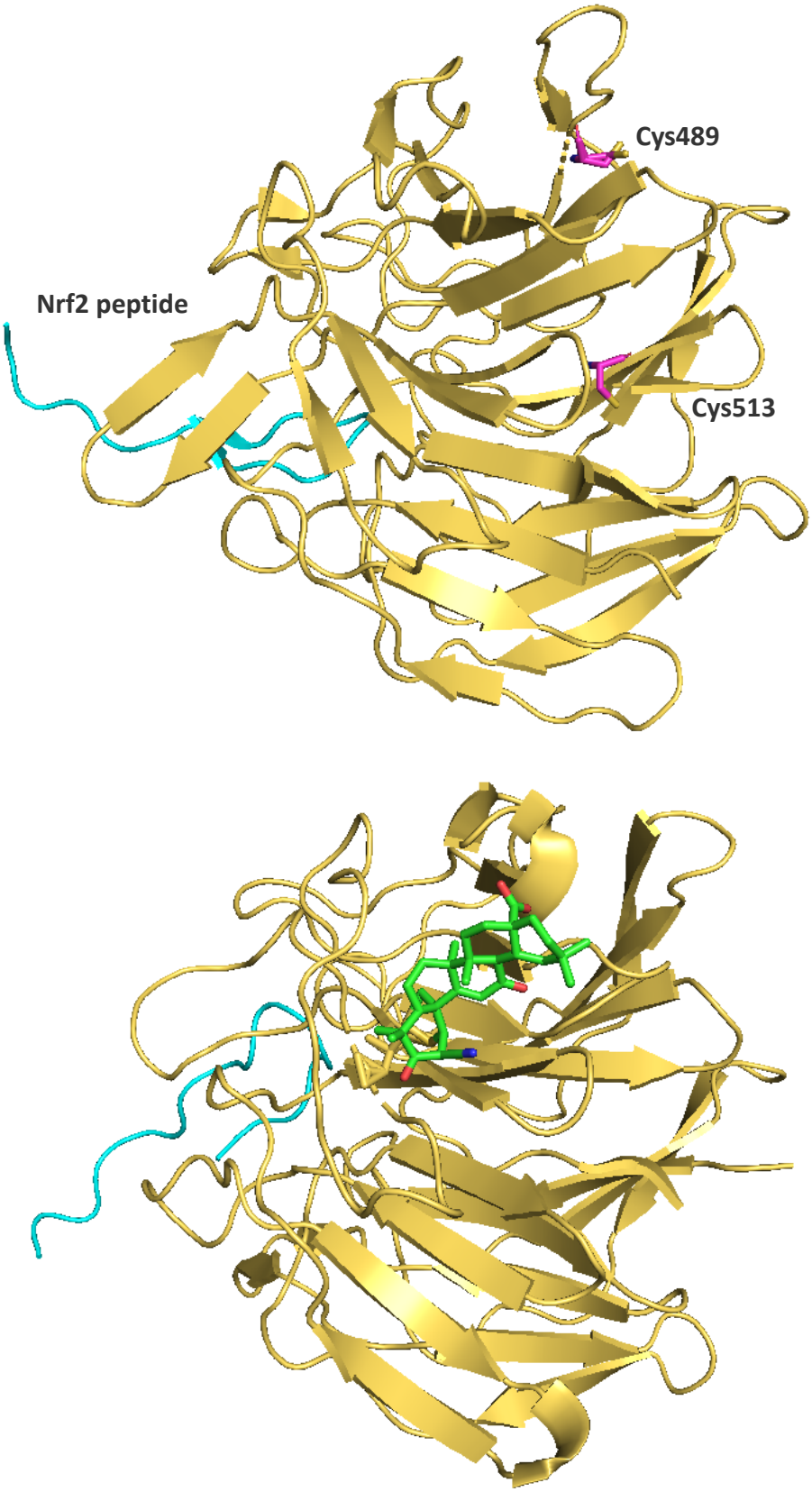


Figure S3. The interaction between Nrf2 and keap1. (A) Crystal structure of Keap1- Kelch (yellow) and bound Nrf2 peptide (Cyan) Cul3(PDB code: 2FUL). (B) Covalent binding of CDDO-Im to cysteine residues in Kelch (modification of Cys489 is shown) is unlikely to affect the Nrf2 binding as they are far away from the Nrf2 binding sites. Images are illustrated by PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.).