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A simple in vitro assay for assessing the reactivity of nitrile containing compounds

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ABSTRACT

A quantitative assay involving the reaction of nitriles with glutathione and cysteine has been used as a simple in vitro screen to assess potential toxicity risk of candidate compounds in drug discovery. Studies have indicated that, when benchmarked with selected compounds, the reaction of the nitriles with glutathione can provide a useful tool for deciding whether or not to progress compounds in the absence of radiolabelling studies.

Compounds containing electrophilic moieties have been found to be inhibitors of several cysteine proteases.[1] The mechanism of action involves the formation of a covalent reversible bond between the electrophile and the active site thiol.[2] In order to limit potential undesirable side effects, non-specific interactions with thiols need to be minimised. If not, toxicity could arise either from increased oxidative stress due to glutathione depletion or adverse drug related reactions, such as irreversible binding of the nitriles to proteins or DNA.[3] Nitriles have been identified as a useful warhead for cysteine protease inhibition and unlike a number of electrophilic motifs show good DMPK properties (see Fig. 1).

Glutathione, a thiol containing tripeptide, plays a major role in protecting cells from oxidative stress via reaction with free radicals, hydrogen peroxide or reactive electrophiles which are inadvertently formed during metabolism of xenobiotics by cytochrome P-450s.[4] It is present in cells at concentrations ranging from 1 to 10 mM, and exists mainly as the reduced form at physiological pH. Conjugation can be catalysed by glutathione-*S*-transferases,[5] although these enzymes were not employed in this study. Reaction with glutathione, either catalysed or un-catalysed, could also play a key role in understanding pharmacokinetic profiles via an increased appreciation of clearance mechanisms and kinetics. Cysteine, although only present at micromolar levels in cells in its native form,[6] can also play a key role in physiological processes and was therefore included in this study alongside glutathione to broaden the assessment of the reactivity. The structures of both thiols are shown in Figure 2.

Because covalent attachment of the nitrile significantly contributes to overall potency, an in vitro risk assessment tool was required by the project to monitor the inherent reactivity of the warhead. An assay was subsequently employed based on the method employed by Clarke et al.[7] Reactions were initiated by the addition of 50 μM of the nitrile to 5 mM thiol in pH 7.4 phosphate buffer containing 1 mM ethylene diamine tetra-acetic acid (EDTA), at 37 °C. The reaction mixture was incubated in a sealed vial, samples taken at regular intervals and analysed by LC-UV. In order to quantify the reaction, the kinetics were monitored through the loss of the parent compound, as studied by UV spectroscopy. If the chromophore was extremely weak, single-ion monitoring mass spectrometry was used. Reaction with *p*-nitrobenzyl

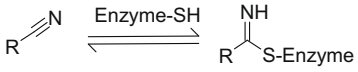

Figure 1. Reversible covalent interaction of a nitrile containing compound with an active site cysteine.

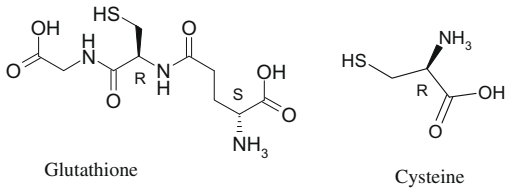

Figure 2. The two thiols used in this study.

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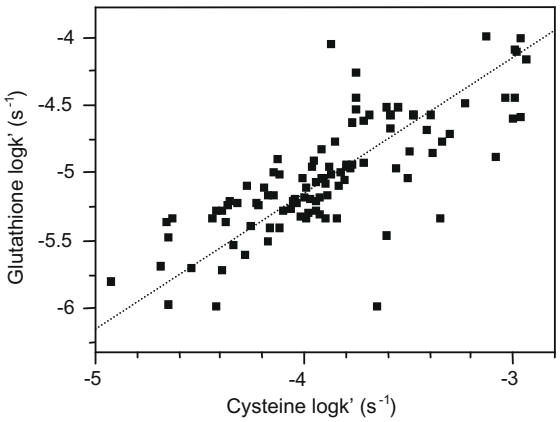
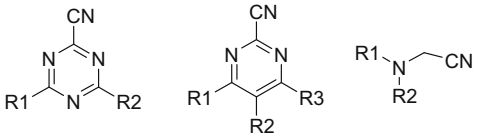


Figure 3. Comparison of the rate of reaction for various nitriles with glutathione and cysteine. The dashed line represents a slope of 1, but where the intercept was fitted and was found to equal −1.2.

Table 1
Typical half-lives for the reaction of various types of nitrile with glutathione and cysteine[a]



1 2 3

Nitrile	Typical half-life with glutathione	Typical half-life with cysteine	Calculated reaction energies[b]
1	2 h	<12 min	−10 kcal/mol
2	20 h	2 h	−8.1 kcal/mol
3	>100 h	>10 h	−3.4 kcal/mol

[a] Values are quoted from the reaction of 50 μM nitrile with 5 mM thiol in pH 7.4 phosphate buffer containing 1 mM EDTA at 37 °C.
[b] Estimated theoretical reaction energies for reaction of nitrile with methanethiol.[12]

chloride (*p*NBC) was employed as a control. Incubation times varied up to 72 h depending on the reactivity of the nitrile. The thiols were found to be still viable under all the experimental conditions that were employed.[8]

Although the reaction has been shown to be reversible,[9] our studies were found to follow pseudo first order reaction kinetics. Figure 3 illustrates the comparison of the first order rate constants for various nitriles with both thiols. The dashed line represents a slope of 1. The RMSE is 0.29, indicating a reasonable link for the reactivity of the two thiols. Extrapolation of the line intercepts the cysteine axis at −1.2 indicating an ~15-fold difference in reactivity between the two thiols. In part, this reactivity difference can be explained by the difference in pK_a values of the two thiols; glutathione 9.3 vs cysteine 8.33.[10] Interestingly, it has also been found that cysteine is superior to glutathione in its ability to protect DNA from radiation induced damage, however this effect was attributed to the increased electrostatic interactions between cysteine and DNA resulting in higher localised concentrations of the radical scavenger.[11] There are several examples where reactivity does not correlate well between the two thiols.

We believe glutathione is the more physiologically relevant thiol and therefore the more pertinent marker for non-specific thiol reactivity. Nevertheless, due to its greater reactivity, cysteine is useful for characterizing more accurately the reactivity of less reactive nitriles. In general, the nitriles were found to react with glutathione and cysteine in accordance with their expected electrophilicities as described by Oballa et al.,[12] although the nature of the substituents R1, R2, and R3 were observed to have a dramatic effect on the level of reactivity. Typical half-lives for three different types of compounds are illustrated in Table 1 together with the estimated theoretical reaction energies as calculated for the reaction with methanethiol. ^{13}C NMR studies were also performed to examine if the chemical shift of the nitrile carbon atom could be used as a direct measurement of electrophilicity. A selection of the above compounds with half-lives for the reaction with glutathione lying between 2 and 100 h, possess ^{13}C NMR chemical shift lying between 117.8 and 113.5 ppm. However, no correlation with the rate of reactivity was observed.[13]

Although the mechanism of action involves the formation of a reversible bond between the nitrile warhead and the active site cysteine, it must be noted that potency against cysteine protease targets does not necessarily correlate with general thiol reactivity. Amino-acetonitrile analogues (**3**) are potent inhibitors of cysteine proteases, even though they display low reactivity towards small molecule thiols: compound **4** (see Fig. 4), a 1.4 nM inhibitor of cathepsin K,[14] having a half-life of 97 h with glutathione and just less than 10 h with cysteine. It is known that the pH at the active site of enzymes such as cathepsin K is in the region of pH 5.5–6.2,[15] and although not measured in this study, the reactivity of the nitriles towards thiols will be reduced at lower pH due a reduced fraction of the thiolate anion, and that the magnitude of this reduction in rate will depend on the pK_a of thiol concerned.[7]

In order to understand the risks associated with compounds of this type in more detail, non-specific binding studies would need be performed using radiolabels, to allow quantification of the amount of drug–protein adduct formed.[16] Incubation of radiolabelled compound with liver proteins in the absence of any metabolic activation, followed by analysis, determines the level of material bound to these proteins due to the reactivity of the parent compound itself, rather than that of a reactive metabolite. The level of binding is typically expressed in terms of pmol drug equivalent bound per mg of total protein after a 1-h incubation. It has been proposed that a target value of less than 50 pmol adduct/mg of protein should be considered an acceptable level.[17] These experiments are conducted in the absence of added NADPH so bioactivation is not induced, hence observed reactivity is due solely to the nature of the parent molecule. This approach is complex and not feasible to run routinely in the early stages of discovery. Therefore, it was decided that an appropriate benchmark needed to be identified to act as a point of reference and determine an acceptable level of chemical reactivity. Testing a series of marketed nitrile containing drugs identified Nilvadipine (Fig. 5) as a suitable marker. The compound is a Calcium channel antagonist that has been on the market since 1991 and has a good safety record.[18] It possesses a half-life of 40 h for the reaction with glutathione, and 2 h with cysteine.[19] Nilvadipine provides a simple benchmark when assessing the reactivity of novel compounds. This approach may be over cautious as the extent of covalent binding to liver protein for **5** was determined to be 70 pmol drug equivalent per mg of protein,[12] and from work carried out in this study it is known that the half-life of the reaction of this compound with glutathione is 7.3 h. Therefore, it could be that the use of Nilvadipine as a bench-

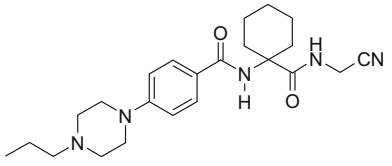


Figure 4. Structure of the cathepsin K inhibitor **4**.

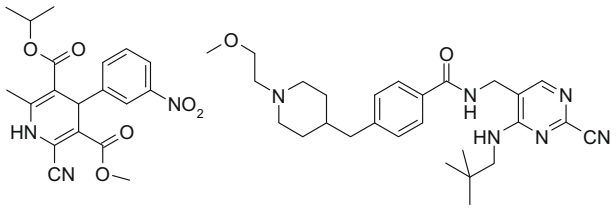


Figure 5. Structures of selected compounds used for benchmarking nitrile reactivity with thiols.

mark is still a conservative approach to assessing the potential toxicity risks. The rate of reaction of compounds with radiolabelled glutathione in vivo has been shown to correlate extremely well with the extent of covalent binding to proteins.[20] Further work is required to understand if the in vitro reactivity of nitriles towards glutathione correlates with the extent of non-specific covalent binding due to the potentially reversible nature of the interaction of the nitrile with the protein thiol.

In summary, the reaction of nitriles with protein thiols can be reversible as seen with enzyme targets, whereas nitriles reacting with cysteine or small cysteine containing peptides can do so in an irreversible fashion. The work described here indicates that the reaction rates of nitriles with small molecule thiols can easily be quantified, and that the reaction rates differ depending on the nature of the thiol. In general, the extent of reactivity is related to the electrophilicity of the nitrile. However, in the absence of an alternative applicable measure of electrophilicity, this assay provides a simple route to rank the inherent reactivity of such nitriles. The reaction sequence with cysteine is much faster than with glutathione, and although there is a general correlation of the rates of reaction, they are not always a good indicator of how a compound will behave in the presence of other thiols. We therefore believe that as glutathione is more physiologically relevant, its rate of reaction should be used preferentially for in vitro assessment. Very rapid reaction of compounds with glutathione may highlight a potential toxicity risk either via depletion of the glutathione or via the irreversible covalent binding of these compounds to proteins. Additionally, this high level of reactivity may also explain the lack of cellular activity for certain compounds.

It is acknowledged that in order to more fully understand the potential for toxic events to be induced by the presence of these compounds, the dose and pharmacokinetic properties need to be taken into account. In the absence of additional radiolabelling studies, the above assay can be used as a simple way of assessing the potential toxicity risk associated with nitrile containing compounds. Based on the reactivity of Nilvadipine, a target half-life of 40 h, or greater, is proposed as a safe guideline for nitriles under the assay conditions described above.

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