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The 1,4-naphthoquinone scaffold in the design of cysteine protease inhibitors

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Abstract—A series of 1,4-naphthoquinone derivatives diversely substituted at C-2, C-3, C-5 and C-8, prepared by reaction of amines, amino acids and alcohols with commercial 1,4-naphthoquinones, has been evaluated against papain and bovine spleen cathepsin B. These 1,4-naphthoquinone derivatives were found to be irreversible inhibitors for both cysteine proteases, with second-order rate constants, k_2, ranging from 0.67 to 35.4 $M^{-1} s^{-1}$ for papain, and from 0.54 to 8.03 $M^{-1} s^{-1}$ for cathepsin B. Some derivatives display a hyperbolic dependence of the first-order inactivation rate constant, k_{obs}, with the inhibitor concentration, indicative of a specific interaction process between enzyme and inhibitor. The chemical reactivity of the compounds towards cysteine as a model thiol is dependent on the naphthoquinone LUMO energy, whereas papain inactivation is not. The 1,4-naphthoquinone derivatives are inactive against the serine protease, porcine pancreatic elastase.

1. Introduction

Quinones play vital roles in the biochemistry of living cells, where they are involved in cellular respiration, photosynthesis and cellular defence against bacteria, fungi and parasites. They became the subject of intense research because of their interesting pharmacological, for example, antitumour, trypanocidal, anti-inflammatory, antiviral and antifungal, properties.[1] Some have reached clinical use as antineoplastic drugs[2] and atovaquone, a hydroxyl-1,4-naphthoquinone, is a broad-spectrum antiprotozoal drug.[3] Unfortunately, the potential usefulness of quinones is limited by their toxicity. Their actions and toxicity are still difficult to understand, although at least two competing mechanisms have been identified: (i) their capacity to undergo redox cycling, generating reactive oxygen species, and (ii) their capacity to act as electrophiles via Michael-type addition, leading to covalent modification of thiols or other vital physiological components, proteins, DNA and RNA.[2] Whether a quinone acts as an electrophile or a redox cycler is closely associated with its substitution pattern. Although the production of reactive oxygen species has been suggested to play a prominent role in their actions, recent investigations have focused on the electrophilicity of quinones, in order to explore the inhibitory potential of quinones against enzymes containing a thiol residue at or near the active site (topoisomerases and protein-tyrosine phosphatases).[4–6] In spite of the potentially high reactivity associated with quinones, it should be possible to modulate this property by the appropriate choice of substituents, as shown by atovaquone, which has a relatively favourable side-effect profile and is tolerated by children.[3]

Among the enzymes dependent on a thiol nucleophile (cysteine) at their active sites, the largest and best studied is the papain superfamily,[7] which encompasses parasite enzymes (falcipain, cruzain and rhodesain) and mammalian enzymes (e.g., cathepsins and calpains). The first group is crucial in the metabolism and reproductive function of *Plasmodium falciparum* and *Trypanosoma* spp., while elevated levels of the second group of enzymes are evident in pathological processes such as muscular dystrophy, osteoporosis, chronic inflammatory diseases and cancer.[8] Therefore, papain-like cysteine proteases represent major therapeutic targets for protease inhibitor development.

Keywords: Quinones; Papain; Cathepsin B; Cysteine proteases; Thiols; Reactivity; LUMO energy.
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Bearing in mind the reactivity of thiols to quinones, we have focused on the evaluation of the 1,4-naphthoquinone scaffold as a potential building block for the design of irreversible inhibitors of cysteine proteases. To achieve this goal, we have determined the inhibition kinetics of papain, the best known cysteine protease and as such a good model for this family of enzymes, with a group of naphthoquinones, **1** (Table 1). For papain-like cysteine proteases, the most important element of substrate specificity is the hydrophobic S2 subsite, which is highly selective for bulky *S*-amino acids such as Phe or the Leu side-chain of the epoxysuccinyl-Leu-*i*-Am inactivator, E64c (*i*-Am = *iso*-amylamine).[9] It is also known that papain favours ligands based on the *N*-Ac-*S*-Phe-Gly motif,[10] and thus Phe, Leu-*i*-Am and Gly moieties were attached to the naphthoquinone core to probe the potential interaction between inhibitors and papain. We have also investigated the inactivation of bovine spleen cathepsin B and the reactivity of compounds **1** towards cysteine to explore the relationship between the intrinsic chemical reactivity and the enzyme inhibition activity.

2. Results and discussion

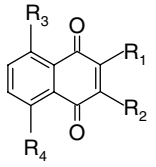
2.1. Chemistry

1,4-Naphthoquinone derivatives substituted at C-2 and C-3 were prepared by condensation of commercially available naphthoquinones with amines, *S*-α-amino acid ethyl esters or EtOH. In the first approach to the synthesis of amino acid derivatives **1a** and **1b** (Scheme 1), 2-bromo-1,4-naphthoquinone **1k** was reacted with *S*-phenylalanine ethyl ester hydrochloride (1 equiv) and TEA (2 equiv) in DCM, but after stirring for 3 h at rt, only starting material was recovered. Changing the solvent to abs EtOH, as previously described,[11,12] allowed the reaction of **1k** with amine to proceed smoothly through conjugate addition chemistry.[13] This reaction led to the formation, in a one-pot procedure, of the mono-substituted 1,4-naphthoquinones (**1a**, **1d** and **1g**) as the major products, along with their bromo analogues (**1b**, **1e** and **1h**). This is a slightly different result from that recently described for the reaction of 2-bromo-1,4-naphthoquinones with *S*-α-amino acid methyl esters, in which the non-bromo derivatives were the only products isolated.[14] However, it must be mentioned that in those investigations reactions were performed under reflux.

2.2. Reactivity studies

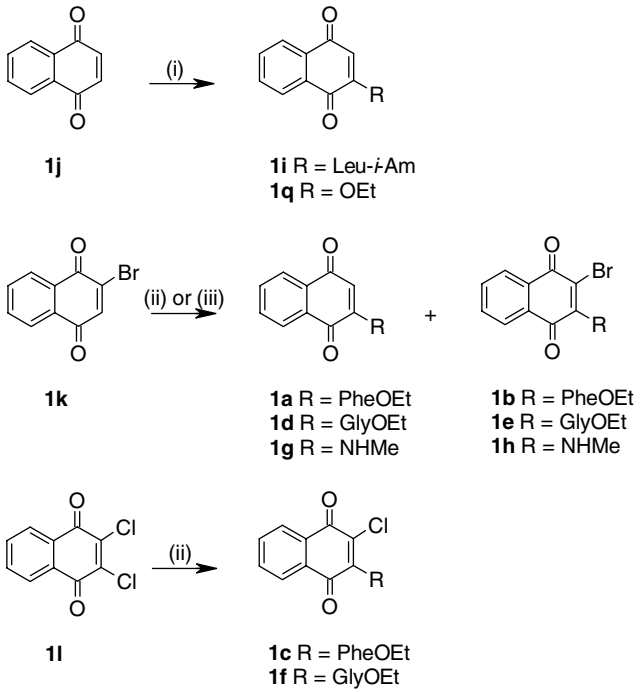
As 1,4-naphthoquinone derivatives may function as covalent-binding inhibitors of cysteine proteases, information on reactivity of these compounds towards thiols is important. In an attempt to get insight into the factors that could affect the chemical reactivity of each compound, the LUMO energies for **1a**–**s** were calculated using density functional theory (DFT). This parameter was chosen on the basis of two reported correlations; first, between the reactivity of quinones and their

Table 1. LUMO energies, cysteine-catalyzed second-order rate constants, k_{cys^-}, and papain inhibition data for naphthoquinones **1**



Compound	R^1	R^2	R^3	R^4	E_{LUMO}/Hartree	k_{cys^-} ($M^{-1}s^{-1}$)	Papain		
							k_2 (M^{-1} s^{-1})	k_{inact} (10^{-3} s^{-1})	K_I (μM)
1a	PheOEt	H	H	H	−0.1146	25.9	NI[a]	—	—
1b	PheOEt	Br	H	H	−0.1214	—	1.92	—	—
1c	PheOEt	Cl	H	H	−0.1216	—	0.67	—	—
1d	GlyOEt	H	H	H	−0.1097	0.49	35.4	0.34 ± 0.05	9.57 ± 4.18
1e	GlyOEt	Br	H	H	−0.1172	—	14.7	1.32 ± 0.03	89.6 ± 7.08
1f	GlyOEt	Cl	H	H	−0.1174	—	5.35	1.42 ± 0.16	266 ± 68.4
1g	NHMe	H	H	H	−0.1070	0.24	0.76	—	—
1h	NHMe	Br	H	H	−0.1149	—	1.03	—	—
1i	Leu-*i*-Am	H	H	H	—	—	1.35	0.30 ± 0.04	222 ± 39.4
1j	H	H	H	H	−0.1245	2851.2	(49.5 ± 4.2)[b]	—	—
1k	Br	H	H	H	−0.1320	—	33.3	2.20 ± 0.31	66.0 ± 20.0
1l	Cl	Cl	H	H	−0.1370	—	6.39	—	—
1m	Morpholinyl	Cl	H	H	−0.1161	93.1	9.29	—	—
1n	Me	H	H	H	−0.1193	114.3	4.90	—	—
1o	Me	H	Me	Me	−0.1133	—	NI[a]	—	—
1p	OH	H	H	H	−0.1170	—	(82.0 ± 4.4)[c]	—	—
1q	OEt	H	H	H	−0.1114	10.9	11.6	1.34 ± 0.18	115 ± 43.7
1r	H	H	OH	H	—	—	(60.4 ± 4.1)[b]	—	—
1s	H	H	OAc	H	−0.1335	—	14.6	—	—

[a] No inhibition when tested up to their solubility limit.
[b] Inhibition % at 65 μM after 30-min incubation.
[c] Inhibition % at 1.3 mM after 20-min incubation.



Scheme 1. Reagents: (i) TEA, abs EtOH for **1q** and TEA, Leu-*i*-Am, abs EtOH for **1i**; (ii) PheOEt or GlyOEt hydrochlorides, TEA, abs EtOH for **1a**–**f**; (iii) R = $MeNH_2$, abs EtOH for **1g**, **h**.

LUMO energy,[6,15,16] and second, between LUMO energies and electronic descriptors[17] thereby providing an alternative approach to QSAR studies.[17–20] In the context of this latter observation, in the present study, we observed a correlation between the LUMO energy of compounds **1d**, **1g**, **1j**, **1k**, **1l**, **1n** and **1q** (Table 1) and the corresponding Hammett σ_p constants for C-2 and C-3 substituents ($r^2 = 0.910$; data not shown).

The reaction between naphthoquinones and cysteine was followed spectrophotometrically at 25 °C. On mixing the reactants, there was a progressive change in the absorbance, followed by a plateau region. Subsequently, the reaction was characterized by continuous spectral changes. Some of the spectra did not present an isosbestic point. These observations are similar to previous reports[18] regarding the reactivity of quinones, which related this pattern to the occurrence of complex secondary reactions involving the hydroquinone product that results from the initial Michael addition. Since the kinetic studies were performed without protection from air, this complexity may arise from the aerobic oxidation of those products.[6,21] In order to simplify the kinetic interpretation of the overall process, some of the previous investigations were performed with exclusion of oxygen. To the best of our knowledge, spectrophotometry has been used only once to investigate naphthoquinones,[21,22] and then under anaerobic conditions.[21] Nevertheless, it has been recognized that these conditions may compromise any application of the results to biological systems.[21]

In the present work, the reaction was followed at a fixed wavelength until completion of the initial spectral changes and before any significant secondary reaction was observed. The UV–visible spectra were also recorded in the presence of buffer lacking cysteine; the lack of change between scans obtained over 19 h excluded any background reaction. For the reactions involving cysteine, plotting the pseudo first-order rate constant, k_{obs}, versus the total cysteine concentration, $[cys]_t$, gave an intercept at $[cys]_t = 0$ indistinguishable from zero and a slope, k_{cat}, which, in principle, contains the contributions of both cysteine and its thiolate to the overall reaction. For **1n**, k_{cat} values were determined at different pH values (7.1, 7.9, 8.0 and 8.8) and then plotted against the fraction of cysteine present as the free base, $\alpha = [cys^-]/[cys]_t$ (Fig. 1). The linear correlation showed an intercept at $\alpha = 0$ indistinguishable from zero, indicating that there is no detectable reaction for the undissociated thiol.[23] The second-order rate constant for the reaction with the thiolate anion, k_{cys^-}, was determined from the intercept at $\alpha = 1$. This result is consistent with previous studies with quinones which claim thiolate anion as the reactive species.[18,21] For the remaining compounds, k_{cys^-} values were determined by $k_{cys^-} = k_{cat}/\alpha$ (Table 1).

Naphthoquinone **1j**, however, revealed a hyperbolic dependence of k_{obs} on the total cysteine concentration (Fig. 2). Such a dependence has been reported for the reaction of dopaminoquinone with cysteine[24] and has been ascribed to an initial formation of a reversible

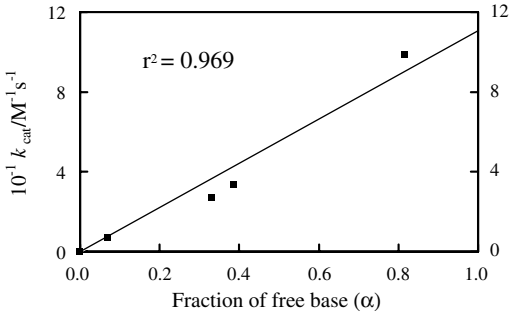


Figure 1. Plot of the catalytic rate constants, k_{cat}, for the reaction of **1n** with cysteine, against the fraction of free base, α, at 25 °C and $I = 0.5$ M (KCl).

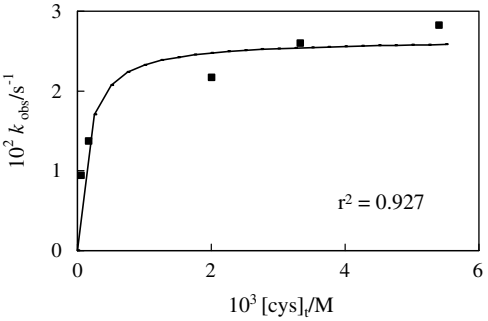


Figure 2. Plot of the pseudo first-order rate constant, k_{obs}, for the reaction of naphthoquinone **1j** with cysteine, against the total cysteine concentration at pH 7.08 ($\alpha = 7.08 \times 10^{-2}$) at 25.0 °C and ionic strength 0.5 M. Points are experimental and line is theoretical for $1/K_{cys}$ $9.26 \times 10^{-6} \pm 3.16 \times 10^{-6}$ M and k_{cys} $2.64 \times 10^{-2} \pm 0.17 \times 10^{-2}$ s^{-1}.

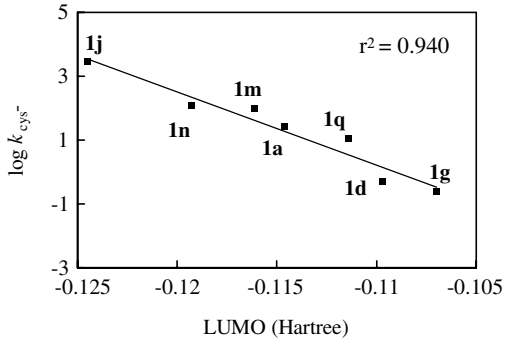
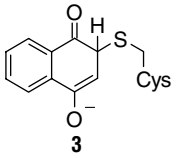


Figure 3. Linear correlation between the LUMO orbital energies and the second-order rate constants, k_{cys^-}, for the reaction of **1** with cysteine.

tetrahedral intermediate, resulting from cysteine conjugate addition to C-6 (dopaminoquinone numbering), which is then followed by the decomposition into the final product. Assuming that reaction of **1j** with cysteine *thiolate* also involves initial reversible formation of an adduct (e.g., **3**), then k_{obs} dependence on $[cys]_t$ follows Eq. 1,

$$k_{obs} = \frac{k_{cys}[cys]_t}{\{[(1/K_{cys})(K_a + [H^+])/K_a] + [cys]_t\}} \quad (1)$$

where K_{cys} is the stability constant for the formation of the adduct, k_{cys} is the first-order rate constant for the decomposition of the adduct and $(K_a + [H^+])/K_a$ is $1/\alpha$. Kinetic data were computer-fitted to Eq. 1 allowing the determination of K_{cys} (1.08×10^5 M^{-1}), of k_{cys} (2.64×10^{-2} s^{-1}) and thus of the second-order rate-constant for the reaction with the thiolate anion k_{cys^-} ($= k_{cys}K_{cys}$).



3

The k_{cys^-} values varied by up to four orders of magnitude, with naphthoquinone **1j** as the most effective acceptor for cysteine (Table 1). Plotting log k_{cys^-} against the corresponding LUMO energies resulted in a linear correlation (Fig. 3), which shows that the lower the LUMO energy the more reactive the molecule. Calculation of the LUMO density for the glycine derivative **1d** (Fig. 4), as well for other aminoacid derivatives, showed that for these compounds this orbital is largely centred on the double bond of the quinone ring. Overall, these observations are consistent with the occurrence of a Michael-type addition, which is dependent on the electrophilicity of the naphthoquinone.

2.3. Enzyme inhibition studies

Kinetic inhibition assays were performed by Kitz and Wilson's pre-incubation method.[25] Accordingly, enzyme inactivation may be represented by the minimal reaction depicted in Eq. 2, where E·I represents the non-covalent enzyme–inhibitor complex, E ~ I the covalently bound complex, K_I represents the dissociation constant of the E·I complex and k_{inact} is the first-order rate-constant for the chemical inactivation step. The compounds were found to inactivate papain, at a rate dependent on the concentration of the inhibitor as well as on the incubation time. This type of kinetic profile has been related recently to the quinone behaving as an electrophile, in contrast with a redox-dependent mechanism which was much more affected by the concentration of the reducing agent dithiothreitol (DTT) than by the inhibitor concentration.[26] Therefore, the present results are consistent with covalent modification of the enzyme. The time dependency of inhibition also suggested an irreversible process, which was confirmed by dialyses carried out after reaction of the Gly derivative **1d** with papain, from which no enzyme activity could be recovered.

$$E + I \underset{K_I}{\rightleftharpoons} E \cdot I \overset{k_{inact}}{\rightarrow} E \sim I \quad (2)$$

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]} \quad (3)$$

For compounds **1d**, **1e**, **1f**, **1i**, **1k** and **1q**, the pseudo first-order inactivation rate constants, k_{obs}, displayed a hyperbolic dependence with [I] (Fig. 5a) indicative of saturation inhibition kinetics, which suggests that the inhibition process is preceded by a specific interaction between enzyme and inhibitor in a Michaelis-type complex (Eq. 3). In the case of derivatives **1b**, **1c**, **1h**, **1i**, **1l**, **1m**, **1n** and **1s**, k_{obs} varied linearly with inhibitor concentration, suggesting that $K_I \gg [I]$, the corresponding slope being the apparent second-order rate constants for the inactivation process k_2 ($=k_{inact}/K_I$) (Fig. 5b). Overall, 1,4-naphthoquinone derivatives inhibited papain with the second-order rate constants for the inactivation process, k_2, ranging from 0.67 to 35.4 M^{-1} s^{-1}, which represents ~50-fold variation in their inhibitory activity (Table 1). Unfortunately, the Phe and Leu-*i*-Am fragments did not improve inhibitory potency, although the observation of a hyperbolic dependence for the Leu-*i*-Am derivative **1i** supports a specific inhibition process. The phenylalanine derivative **1a** did not inactivate papain. The lack of activity of compounds bearing a phenylalanine residue directly attached to the reactive site has been previously observed for vinylogous amino acid esters and ascribed to a binding mode where the Michael acceptor is far from the reach of the sulfur atom of Cys25 due to the interaction between phenylalanine and the S2 hydrophobic subsite of papain.[27] The glycine derivative **1d** ($k_2 = 35.4$ M^{-1} s^{-1}) and 2-bromo-1,4-naphthoquinone **1k** ($k_2 = 33.3$ M^{-1} s^{-1}) were the most potent inhibitors. Comparing the kinetic parameters of **1d** and **1k**, it is clear that the first-order rate constant for the chemical inactivation step, k_{inact}, plays an important role in the inhibitory activity of **1k**. In fact, this compound showed the highest k_{inact} of all, consistent with the propensity of halo-quinones to undergo nucleophilic substitution, ascribed to the increased electron affinity provided by the substituent.[13]

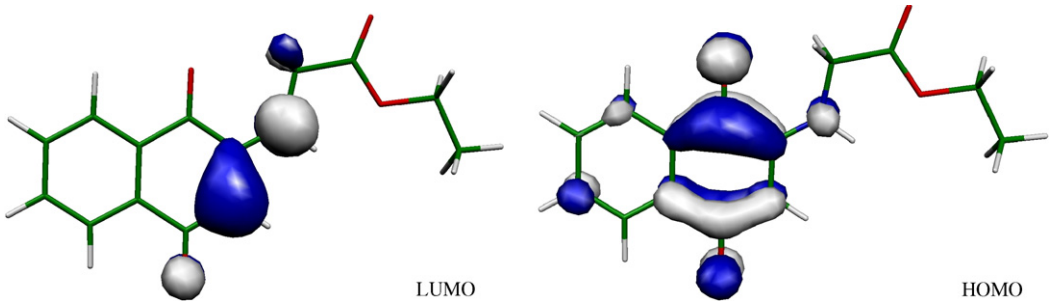


Figure 4. Frontier molecular orbitals of a low-energy conformation of the glycine derivative **1d**.

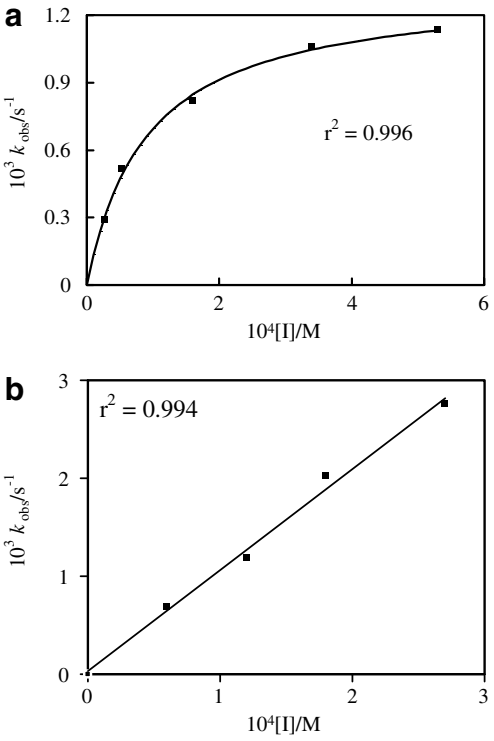


Figure 5. (a) Plot of the pseudo first-order inactivation rate constant, k_{obs}, against inhibitor concentration for the inactivation of papain by derivative **1e**; (b) plot of the pseudo first-order inactivation rate constant, k_{obs}, for the inactivation of papain by derivative **1h** against inhibitor concentration.

The expected contribution of an electron-withdrawing group to the increase of the activity is also clear in the Phe and NHMe series, particularly in the case of the brominated phenylalanine derivative **1b** (1.92 M^{-1} s^{-1}) compared to its inactive non-halo counterpart **1a**. In contrast, the potency of the glycine derivative **1d** is connected with its K_I (9.57 μM), the lowest in the series. This suggests that the Gly residue is able to promote stabilization of the non-covalent inhibitor–enzyme complex, directing the naphthoquinone moiety to the active cysteine residue thereby enhancing the inhibitory potency, even at low alkylation rate constants. Modelling the interaction of this compound with papain, using the program GOLD, revealed that all 10 inspected conformations presented hydrogen bonding interactions between the carbonyl oxygen atom at C-4 and NH of Gln19 (Fig. 6). This orientation presents C-3 close (2.8–2.9 Å) to the sulfur atom of Cys25, as would be expected for Michael addition chemistry. The ethyl glycinate moiety is pointing towards the S2 pocket, although it does not reach this binding site.

The suggestion that the ability of the inhibitor to fit in the active site of papain may be much more important than the intrinsic electrophilicity of the naphthoquinone is further supported by the comparison of the kinetic parameters for the glycine derivatives (**1d–f**), for which the halo compounds **1e** and **1f** are weaker inhibitors than the non-halo counterpart **1d** (largely, though not exclusively, due to an increase in the K_I value). These observations also suggest that substitution both in the C-2 and C-3 positions of the naphthoquinone core may be detrimental to inhibitory activity. In fact, a similar situation seems to occur in the case of the methylamino bromo derivative **1h** which, in contrast to 2-bromo-1,4-naphthoquinone **1k**, did not exhibit saturation kinetics.

Naphthoquinone **1j**, lawsone **1p** and juglone **1r** were shown to be time-dependent inhibitors but, unfortunately, graphical representation of v/v_0 versus incubation time presented a scattering of points which were poorly fitted to a pseudo-first-order kinetics, so their inhibitory activity was evaluated at fixed time and concentration of the inhibitor. For compounds **1j** and **1r**, this departure from the usual behaviour could result

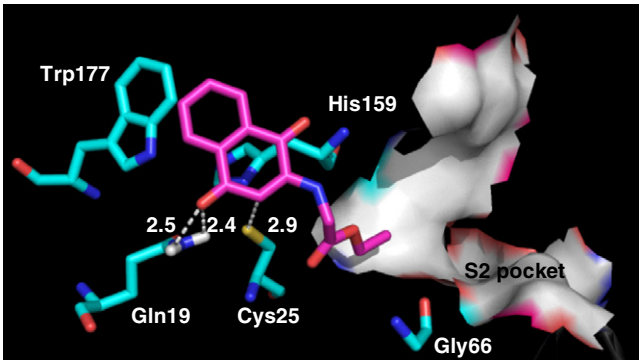


Figure 6. GOLD-generated active site interaction of papain and **1d** (magenta). Figure generated using Pymol software: DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA. http://www.pymol.org.

from instability of compounds in the reaction buffer with DTT, which is consistent with the report that these compounds have a high reactivity towards simple thiols,[22] and with observation that the incubation mixture turned from pale yellow to brown, this colour change only occurring in the presence of DTT. It is known that quinones can transfer electrons from DTT to oxygen.[26] However, inhibition by **1r** was not prevented by keeping the incubation mixture under nitrogen, or by the addition of ascorbic acid to the reaction buffer. Therefore, the possible existence of significant redox cycling can be excluded.

In an attempt to assess how the electronic properties of the substituents affect the rate of enzyme inhibition, we analysed LUMO energy values along with the kinetic parameters of papain inhibition (Table 1). One interesting finding from compounds **1d**–**f**, **1k** and **1q** is a trend in which k_{inact} values increase as the corresponding LUMO energies decrease (Table 1), suggesting that the chemical inactivation step of papain might be a function of the LUMO energy of the inhibitor. Even so, as k_{inact} values vary only 7-fold (**1k** versus **1i**), whereas K_I values vary ~30-fold (**1d** vs **1f**), it would seem that the overall activity of the compounds towards papain is governed to a large extent by the molecular recognition of the inhibitor prior to the irreversible reaction step. Indeed, it was not possible to establish any correlation between the apparent second-order rate constants, k_2, and LUMO energies, indicating that inhibitory activity does not parallel chemical reactivity.

The best inhibitors were tested against bovine spleen cathepsin B, which shares about 80% homology with the human liver variety.[28] Cathepsin B, one of the best characterized mammalian cysteine proteases, is implicated in a variety of diseases.[29] The presently reported compounds were found to be time-dependent inhibitors, with second-order rate constant k_2 values 3–20 times smaller than those for papain (Table 2). The most potent inhibitor for papain, the glycine derivative **1d**, was also the most active against cathepsin B. Its dissociation constant K_I is also the lowest in the series, with a similar value to that with papain. In contrast, 2-bromo-1,4-naphthoquinone **1k** exhibited a linear dependence between k_{obs} and [I], suggesting a drastic increase in the K_I value. Against a serine protease, porcine pancreatic elastase (PPE), this group of derivatives was shown to have no activity when incubated for 2 h over the same range of concentrations used against papain and cathepsin B; this suggests that 1,4-naphthoquinone derivatives may be selective inhibitors for cysteine proteases.

Table 2. Second-order rate constants, k_2, for inactivation of cathepsin B by **1**

Compound	Bovine spleen cathepsin B		
	k_2 (M^{-1} s^{-1})	k_{inact} (10^{-4} s^{-1})	K_I (μM)
1d	8.03	1.12 ± 0.06	14.0 ± 3.07
1e	4.37	2.44 ± 0.24	55.9 ± 14.1
1k	5.65	—	—
1q	0.54	4.28 ± 0.63	797 ± 228

3. Conclusion

It has been shown that 1,4-naphthoquinone derivatives are able to inhibit the cysteine proteases papain and bovine spleen cathepsin B in an irreversible manner. The results suggest that the inactivation process is more affected by the specific recognition interactions between inhibitor and enzyme than by the electrophilicity of the naphthoquinone moiety. This observation is quite important considering that intrinsic chemical reactivity of naphthoquinones may be cited as a potential limitation to their applicability under biological conditions, where other thiol species are expected to occur at high concentrations, for example, glutathione, promoting unspecific undesirable conjugations. Thus, the 1,4-naphthoquinone scaffold appears to be a reliable approach to the development of papain-like cysteine protease inhibitors. By adjusting the inherent chemical reactivity downwards and exploiting the specific binding requirements of the nucleophilic attack it should be possible to tailor quinone and quinone-like inhibitors to a variety of cysteine enzymes that are potential therapeutic targets.

4. Experimental

4.1. General information

All materials were used without further purification. Reagent grade chemicals were bought from Sigma–Aldrich (naphthoquinones **1j**, **1k**, **1l**, **1m**, **1n** and **1r**, amino acid ethyl esters, Boc-protected amino acid *N*-hydroxysuccinimide ester, *iso*-amylamine) and Merck (methylamine and solvents). Papain (Sigma P4762), bovine spleen cathepsin B (Sigma C6286, 10 U), PPE (Sigma E0258), benzoyl-*S*-arginine-4-nitroanilide (BAPA), Z-*S*-Lys-ONp, Suc-Ala-Ala-Ala-4-nitroanilide and 0.1 M Hepes buffer (H9897) were obtained from Sigma–Aldrich. DMSO and buffer materials for kinetics measurements were of analytical grade and were purchased from Merck (Germany). Derivatives **1o** and **1s** resulted from previous investigations.[30] Dialysis experiments were carried out with Spectra/Por 4 dialysis membranes, 12000–14000 MW (Spectrum Laboratories Inc. 132697). Column chromatography was performed with silica gel (Merck, 230–400 mesh ASTM). TLC was performed on pre-coated silica gel 60 F_{254} (Merck) and visualized under UV light. The IR spectra were determined using NaCl solution cells on a Nicolet Impact 400 FTIR spectrophotometer and only the most significant absorption bands are reported. Low-resolution mass spectra were recorded using VG Quattro LCMS instruments. Elemental analyses were performed using an EA 1110 CE Instruments automatic analyser. The ^1H and ^{13}C NMR were recorded on a Bruker Avance 400 NMR spectrometer (^1H 400 MHz; ^{13}C 100.62 MHz) in $CDCl_3$ solution; chemical shifts, δ, are expressed in ppm. Signal assignments were effected using ^1H–^1H COSY, HMQC and HMBC 2D NMR techniques. Coupling constants (*J*) are reported in hertz (Hz). Kinetic measurements were carried out using either Shimadzu UV UV-2100 or Shimadzu UV-1603

spectrophotometers, equipped with a temperature controller (CPS).

4.2. Synthesis

S-Phenylalanine ethyl ester hydrochloride (97 mg; 0.4 mmol) and TEA (85 mg; 0.8 mmol) were added to a solution of 2-bromo-1,4-naphthoquinone (102 mg; 0.4 mmol) in abs EtOH (15 mL). After stirring at rt for 1 h, the solvent was evaporated to yield crude product (300 mg). Compounds **1a** (61 mg) and **1b** (16 mg) were separated by column chromatography on silica gel (30 g) using DCM as eluent (0.5 L). Compounds **1c**, **1d**, **1e** and **1f** were prepared using the same method from the appropriate naphthoquinone and *S*-amino acid ethyl ester.

4.2.1. *N*-(1,4-Naphthoquinon-2-yl)-*S*-phenylalanine ethyl ester (1a). Light orange gummy solid (42%); IR (film): ν_{max} = 3374, 1737, 1673, 1571 cm^{-1}. δ ^{1}H NMR 1.24–1.27 (m, 3H, CH_2CH_3), 3.19 (dd, 1H, *J* = 6.6, 14.0, C*H*HPh), 3.28 (dd, 1H, *J* = 5.6, 14.0, CH*H*Ph), 4.19–4.24 (m, 2H, CH_2CH_3), 4.32 (br ddd, 1H, *J* = 5.6, 6.6, 7.6, CH), 5.71 (s, 1H, H-3), 6.33 (d, 1H, *J* = 7.6, NH), 7.16–7.18 (m, 2H, *Ph*), 7.25–7.34 (m, 3H, *Ph*), 7.64 (dt, 1H, *J* = 1.2, 7.6, H-7), 7.74 (dt 1H, *J* = 1.2, 7.6, H-6), 8.06 (brdd, 1H, *J* = 0.8; 7.6, H-8), 8.09 (brdd, 1H, *J* = 0.4, 7.6, H-5). δ ^{13}C NMR 14.1 (CH_2CH_3), 37.5 (CH_2Ph), 56.1 ($CHCH_2$Ph), 62.0 (CH_2CH_3), 102.1 (C-3), 126.2 (C-8), 126.4 (C-5), 127.5 (CH), 128.8 (CH), 129.2 (CH), 130.5 (C-8a), 132.2 (C-7), 133.2 (C-4a), 134.8 (C-6), 135.1 (C), 146.6 (C-2), 170.3 (CO), 181.2 (C-1), 183.2 (C-4). ESI-MS *m*/*z* (rel int.) 350 (MH^+) (100). Anal. Calcd ($C_{21}H_{19}NO_4$): C, 72.19; H, 5.48; N, 4.01. Found: C, 72.45; H, 5.61; N, 4.13.

4.2.2. *N*-(3-Bromo-1,4-naphthoquinon-2-yl)-*S*-phenylalanine ethyl ester (1b). Orange oil (9%); IR (film): ν_{max} = 3317, 1737, 1673, 1571 cm^{-1}. δ ^{1}H NMR 1.26–1.30 (m, 3H, CH_2CH_3), 3.20–3.31 (m, 2H, CH_2Ph), 4.21–4.27 (m, 2H, CH_2CH_3), 5.58 (m, 1H, CH), 6.28 (br s, 1H, NH), 7.20–7.22 (m, 2H, *Ph*), 7.24–7.34 (m, 3H, *Ph*), 7.65 (dt, 1H, *J* = 1.2, 7.6, H-7), 7.72 (dt, 1H, *J* = 1.2, 7.6, H-6), 8.00 (br d, 1H, *J* = 7.6, H-8), 8.14 (br d, 1H, *J* = 6.8, H-5). δ ^{13}C NMR 14.1 (CH_2CH_3), 40.0 (CH_2Ph), 57.6 ($CHCH_2$Ph), 61.9 (CH_2CH_3), 126.9 (C-5 and C-8), 127.5 (CH), 128.8 (CH), 129.4 (CH), 131.9 (C-4a), 132.6 (C-7), 134.7 (C-6), 135.0 (C), 171.2 (CO), 176.4 (C-4), 179.8 (C-1); the following ^{1}H and ^{13}C signal assignments are interchangeable: C-1 with C-4, C-4a with C-8a, H-5 with H-8 and H-6/C-6 with H-7/C-7. ESI-MS *m*/*z* (rel int.) 428 (MH^+) (83), 430 (MH^++2) (100). Anal. Calcd ($C_{21}H_{18}BrNO_4$): C, 58.89; H, 4.24; N, 3.27. Found: C, 58.77; H, 4.44; N, 3.42.

4.2.3. *N*-(3-Chloro-1,4-naphthoquinon-2-yl)-*S*-phenylalanine ethyl ester (1c). Compound **1c** (18 mg) was prepared from 2,3-dichloro-1,4-naphthoquinone and *S*-phenylalanine ethyl ester hydrochloride; orange oil (11%); IR (film): ν_{max} = 3315, 1737, 1673, 1571 cm^{-1}. δ ^{1}H NMR 1.27 (t, 3 H, *J* = 7.2, CH_2CH_3), 3.24-3.27 (m, 2H, CH_2Ph), 4.20–4.28 (m, 2H, CH_2CH_3), 5.51 (ddd, 1H, *J* = 2.0, 6.0; 12.2, CH), 6.34 (br s, 1H, NH), 7.19–7.21 (m, 2H, *Ph*), 7.27–7.34 (m, 3H, *Ph*), 7.66 (dt, 1H, *J* = 1.2, 7.6, H-7), 7.74 (dt, 1H, *J* = 1.2, 7.6, H-6), 8.03 (br d, 1H, *J* = 7.6, H-8), 8.15 (br d, 1H, *J* = 7.6, H-5). δ ^{13}C NMR 14.2 (CH_2CH_3), 40.2 (CH_2Ph), 57.3 ($CHCH_2$Ph), 61.9 (CH_2CH_3), 126.8 (C-8), 126.9 (C-5), 127.6 (CH), 128.9 (CH), 129.4 (CH), 130.0 (C-8a), 132.3 (C-4a), 132.7 (C-7), 134.9 (C-6), 135.1 (C), 171.3 (CO), 176.8 (C-4), 180.1 (C-1); the following ^{1}H and ^{13}C signal assignments are interchangeable: C-1 with C-4, C-4a with C-8a, H-5/C-5 with H-8/C-8 and H-6/C-6 with H-7/C-7. ESI-MS *m*/*z* (rel int.) 384 (MH^+) (100), 386 (MH^++2) (30). Anal. Calcd ($C_{21}H_{18}ClNO_4$): C, 65.71; H, 4.73; N, 3.65. Found: C, 65.73; H, 4.45; N, 3.66.

4.2.4. *N*-(1,4-Naphthoquinon-2-yl)glycine ethyl ester (1d). Compound **1d** (19 mg) was prepared from 2-bromo-1,4-naphthoquinone and glycine ethyl ester hydrochloride; orange amorphous solid (9%); IR (film): ν_{max} = 3373, 1743, 1677, 1573 cm^{-1}. δ ^{1}H NMR 1.34 (t, 3H, *J* = 7.2, CH_2CH_3), 3.94 (d, 2H, *J* = 5.2, NCH_2), 4.31 (q, 2H, *J* = 7.2, CH_2CH_3), 5.66 (s, 1H, H-3), 6.41 (br s, 1H, NH), 7.64 (br t, 1H, *J* = 7.6, H-7), 7.73 (br t, 1H, *J* = 7.6, H-6), 8.05–8.10 (m, 2H, H-5 and H-8). δ ^{13}C NMR 14.2 (CH_2CH_3), 43.9 (NCH_2), 62.1 (CH_2CH_3), 102.1 (C-3), 126.2 (C-8), 126.4 (C-5), 130.5 (C-8a), 132.3 (C-7), 133.3 (C-4a), 134.8 (C-6), 147.2 (C-2), 168.3 (CO), 181.2 (C-1), 183.2 (C-4). ESI-MS *m*/*z* (rel int.) 260 (MH^+) (50). Anal. Calcd ($C_{14}H_{13}NO_4$): C, 64.86; H, 5.05; N, 5.40. Found: C, 64.59; H, 5.01; N, 5.60.

4.2.5. *N*-(3-Bromo-1,4-naphthoquinon-2-yl)glycine ethyl ester (1e). Compound **1e** (18 mg) was obtained together with compound **1d**; orange amorphous solid (6%); IR (film): ν_{max} = 3317, 1741, 1677, 1569 cm^{-1}. δ ^{1}H NMR 1.34 (t, 3H, *J* = 7.2, CH_2CH_3), 4.31 (q, 2H, *J* = 7.2, CH_2CH_3), 4.62 (d, 2H, *J* = 5.6, NCH_2), 6.54 (br s, 1H, NH), 7.66 (dt, 1H, *J* = 1.0; 7.6, H-7), 7.73 (dt, 1H, *J* = 1.2; 7.6, H-6), 8.05 (br d, 1H, *J* = 7.6, H-8), 8.15 (br d, 1H, *J* = 7.6, H-5). δ ^{13}C NMR 14.2 (CH_2CH_3), 46.7 (NCH_2), 62.1 (CH_2CH_3), 127.0 (C-5 and C-8), 129.9 (C-8a), 132.0 (C-4a), 132.6 (C-7), 134.8 (C-6), 146.2 (C-2), 169.5 (CO), 176.5 (C-4), 179.8 (C-1); the following ^{1}H and ^{13}C signal assignments are interchangeable: C-1 with C-4, C-4a with C-8a, H-5 with H-8 and H-6/C-6 with H-7/C-7. ESI-MS *m*/*z* (rel int.) 338 (MH^+) (78), 340 (MH^++2) (100). Anal. Calcd ($C_{14}H_{12}BrNO_4$): C, 49.73; H, 3.58; N, 4.14. Found: C, 50.30; H, 3.60; N, 4.15.

4.2.6. *N*-(3-Chloro-1,4-naphthoquinon-2-yl)glycine ethyl ester (1f). Compound **1f** (11 mg) was prepared from 2,3-dichloro-1,4-naphthoquinone and glycine ethyl ester hydrochloride; orange amorphous solid (7%); IR (film): ν_{max} = 3315, 1737, 1673, 1571 cm^{-1}. δ ^{1}H NMR 1.34 (t, 3H, *J* = 7.2, CH_2CH_3), 4.31 (q, 2H, *J* = 7.2, CH_2CH_3), 4.61 (d, 2H, *J* = 6.0, NCH_2), 6.53 (br s, 1H, NH), 7.66 (dt, 1H, *J* = 0.8; 7.6, H-7), 7.75 (dt, 1H, *J* = 0.8; 7.6, H-6), 8.06 (br d, 1H, *J* = 7.6, H-8), 8.16 (br d, 1H, *J* = 7.6, H-5). δ ^{13}C NMR 14.2 (CH_2CH_3), 46.3 (NCH_2), 62.1 (CH_2CH_3), 126.8 (C-8), 126.9 (C-5), 129.9 (C-8a), 132.3 (C-4a), 132.7 (C-7), 134.9 (C-6),

143.9 (C-2), 169.5 (CO), 176.9 (C-4), 180.1 (C-1); the following ^{1}H and ^{13}C signal assignments are interchangeable: C-1 with C-4, C-4a with C-8a, H-5/C-5 with H-8/C-8 and H-6/C-6 with H-7/C-7. The ^{1}H NMR spectra of **1f** are in agreement with those reported in the literature.[31] ESI-MS *m*/*z* (rel int.) 294 (MH^+) (100), 296 (MH^++2) (37). Anal. Calcd ($C_{14}H_{12}ClNO_4$): C, 57.25; H, 4.12; N, 4.77. Found: C, 57.30; H, 4.30; N, 4.60.

4.2.7. 2-(Methylamino)-1,4-naphthoquinone (1g) and 2-(methylamino)-3-bromo-1,4-naphthoquinone (1h). To a solution of 2-bromo-1,4-naphthoquinone (283 mg; 1.2 mmol) in abs EtOH (40 mL) was added an excess of aqueous methylamine solution (40%; 182 mg; 2.3 mmol). The mixture instantly turned orange. After vigorous stirring at rt for 40 min, the solvent was evaporated to yield crude product (330 mg). Compounds **1g** (170 mg) and **1h** (19 mg) were separated by column chromatography on silica gel (40 g) using as eluents DCM (0.2 L) and a mixture of DCM/EtOAc 3:1 (0.2 L).

Compound **1g**; red amorphous solid (78%); IR (film): ν_{max} = 3335, 1678, 1566 cm^{-1}. δ ^{1}H NMR 2.96 (d, 3H, *J* = 5.6, CH_3), 5.74 (s, 1H, H-3), 5.97 (br s, 1H, NH), 7.64 (dt, 1H, *J* = 1.2, 7.6, H-7), 7.75 (dt, 1H, *J* = 1.2, 7.6, H-6), 8.06 (brdd, 1H, *J* = 0.8; 7.6, H-8), 8.12 (brdd, 1H, *J* = 0.8, 7.6, H-5). δ ^{13}C NMR 29.2 (CH_3), 100.7 (C-3), 126.3 (C-5 and C-8), 130.5 (C-8a), 132.0 (C-7), 133.7 (C-4a), 134.8 (C-6), 149.0 (C-2), 181.9 (C-1), 182.9 (C-4). ESI-MS *m*/*z* (rel int.) 188 (MH^+) (100). Anal. Calcd ($C_{11}H_9NO_2$): C, 70.58; H, 4.85; N, 7.48. Found: C, 70.96; H, 4.88; N, 7.43.

Compound **1h**; red amorphous solid (6%); IR (film): ν_{max} = 3315, 1678, 1571 cm^{-1}. δ ^{1}H NMR 3.47 (d, 3H, *J* = 5.6, CH_3), 6.17 (br s, 1H, NH), 7.64 (dt, 1H, *J* = 1.2, 7.6, H-7), 7.73 (dt, 1H, *J* = 1.2, 7.6, H-6), 8.04 (brdd, 1H, *J* = 0.8; 7.6, H-8), 8.17 (brdd, 1H, *J* = 0.8, 7.6, H-5). δ ^{13}C NMR 33.1 (CH_3), 126.9 (C-8), 127.1 (C-5), 129.8 (C-8a), 132.4 (C-4a), 132.5 (C-7), 134.9 (C-6), 147.0 (C-2), 176.5 (C-4), 180.2 (C-1); the following ^{1}H and ^{13}C signal assignments are interchangeable: C-1 with C-4, C-4a with C-8a, H-5/C-5 with H-8/C-8 and H-6/C-6 with H-7/C-7. ESI-MS *m*/*z* (rel int.) 266 (MH^+) (86), 268 (MH^++2) (100). Anal. Calcd ($C_{11}H_8BrNO_2$): C, 49.65; H, 3.03; N, 5.26. Found: C, 49.87; H, 2.98; N, 5.21.

4.2.8. 1-[(1,4-Naphthoquinon-2-yl)-*S*-leucylamido]-3-methylbutane (1i). The side-chain Leu-*i*-Am was prepared under standard peptide coupling conditions from the corresponding Boc-protected amino acid *N*-hydroxysuccinimide ester and *iso*-amylamine, followed by the removal of the *N*-Boc group with trifluoroacetic acid.[32,33] The resultant amine (131 mg; 0.7 mmol) was added to a solution of 1,4-naphthoquinone (110 mg; 0.7 mmol) in abs EtOH (10 mL) with TEA (66 mg; 0.7 mmol). The reaction mixture was vigorously stirred at rt for 1 h and then the solvent was evaporated. Compound **1i** (42 mg) was purified by column chromatography on silica gel (40 g) using as eluents DCM (0.2 L) and a mixture of DCM/EtOAc 19:1 (0.5 L): brown oil (18%); IR (film): ν_{max} = 3335, 1675, 1572, 1640 cm^{-1}. δ ^{1}H NMR 0.90 [d, 6H, *J* = 6.8, 2 × CH_3 (*i*-Am)], 0.95 [d, 3H, *J* = 6.4, CH_3 (Leu)], 1.02 [d, 3H, *J* = 6.0, CH_3 (Leu)], 1.37–1.42 [m, 2H, CH_2CH (*i*-Am)], 1.55–1.63 [m, 1H, CH (*i*-Am)], 1.70–1.79 [m, 2H, C*H*H and C*H*(CH_3)$_2$ (Leu)], 1.88–1.92 [m, 1H, CH*H* (Leu)], 3.28–3.33 [m, 2H, NHCH_2 (*i*-Am)], 3.89–3.92 [m, 1H, C*H*NH (Leu)], 5.82 (s, 1H, H-3), 6.13 [d, 1H, *J* = 6.8, NH (Leu)], 6.28 [m, 1H, NH (*i*-Am)], 7.66 (dt, 1H, *J* = 1.2, 7.6, H-7), 7.76 (dt, 1H, *J* = 1.2, 7.6, H-6), 8.07-8.10 (m, 2H, H-5 and H-8). δ ^{13}C NMR 21.9 [CH_3 (Leu)], 22.4 [2 × CH_3 (*i*-Am)], 22.9 [CH_3 (Leu)], 25.1 [*C*H(CH_3)$_2$ (Leu)], 25.9 [CH (*i*-Am)], 38.0 [NH*C*H_2 (*i*-Am)], 38.3 [*C*H_2CH (*i*-Am)], 41.8 [CH_2 (Leu)], 55.9 [CHNH (Leu)], 102.4 (C-3), 126.3 (C-8), 126.5 (C-5), 130.4 (C-8a), 132.4 (C-7), 133.2 (C-4a), 135.0 (C-6), 147.4 (C-2), 170.3 [CO (Leu)], 181.2 (C-1), 183.2 (C-4). ESI-MS *m*/*z* (rel int.) 357 (MH^+) (100). Anal. Calcd ($C_{21}H_{28}N_2O_3$): C, 70.76; H, 7.92; N, 7.86. Found: C, 70.95; H, 7.45; N, 7.53.

4.2.9. 2-Ethoxy-1,4-naphthoquinone (1q). A solution of 1,4-naphthoquinone (311 mg; 1.9 mmol) in abs EtOH (40 mL) with TEA (192 mg; 1.9 mmol) was vigorously stirred at rt for 1 h, during which time a red colour was found to develop. After stirring for another 12 h, the solvent was evaporated to yield crude product (529 mg), which was fractionated by column chromatography on silica gel (40 g) using DCM (0.45 L) as eluent to afford 110 mg of **1q**: yellow amorphous solid (29%); IR (film): ν_{max} = 1682, 1656, 1610 cm^{-1}. δ ^{1}H NMR 1.55 (t, 3H, *J* = 6.8, CH_2CH_3), 4.12 (q, 2H, *J* = 6.8, CH_2CH_3), 6.17 (s, 1H, H-3), 7.73 (dt, 1H, *J* = 1.6, 7.6, H-7), 7.77 (dt, 1H, *J* = 1.6, 7.6, H-6), 8.10 (brdd, 1H, *J* = 1.6; 7.4, H-8), 8.15 (brdd, 1H, *J* = 1.6, 6.8, H-5). δ ^{13}C NMR 13.9 (CH_2CH_3), 65.4 (CH_2CH_3), 110.2 (C-3), 126.1 (C-8), 126.7 (C-5), 131.2 (C-8a), 132.0 (C-7), 133.3 (C-4a), 134.3 (C-6), 159.7 (C-2), 180.2 (C-1), 185.1 (C-4). ESI-MS *m*/*z* (rel int.) 422 ($M_2NH_4^+$) (82). Anal. Calcd ($C_{12}H_{10}O_3$): C, 71.28; H, 4.98. Found: C, 71.30; H, 4.58.

4.3. Kinetics of reaction with cysteine

Thiol solutions were prepared immediately before use, by dissolution of cysteine in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.1, containing KCl to maintain ionic strength at 0.5 M. Solutions with a change in the final pH value greater than 0.05 were rejected. The concentration of thiol in solution was measured just before use by Ellman's method.[34] In the case of menadione, **1n**, the rates were measured at three additional pH values using sodium borate/HCl buffer (pH 7.9, 8.0 and 8.8).[35] The kinetic measurements were carried out at 25.0 ± 0.1 °C without protection from air. The naphthoquinone stock solutions were prepared in DMSO. Reactions were initiated by addition of an aliquot of naphthoquinone stock (150 μL) to the buffer containing cysteine (2.85 mL), and recorded against a blank solution of the buffer with cysteine and DMSO. Cysteine concentration ranged between 0.5 and 5 mM except in the kinetics of naphthoquinone **1j**, in which the range was 0.06–5 mM. The initial concentration of the naphthoquinone in the cuvette and the wavelengths used to monitor the

kinetic reactions were as follows: **1j** (50 μM; increase at 360 nm), **1q** (50 μM; decrease at 282 nm),**1 g** (50 μM; increase at 300 nm), **1a** (32 μM; decrease at 265 nm), **1d** (50 μM; increase at 277.3 nm), **1m** (10 μM; decrease at 500 nm), **1n**(50 μM; increase at 279.8 nm). The pseudo first-order rate constants, k_{obs}, were obtained from the slopes of the plots of $\ln(\delta A)$ versus time, where δA represents the difference in absorbance between time t and time infinity.

4.3.1. Determination of free thiol content. The free thiol content was measured by the method of Ellman.[34] In general, aliquots of the thiol solutions (⩽50 μL) were added to a cuvette containing a 0.1 mM solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.4, to give a total volume of 1 mL. Under these conditions, thiol concentrations in the cuvette ranged between 20 and 120 μM (absorbance values between 0.3 and 1.7). The absorbance at 412 nm was measured after 5 min, against a blank solution lacking thiol. A new blank was prepared for every measurement. Thiol concentration was calculated through the formation of the yellow dianion of 5-thio-2-nitrobenzoic acid ($\varepsilon = 14.15 \times 10^3\ M^{-1}\ s^{-1}$).[34]

4.4. Papain, cathepsin B and PPE inhibition studies

4.4.1. Kinetic studies. The inhibitory activity was measured by Kitz and Wilson's pre-incubation method,[25] using procedures based on the literature.[36–38] Assays of control incubations were performed at the same time as inhibitor incubations. Papain[36] (1 mg/mL) was activated by incubation at 25 °C, during 30 min, in 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0, containing 2.5 mM EDTA and 2.0 mM DTT. Reactions were started by the addition of the inhibitor stock solution in DMSO (50 μL) to the incubation solution containing papain (300 μL of the stock solution 1 mg/mL) and 650 μL of 50 mM K_2HPO_4/KH_2PO_4, pH 7.0, containing only 2.5 mM EDTA. At different times, aliquots (100 μL) from the incubation mixture were withdrawn and transferred to a cuvette containing 100 μL of BAPA stock solution (10 mM in DMSO) and 800 μL of 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0, containing 2.5 mM EDTA and 2.0 mM DTT. Enzyme activity was determined at 25.0 ± 0.1 °C, by following the release of *p*-nitroaniline from BAPA at 410 nm during 4 min, against a blank of 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0, containing 2.5 mM EDTA and 2.0 mM DTT. The assays in the presence of ascorbic acid were carried out as described above, except that the incubation buffer included ascorbic acid (2.0 and 30 mM). Cathepsin B[37] (10 U/2 mL) was incubated at 25 °C, during 15 min in 20 mM sodium acetate buffer, pH 5.0, containing 1.0 mM EDTA and 30 mM DTT. Reactions were started by addition of the inhibitor stock solution in DMSO (50 μL) to the incubation solution containing cathepsin B (20 μL of the stock solution 10 U/2 mL) and 930 μL of 20 mM sodium acetate buffer, pH 5.0, containing only 1.0 mM EDTA. At various time intervals, cathepsin B activity was assayed by taking a 100 μL aliquot of the incubation solution and adding it to a cuvette containing 100 μL of the Z-*S*-Lys-ONp stock solution (0.8 mM in DMSO) and 800 μL of 20 mM sodium acetate buffer, pH 5.0, containing 1.0 mM EDTA and 2.0 mM DTT. Enzyme activity was monitored at 25.0 ± 0.1 °C by following the appearance of the *p*-nitrophenol product at 326 nm, over a period of 3 min, against a blank of 20 mM sodium acetate buffer, pH 5.0, containing 1.0 mM EDTA and 2.0 mM DTT. For PPE,[38] the inhibitor (50 μL of a stock solution in DMSO) was mixed with 750 μL of 0.1 M Hepes buffer, adjusted to pH 7.2, and 200 μL of a stock solution of enzyme (50 μM in 0.1 M Hepes buffer, pH 7.2). Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette containing 5 μL of the Suc-Ala-Ala-Ala-4-nitroanilide stock solution (12.5 mM in DMSO) and 895 μL of 0.1 M Hepes buffer. The absorbance change was followed for 50 s at 390 nm against a blank of 0.1 M Hepes buffer. Pseudo first-order inactivation rate constants, k_{obs}, were determined from plots of $\ln(v/v_0)$ versus incubation time. Four to six inhibitor concentrations were used to determine k_2, K_I and k_{inact} values, with the exception of the k_2 value for **1c**, which was calculated using a single inhibitor concentration due to solubility problems. In the case of hyperbolic dependences, K_I and k_{inact} values were determined by computer-fit (Sigma Plot®) using least squares nonlinear regression analysis to Eq. 3.

4.4.2. Dialysis experiments. Papain was inactivated to the extent of about 80% by the glycine derivative **1d** in an incubation mixture (2 mL) prepared as described for the inhibition studies. This solution was then dialysed at 4 °C over a period of 24 h, against 0.8 L of 50 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0, containing 2.5 mM EDTA and 2 mM DTT. This thiol was used to maintain the reducing conditions essential for papain activity. The enzyme activity was periodically assayed during the 24 h of dialysis, along with the determination of papain concentration by the Bradford Reagent[39] (Bio-Rad) using a microassay procedure. For comparison between the assays, the enzyme activity was expressed as $(\Delta A/\Delta t)/(\mu g\ papain)$, where ΔA represents the increase in absorbance during the reading time (Δt), due to the release of *p*-nitroaniline. No recovery of enzyme activity was detected, while the control sample retained its activity. The protein recovery was always superior to 80%.

4.5. Molecular orbital calculations

Density functional theory (DFT)[40] calculations were carried out with the Gaussian 03 suite of programs.[41] 1,4-Naphthoquinone structures were optimised without symmetry constraints using the hybrid B3LYP functional, which is a combination of the Becke's three-parameter (B3) exchange functional[42] with the Lee, Yang and Parr (LYP) correlation functional,[43] in conjunction with 6-311G(d,p)[44] basis set. All geometries were found to be minima on the respective potential energy surfaces. Lowest unoccupied molecular orbital (LUMO) energies were calculated at this level of theory.

4.6. Molecular modelling

Molecular docking enzyme-inhibitor studies were performed with the flexible GOLD 3.0 (Genetic Optimisation for Ligand Docking) package.[45] GOLD program uses a genetic algorithm (which mimics the process of evolution by applying genetic operators) to explore the preferred orientations and conformations of the ligand in relation to the receptor, and has been fully validated against 305 diverse and extensively checked protein–ligand complexes from the PDB database. The docking results were ranked based on the goldscore scoring function[46] (fitness function). The papain crystal structure used in the calculations (PDB code 1PPN)[47] was obtained by deletion of one oxygen and the methanol present in the crystal structure. The structure was energy-minimized. For each ligand (1,4-naphthoquinone geometries were previously energy-minimized with DFT) 50 docking runs were performed (100,000 genetic algorithm operations, 5 islands).

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