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A generally applicable method for assessing the electrophilicity and reactivity of diverse nitrile-containing compounds

Renata M. Oballa,* Jean-François Truchon, Christopher I. Bayly, Nathalie Chauret, Stephen Day, Sheldon Crane and Carl Berthelette

Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, 16711 TransCanada Highway, Kirkland, Que., Canada H9H 3L1

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Abstract—Nitrile-based inhibitors of cathepsin K have been known for some time and mechanism-of-action studies have demonstrated that cysteinyl proteases interact with nitriles in a reversible fashion. Three main classes of nitrile-containing inhibitors have been published in the cathepsin K field: (i) cyanamides, (ii) aromatic nitriles, and (iii) aminoacetonitriles. A computational approach was used to calculate the theoretical reactivities of diverse nitriles and this was found to correlate with their extent of reactivity with free cysteine. Moreover, there is a tentative link between high reactivity with cysteine and the potential to lead to irreversible covalent binding to proteins.

Cathepsin K is a lysosomal cysteine protease that is highly expressed in osteoclasts, cells that resorb bone. It is believed to be the principal enzyme responsible for degradation of Type I collagen in osteoclastic bone resorption. Thus, inhibition of cathepsin K represents a potential therapeutic approach for diseases characterized by excessive bone resorption such as osteoporosis.[1]

Many chemotypes have been described as inhibitors of cysteine proteases.[2] The majority are dependent for their activity on the presence of an electrophilic moiety which can form either a reversible or an irreversible covalent bond with the active site cysteine of the enzyme. Peptidic nitriles are known to be potent and reversible inhibitors of cysteine proteases of the papain family.[3] ^{13}C NMR studies with papain have demonstrated that nitriles form a reversible thioimidate ester adduct with papain.[3d,4] Furthermore, mechanism-of-action studies have demonstrated that cathepsin K interacts with nitriles in a reversible fashion (Fig. 1).[2b,4,7a] Three classes of nitrile-containing inhibitors have been published in the cathepsin K field: (i) cyanamides,[4,5] (ii) aromatic nitriles,[6] and (iii) aminoacetonitriles[7] (Fig. 2).

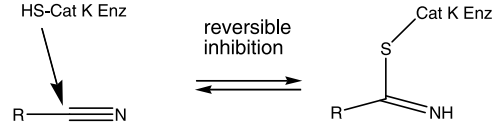

Figure 1. Activity of a nitrile-based cathepsin K inhibitor is the result of a reversible covalent bond with the active site cysteine of the enzyme.

While each class of compounds include potent inhibitors of cathepsin K, our own experiences with cyanamides suggested that there could be profound differences in the electrophilicities of the nitrile moieties. We hypothesized that increased electrophilicity and reactivity could impact the reversibility of the enzyme–inhibitor complex which would result in irreversible covalent binding to thiol-containing proteins.

Calculated nitrile electrophilicity. We devised a generally applicable method for assessing the electrophilicity of diverse nitrile-containing compounds by calculating the reactivity of methanethiol with said nitriles. We are considering 'reactivity' here in the thermodynamic sense, that is, how the equilibrium in Figure 1 will lie more to the right as the R-group makes the nitrile

Keywords: Cathepsin K; Nitrile; Osteoporosis; Cyanamide; Aminoacetonitrile; Nitrile warhead; Irreversible covalent binding; Cysteine adduct; Calculated electrophilicity.
* Corresponding author. Tel.: +1 514 428 3617; fax: +1 514 428 4900; e-mail: renata_oballa@merck.com

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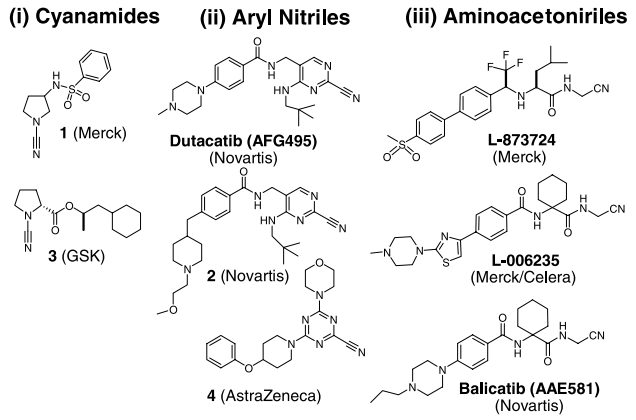


Figure 2. Three classes of nitrile-based cathepsin K inhibitors: (i) cyanamdies, (ii) aryl nitriles and (iii) aminoacetonitriles.

carbon more electron poor. Density functional theory calculations can be used to generate a reliable reactivity index of diverse nitrile-containing molecules. In this paper, we approximate the free energy of formation of the thioimidate by calculating the energy difference between the thioimidate adduct and the precursor methanethiol and the nitrile molecule. We first fully optimized the geometry of the reagents and the products in gas phase with the B3LYP/6-311G(d,p)[8a–c] level of theory using the Gaussian 2003 software.[8d] This is followed by an energy single point calculation with the PCM[8e] method within water.[8d] Including water solvation, even in an approximate way, was important to avoid overestimating the benefit of an intramolecular H-bond that the thioimidate can form. The reactivity index, given in kcal/mol, is simply calculated: E(adduct) − E(nitrile compound) − E(methanethiol). While the values obtained are, in some sense, an approximation to the theoretical free energy of formation of the thioimidate in water, they do not take into account other factors such as zero point vibrational energy, basis set superposition error, and temperature effects. The actual values of the relative energies should not be interpreted but rather the ranking between the compounds.

Shown in Figure 3 is a scale of reactivity values of diverse aliphatic and aromatic nitrile moieties as a result of their reaction with methanethiol. As expected, most nitriles are more electrophilic than simple, unsubstituted ketones, another reported class of cathepsin K inhibitors.[2] A simple benzonitrile shows relatively poor electrophilicity, with a calculated reactivity with methanethiol of +0.4 kcal/mol (Fig. 3). If, however, the phenyl is replaced with an electron-withdrawing heterocycle, an increase in nitrile electrophilicity is observed. For example, a 2-cyanopyridine (i.e., **5**, −3.4 kcal/mol) is more electrophilic than its benzonitrile counterpart and a 2-cyanopyrimidine (i.e., **6**, −8.1 kcal/mol) shows an even further enhancement in electrophilicity. With regard to aminoacetonitriles (i.e., **7**, −3.4 kcal/mol), an intermediate electrophilicity is obtained between that of a benzonitrile and the more electron-deficient aromatic nitriles. Another class of nitrile-based cathepsin K inhibitors are cyanamides. Again, the electron-withdrawing influence of a nitrogen directly attached to the nitrile moiety serves to increase

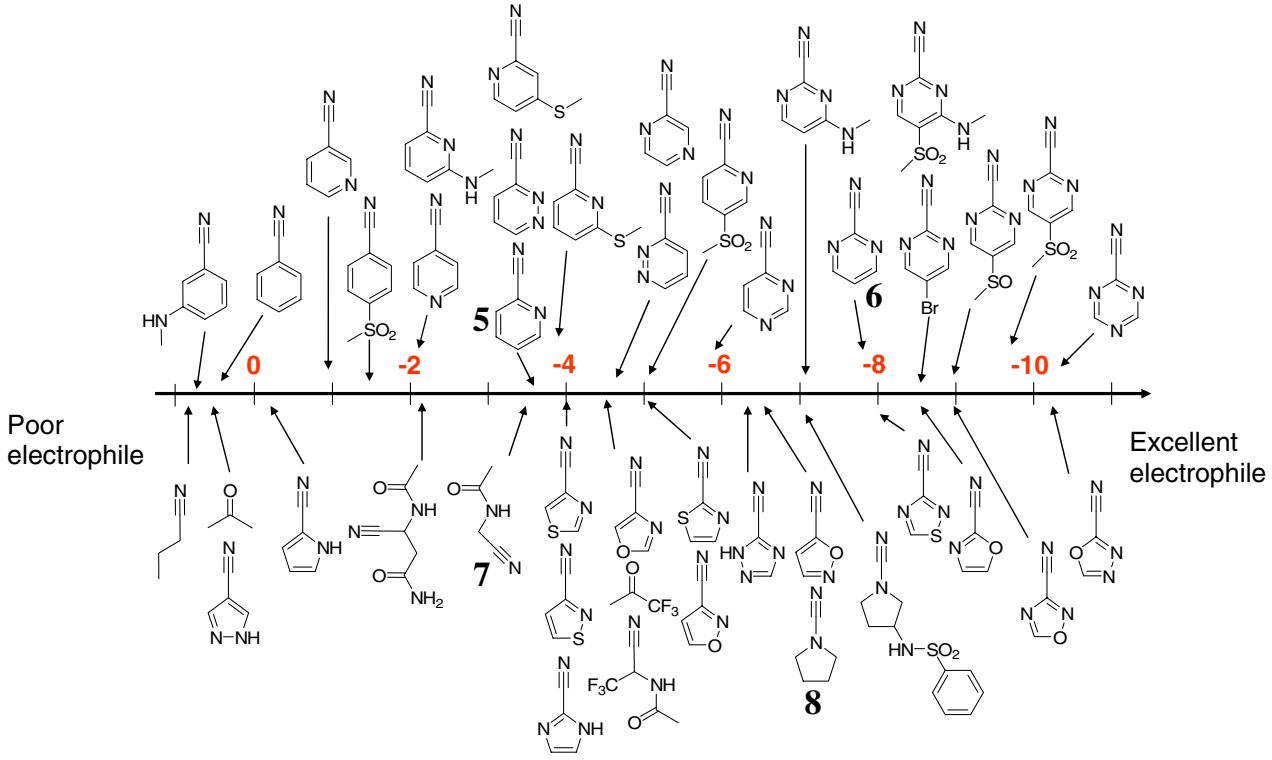


Figure 3. Calculated reaction energies of diverse nitrile moieties with methanethiol are shown in kcal/mol. A very negative value indicates high electrophilicity, whereas small negative values are attributed to poorer electrophiles.

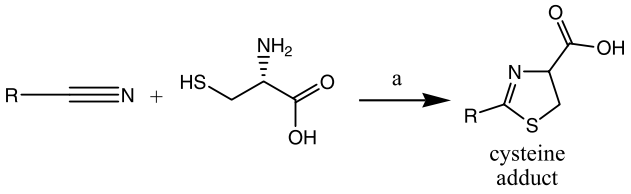
the electrophilicity of the nitrile (i.e., **8**, −6.7 kcal/mol). Of the reported classes of nitrile-based cathepsin K inhibitors, aminoacetonitriles possess the least electrophilic nitrile group, whereas both the aryl nitriles (pyrimidine and triazine nitriles) and cyanamides have significantly more electrophilic nitrile moieties.

Experimental nitrile reactivity. It has been reported that aliphatic and aromatic nitriles can react with cysteine to form thiazoline derivatives (Scheme 1).[9] These cysteine adducts are formed in an irreversible fashion and can be readily characterized. In order to determine if the calculated electrophilicity of diverse nitrile moieties correlates with the actual reactivity of the nitrile with a thiol nucleophile, the extent of thiazoline adduct formed upon reaction with cysteine was monitored. While this provides a kinetic measure of reactivity as opposed to the thermodynamic assessment calculated theoretically and desired for the enzyme equilibrium given in Figure 1, we felt that this experimental approach would still provide a useful relative measure of the relative electrophilicity of the nitriles tested.

As shown in Table 1, a poorly electrophilic naphthyl nitrile **9**[10] (+0.4 kcal/mol) does not form a cysteine adduct following a 30-min incubation with 1 mM cysteine, as judged by LC–MS. However, a slightly more reactive 2-cyanopyridine **10** (−2.0 kcal/mol) does form a small amount of the thiazoline adduct (2%). The aminoacetonitriles L-873724,[7e] L-006235,[7f] and Balicatib,[11] which are of intermediate electrophilicity (−3.4 kcal/mol), did form a small amount of cysteine adduct under the 30-min reaction conditions (4–9%). In order to verify that the new peak by LC–MS (+104 amu) correlates to the thiazoline adduct, an authentic standard of the thiazoline adduct of L-873724 was synthesized (L-873724, cysteine, K_2CO_3, MeOH/H_2O, rt) and was found to co-elute with the new peak (Fig. 4).

With regard to the moderately electrophilic 2-cyanopyridines, the extent of cysteine adduct formation was very dependent on the nature of the pyridine substituents. For example, an electron-donating 6-amino group serves to decrease the calculated electrophilicity (**10**, −2.0 kcal/mol) and this correlated with a decreased reactivity with cysteine (2% adduct formation). When the amino group is replaced with a sulfur (compound **11**), the calculated electrophilicity increases (−3.8 kcal/mol) as does the extent of adduct formation (29%).

At the more electrophilic end of the spectrum (Table 1), reactivity of cyanamide **1**[4] and pyrimidine nitriles **2**[6b]



Scheme 1. Reaction of a nitrile moiety with cysteine. Reagents and conditions: (a) 100 μM nitrile, 1 mM cysteine, PO_4 buffer, pH 7.4, 37 °C, 30 min.

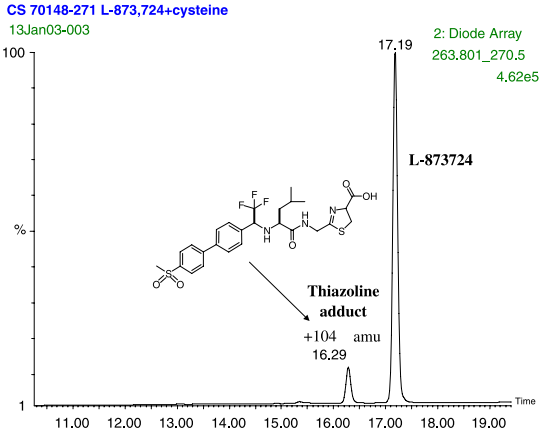
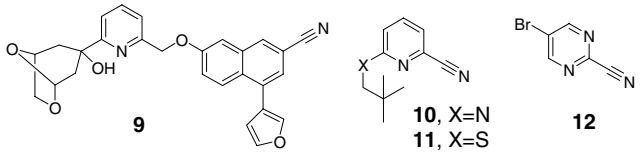


Figure 4. LC–MS UV trace and quantification of the thiazoline adduct formed upon reaction of L-873724 with cysteine, PO_4 buffer, pH 7.4, 37 °C, 1 h.

Table 1. Correlation of extent of cysteine adduct formation with calculated electrophilicity of the nitrile moiety



Structure	Nitrile classification	Calcd electrophilicity[a]	% cysteine adduct formed[b] (%)
9	Naphthyl nitrile	+0.4	0
10	Pyridine nitrile	−2.0	2
L-873724	Aminoacetonitrile	−3.4	5
L-006235	Aminoacetonitrile	−3.4	4
Balicatib	Aminoacetonitrile	−3.4	9
11	Pyridine nitrile	−3.8	29
1	Cyanamide	−6.9	71
2	Pyrimidine nitrile	−7.2	79
12	Pyrimidine nitrile	−8.6	100

[a] As determined by theoretical reaction with methanethiol, reaction energy in kcal/mol.
[b] 100 μM nitrile, 1 mM cysteine, PO_4 buffer, pH 7.4, 37 °C, 30 min.

and **12** correlated with a significantly higher amount of cysteine adduct formation (71–100%). For all compounds except the poorly electrophilic **9**, a time course study indicated that the amount of adduct formed gradually increased to 100%.

The data in Table 1 clearly indicates that the calculated electrophilicity is a very good predictor of the reactivity of nitriles with a thiol nucleophile. The correlation bears the expected titration curve-like character suggesting that 50% cysteine adduct would occur when the calculated electrophilicity is approximately −5 kcal/mol. With regard to the various classes of nitrile-based cathepsin K inhibitors, both the cyanamide and pyrimidine nitrile series possess a highly reactive nitrile moiety. On the other hand, the aminoacetonitriles clearly possess a much less reactive nitrile moiety.

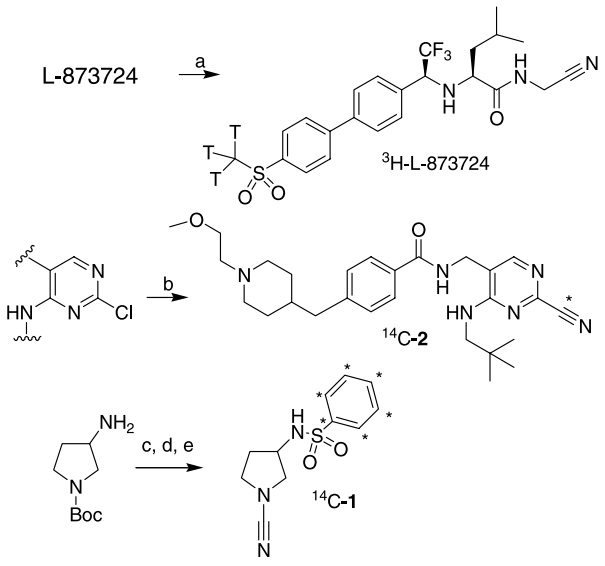
Potential for irreversible covalent binding. Since two classes of cathepsin K inhibitors possess highly reactive, electrophilic nitrile moieties (**1** and **2** in Table 1), we decided to investigate the potential for irreversible covalent binding to thiol-containing proteins. Drug–protein adducts (irreversible covalent binding) formed in humans may have the potential to act as haptens and elicit an immune-mediated adverse event. To assess in vitro covalent binding, a radiolabeled analogue of a compound is incubated in liver preparations from animals and humans followed by analysis of the precipitated protein for radioactive content.[12] Covalent binding is expressed as pmol drug equiv/mg total protein after a one-hour incubation.

To test the potential for irreversible covalent binding, one radioactive analogue from each class of the nitrile-based cathepsin K inhibitors was synthesized (Scheme 2). In the aminoacetonitrile class, a radioactive analogue was synthesized by performing a tritium exchange on the methyl sulfone of L-873724.[13] In the pyrimidine nitrile class, a ^{14}C-nitrile was introduced via a cyanide displacement reaction. And finally, in the cyanamide series, a ^{14}C-phenyl was introduced by reacting an amine precursor with the required radioactive phenyl sulfonyl chloride.

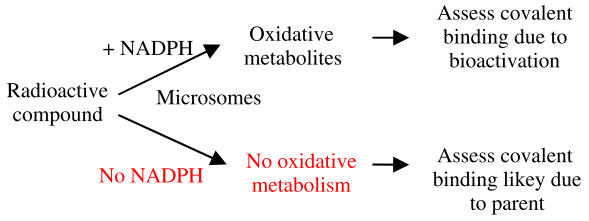
Normally, microsomal incubations are carried out in the presence of NADPH to monitor bioactivation that results in reactive electrophiles which, in turn, may irreversibly bind to proteins. Since we were interested in monitoring the potential of the parent nitrile moiety to react irreversibly with proteins, the incubations described below were done in the absence of NADPH (no oxidative metabolism, see Scheme 3).

The results from the covalent binding studies in human liver microsomes (in the absence of NADPH) are depicted in Table 2. The compound bearing the least reactive nitrile, ^{3}H-L-873724, shows negligible levels of covalent binding.

However, the more reactive pyrimidine nitrile ^{14}C-**2** and cyanamide ^{14}C-**1** were found to exhibit significant levels of covalent binding.[14] In particular, the cyanamide ^{14}C-**1** shows high levels of covalent binding in human liver microsomes (460 pmol equiv/mg @ 1 h). Similarly high levels were also obtained with ^{14}C-**1** when incubated in rat liver microsomes (550 pmol equiv/mg @ 1 h). The moiety responsible for the covalent binding is assumed to be the nitrile. As with the correlation between calculated electrophilicity and reactivity with cysteine, here too there is a loose correlation between reactivity of the nitrile moiety and its potential to lead to irreversible covalent binding with proteins. Clearly, the less reactive aminoacetonitrile L-873724 has a very low potential to cause covalent binding, whereas the more reactive nitriles **1** and **2** do. However, the extent of irreversible



Scheme 2. Synthesis of radioactive analogues for covalent binding studies. Reagents and conditions: (a) T_2O, DBU, THF, rt, overnight; (b) $Na^{14}CN$, DABCO, DMSO, H_2O, 85 °C; (c) $ClSO_2$–^{14}C-phenyl, Et_3N, CH_2Cl_2, rt; (d) TFA, CH_2Cl_2, rt; (e) BrCN, Et_3N, CH_2Cl_2, rt.



Scheme 3. Microsomal studies done in the presence of NADPH assess covalent binding due to bioactivation, whereas if NADPH omitted, only the covalent binding due to parent is monitored.

Table 2. Correlation of nitrile reactivity with irreversible covalent binding in human liver microsomes

Compound	% cysteine adduct formed[a] (%)	Covalent binding in human liver microsomes (no NADPH) % bound (pmol equiv/mg @ 1 h)[b]
^{3}H-L-873724	5	0.06% (7)
^{14}C-**2**	79	0.5% (70)
^{14}C-**1**	71	3.6% (460)

[a] 100 μM nitrile, 1 mM cysteine, PO_4 buffer, pH 7.4, 37 °C, 30 min.
[b] 10 μM nitrile, 1 mg/mL protein, 37 °C, 1-h incubation.

covalent binding does not correlate with extent of cysteine adduct formation. On the basis of electrophilicity and reactivity for **1** and **2**, one would expect similar levels of covalent binding but as seen in Table 2, the cyanamide **1** causes 7-fold higher levels of covalent binding relative to the pyrimidine nitrile **2**. While these results are consistent with the possibility that the nitrile reactivity may be loosely correlated with irreversible covalent binding, a larger data set including more compounds and more protein sources would be required to determine whether there is, indeed, a relationship.

In summary, a simple theoretical calculation (reactivity of nitriles with methanethiol) was used to predict the electrophilicity of diverse nitrile-containing compounds. This theoretical method is powerful in its simplicity and generality, allowing the direct comparison of electrophilicities between nitriles of different classes. These calculated electrophilicities were found to correlate with the extent of thiazoline adduct formed upon incubation of the nitriles with cysteine. Three classes of nitrile-based cathepsin K inhibitors were studied and it was found that the inhibitors which possess a very electrophilic nitrile (as judged by cysteine adduct formation) also undergo irreversible covalent binding to liver microsomes in the absence of NADPH. The two classes which exhibited such binding were the cyanamides and pyrimidine nitriles. A larger data set would be required to determine if there is a relationship between highly reactive nitriles and irreversible covalent binding to proteins.

However, aminoacetonitriles such as L-873724 possess low reactivity and negligible covalent binding. Thus, the potential for adverse effects in humans due to irreversible covalent binding would be minimized with this class of inhibitors.

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14. In the presence of NADPH, the following results were obtained following a 1-h incubation with human microsomes: 23 pmol equiv/mg@1 h for ^{3}H-L-873724; 1665 pmol equiv/mg @ 1 h for ^{14}C-**2**; and 2400 pmol equiv/mg @ 1 h for ^{14}C-**1**.