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Graphical Abstract

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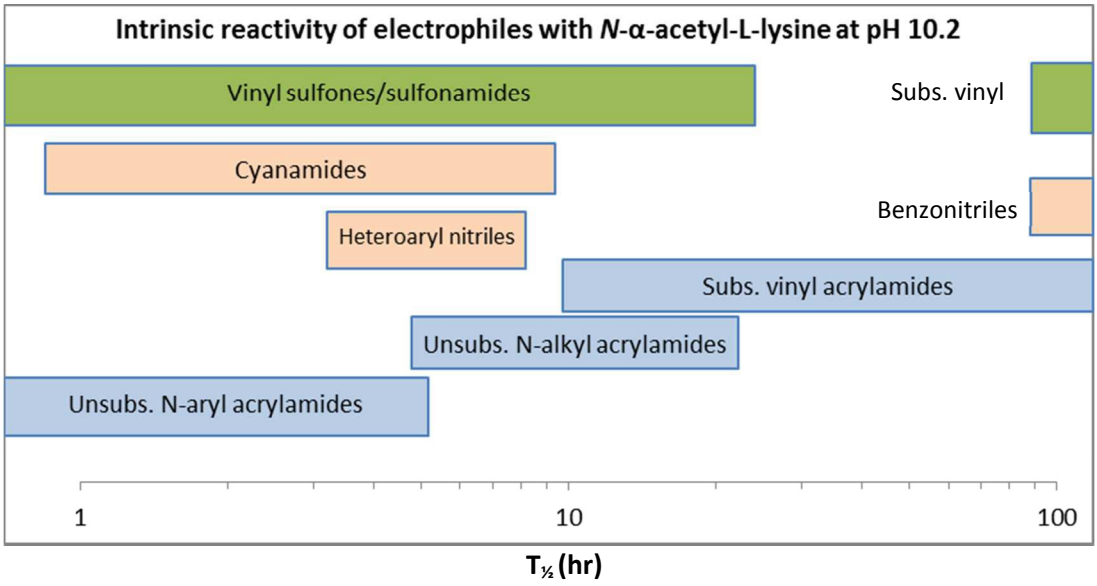

Intrinsic reactivity profile of electrophilic moieties to guide covalent drug design: *N*-α-acetyl-L-lysine as an amine nucleophile†

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Abstract

Covalent drugs contain a reactive electrophilic moiety or covalent reactive group (CRG), which forms an irreversible bond between the drug and a biological target. Consequently, the intrinsic reactivity of the CRG is an important consideration in the design of irreversible inhibitors. Although reactivity assessments of CRGs with sulfur nucleophiles, such as glutathione and cysteine have been reported, reactivity of these moieties with amine-containing nucleophiles is not well described. In this study, intrinsic reactivities were determined for a series of electrophiles (acrylamides, nitriles, cyanamides, sulfones, and sulfonamides) using N-α-acetyl-L-lysine as a model amine-based nucleophile and compared with results using glutathione (GSH). Since the ε-amine of *N*-α-acetyl-L-lysine is protonated at neutral pH, reactions were carried out at pH 10.2. In addition to reporting rate data for reactions of CRGs with N-α-acetyl-L-lysine, elements of selectivity relative to thiol-containing nucleophiles are also be discussed.

Introduction

As of 2011, there were 39 FDA approved covalent drugs[1] with additional examples having been approved since then. However, most of these compounds were discovered accidentally as opposed to being conceived of by rational design. Covalent drugs bind to biological targets via chemical modification using a stable bond. In most cases functional activity of the biological target is only restored when the target has been resynthesized. This covalent or irreversible modification of a biological target can offer several important advantages such as a) establishing non-equilibrium binding kinetics,[2, 3] b) producing a prolonged pharmacodynamic effect if the biological target resynthesis rate is slow, c) achieving complete target occupancy/total inhibition of biological activity, and d) achieving exquisite selectivity if the target possess a unique nucleophilic residue within its active site that is lacking in closely related proteins. Despite these advantages, there remains a reluctance to develop covalent drugs due to the potential for toxicities associated with immunogenic responses to covalently modified proteins.[4, 5] In theory, this risk can be mitigated by increasing the selectivity through manipulation of K_i for binding and k_{inact} for reactivity. Therefore, having an understanding of the reactivity of various CRGs with biologically relevant nucleophiles and a "toolbox" of well characterized CRGs from which to select in drug design are essential components in the discovery of covalent inhibitor drugs.

There are three main types of nucleophilic moieties present in proteins: thiol (cysteine): hydroxyl (serine, threonine and tyrosine), and amine (lysine and histidine). Reactivity profiling of CRGs with these biological nucleophiles can be a valuable exercise.[6] Previous studies in this regard have typically focused on thiol reactivity, using either cysteine or glutathione as the nucleophile.[7-14] However there are very few reports of the reactivity of an amine, such as lysine, as the nucleophile.[11] In most enzymes, lysines are found on the surface of the proteins. There are also many enzymes containing lysine in their active sites, where they play a crucial role in catalysis[15-19] forming hydrogen bonds, acting as nucleophiles or behaving as general bases. For example Lys303 of the pyridoxal 5'-phosphate dependent-enzyme DOPA decarboxylase, which is responsible for the irreversible conversion of l-DOPA to dopamine, is involved in

external aldimine formation and hydrolysis as well as in product release which affects the rate-determining step of decarboxylation.[17] Similarly Lys98 and Lys100 of dihydroneopterin aldolase, an enzyme responsible for the conversion of 7,8-dihydroneopterin to 6-hydroxymethyl-7,8-dihydropterin in the folate biosynthetic pathway, plays an important role in catalysis and substrate binding.[19] The ε-amine of surface lysines usually have pK_a values of ~10.8,[20] hence they are almost fully protonated at physiological pH and are thus very weak nucleophiles. However, it has been reported that active site lysines can have pK_a's as low as 5.7. [21] In such cases, they would likely exhibit adaquate nucleophilicity to be modified by non-selective and/or highly reactive covalent inhibitors. For this study, we determined the reactivity of a series of electrophilic moieties (CRGs) with *N*-α-acetyl-L-lysine as a surrogate, amine-based nucleophile. The electrophiles studied were selected from groups consisting of α,β-unsaturated sulfones and sulfonamides, acrylamides, nitriles and cyanamides – common moieties found in many covalent inhibitors that react with Lys residues on proteins despite not being selective for Lys. Recent publications have highlighted electrophiles that show specificity for Lys, and a fuller study covering multiple examples of these electrophiles would certainly be of value.[22 ,23, 24] Reactivity assessments (experimental details are presented in Supplementary Content) were carried out to determine half lives ($T_{1/2}$) and pseudo-first order rate constants.

Results and Discussion

Michael accepters are prevalent in bioactive molecules. Hence acrylamides were considered as an important class of electrophilic moieties, and a small library of acrylamide electrophiles was constructed varying both the substituents on the vinyl group as well as the substituents on the amide nitrogen. In addition, other electrophilic moieties classes such as nitriles, cyanamides, vinyl sulfones and vinyl sulfonamides were included in the study. Common scaffolds of the electrophilic moieties selected for the study are depicted in Figure 1.

<Insert Figure 1>

N-α-acetyl-L-lysine is a simple model compound to mimic lysine within protein. In order to mimic the lowered pK_a of a biologically relevant nucleophilic lysine, we decided to study *N*-α-acetyl-L-lysine reactivity at pH 10.2 where the ε-amine of lysine would be deprotonated. We initially attempted to perform reactivity assessments at a more physiologically relevant pH of 7.4, but we saw no reactions between N-α-acetyl-L-lysine and even the most reactive electrophiles in our study. This result also indicates that the carboxylate moiety of *N*-α-acetyl-L-lysine is unreactive even though it is deprotonated. To ensure pseudo first order conditions, reactions used 1 equivalent of electrophile and 50 equivalents of *N*-α-acetyl-L-lysine. Since some of the electrophiles were found to be insoluble in aqueous solution; 10 percent *N,N*-dimethylacetamide aqueous solution was used for all the assays. All the reactivity assessments were quantified by MS detection except for four very reactive electrophiles which were analyzed by ^{1}H NMR. For every electrophile, the relative concentrations (analyte to internal standard peak area ratio for MS and sensitive proton peak area for NMR) of the electrophile and the *N*-α-acetyl-L-lysine/electrophile product were plotted against time (Figure 2A). It is important to note that the signals from both the electrophile and the product cannot be compared directly as they possibly have different MS responses due to different ionization potentials.[25] The natural logarithms of the relative concentration of the reactants were plotted against time to determine the pseudo first order rate constants as a negative slope of the straight line (Figure 2B). The pseudo first order rate constant was used to determine half-life using the following equation.

$$T_{1/2} = \ln (2)/(\text{pseudo first order rate constant})$$

< Insert Figure 2>

The measured half-lives of unsubstituted acrylamides reacting with *N*-α-acetyl-L-lysine is presented in Table 1. A number of different acrylamides were studied including those with aromatic and aliphatic N-substituents. We felt it was important to determine exact reactivity of electrophiles caused by

subtle changes in substituents so that the results could be applied confidently during drug design. As expected, pyridine acrylamide **1** reacted more quickly with *N*-α-acetyl-L-lysine than the corresponding phenyl analog **4** due to inductive effect of the pyridine on the electron density of the vinyl group. Aryl substituted acrylamides generally reacted faster than alkyl substituted compounds. As one might expect, the presence of electron withdrawing groups in a benzene ring increased reactivity whereas attaching electron releasing group in the benzene ring decreased reactivity (electron withdrawing substituents **2**, **3**; electron donating substituents **6, 7, 9** compared to **4**). The analogs **6**, **7**, and **9** also show that the effect of the electron-donating OMe group lessens as the group is moved from the *o*- ($T_{1/2}$: 2.85 h) to the *m*- ($T_{1/2}$: 3.58 h) to the *p*- ($T_{1/2}$: 5.94 h) position. The *m*-halo phenyl compounds (**3** and **5**) show similar half-lives to the phenyl analog **4**. Ring strain in the N-substituent plays a significant role in reactivity as observed in compounds **11, 12**, **13**, **15** and **16,** with small ring azetidine amide **11** showing greater reactivity than the corresponding 5- and 6-membered ring analogs. That the piperazine analog **10** reacts more than 7 times faster than the piperidine analog **16** is likely due to electron withdrawing nature of the N-Me piperazine nitrogen. Overall, these results demonstrate that subtle changes in substituents distal from the reaction site can be utilized to modulate reactivity.

<Insert Table 1>

To test the reactivity of α, β-substituted acrylamides with N-α-acetyl-L-lysine, a compound-set was assembled where either the α- and/or a β- position were substituted. Most of these substituted acrylamides were found to be unreactive with *N*-α-acetyl-L-lysine in our experimental conditions. For a few of compounds (**17-22**), half-lives could be measured, while for the others the intrinsic reactivity with lysine was too slow to determine (Table 2). Unreactive compounds were predicted to have half-lives greater than 100 hours, and reactions were not run longer than 21 hours to determine accurate half lives. It was found that placing a methyl group either at the α- or β-position of compound **4** (**20** and **22**) increased half-lives by greater than 30-fold. The effect of stereochemistry of the acrylamide β-substituent can be seen comparing compounds **19** and **34**. The E-stereoisomer (**19**) of 3-methoxy-*N*-

methylacrylamide reacts with *N*-α-acetyl-L-lysine with a half-life of ~48 hours whereas (Z)-analog (**34**) did not react with *N*-α-acetyl-L-lysine under the experimental conditions employed. The δ-lactam analog 3-methylenepiperidin-2-one (**24**) did not react with *N*-α-acetyl-L-lysine but the corresponding N-Me (**18**) and N-Ph analogs (**17**) show increased reactivity again demonstrating the electronic effect of the N-substituent.

<Insert Table 2>

Intrinsic reactivity results of *N*-α-acetyl-L-lysine and a limited number of cyanamides and nitriles is presented in Table 3. As seen with the acrylamide reactivities, ring strain can accelerate cyanamide reactivity. Also a spiro-fused azetidine and piperidine reacted more rapidly than the corresponding monocyclic analogs (**36** vs. **37** and **40** vs. **41**). Aliphatic nitriles did not react under the conditions used (data not shown), but aromatic nitriles were reactive enough to generate half-lives. The influence of an electronegative nitrogen on reactivity was also observed. While benzonitrile **46** did not react, pyridyl nitriles (**43**, **44**, and **45**) did react with *N*-α-acetyl-L-lysine.

<Insert Table 3>

The reactivity of vinyl sulfones and sulfonamides with *N*-α-acetyl-L-lysine is presented in Table 4. The α, β-unsubstituted vinyl sulfones (**47** and **48**) reacted so quickly under the reaction conditions that half-lives could not be assessed using HPLC-MS. However, the half-lives of the reactive sulfones could be determined by decreasing the lysine concentration to 10 equivalents and using ^{1}H NMR to monitor reaction progress. Alkyl substitution on the vinyl moiety decreased reactivity of methyl vinyl sulfones compared to the corresponding unsubstituted analogs (**48** vs. **52** and **53**). Replacement of the N-aryl group in **47** by cyclohexyl group in **50** decreased reactivity more than 10-fold. Vinyl sulfonamides reacted more slowly than vinyl sulfones. Analogous to acrylamides, ring strain, electronic and substitution effects were evident on reactivity. However higher reaction rates were observed for reaction of *N*-α-acetyl-L-lysine with sulfones and sulfonamides compared to acrylamides. For example, most of

the vinyl substituted acrylamides were inert with *N*-α-acetyl-L-lysine while most of the substituted sulfones and sulfonamides showed half lives of less than 30 hours under similar experimental conditions (*vide infra*). This observation compelled us to compare reactivity trends of the electrophiles between glutathione, a biologically relevant thiol-nucleophile, and *N*-α-acetyl-L-lysine with different classes of electrophiles to distinguish if sulfones and sulfonamides are relatively selective in terms of lysine reactivity. Glutathione was found to react with our electrophiles exclusively at the thiol sulfur. The amine in glutathione has a BpKa ~ 9.6 and is thus almost fully protonated at pH 7.4. Thus it's not reactive and does not compete with the more nucleophilic sulfur nucleophile is electrophile modification.

<Insert Table 4>

The glutathione reactivity assays were carried out at pH 7.4 using 10 equivalents of glutathione in 100 mM potassium phosphate buffer while the *N*-α-acetyl-L-lysine reactivity assays were performed at pH 10.2 using 100 mM sodium borate buffer. Despite the differences in pH and nucleophile concentration between glutathione and *N*-α-acetyl-L-lysine assays, a comparison can be made. Using the Henderson-Hasselbach equation (pH = pKa + log ([base]/[acid])), the percent of unprotonated ε-amine can be determined at different pH conditions. At pH 7.4 almost 0% of the ε-amine of lysine will be unprotonated (using pKa of ε-amine 10.8[20]). If the pH is increased to 10.2, approximately 20% of ε-amine will be unprotonated. If we consider only unprotonated ε-amine as a nucleophile, 10 equivalent out of 50 equivalents of *N*-α-acetyl-L-lysine used in reaction condition would be nucleophilic. Hence it can be roughly assumed that there will be similar effective concentrations of the nucleophiles in the *N*-α-acetyl-L-lysine and the glutathione reactivity assays.

The intrinsic reactivity of selected electrophilic compounds with *N*-α-acetyl-L-lysine and glutathione is presented in Table 5 comparing half-lives with *N*-α-acetyl-L-lysine and glutathione respectively. Under our reaction conditions, it is observed that acrylamides, cyanamides and nitriles are slightly less reactive towards *N*-α-acetyl-L-lysine compared to glutathione. Vinyl unsubstituted

acrylamide compounds **1**, **4** and **7** reacted two to five-fold faster with glutathione than with *N*-α-acetyl-L-lysine. Similarly, cyanamide **39** and nitrile **42** react 2- and 9-fold faster respectively. Interestingly all sulfones and sulfonamides electrophilic moieties reacted faster with *N*-α-acetyl-L-lysine. For example, sulfone **52** reacted with *N*-α-acetyl-L-lysine 9-fold faster than with glutathione. Similarly, sulfonamides **51**, **54**, and **57** reacted 1.6 to 4-fold faster with *N*-α-acetyl-L-lysine than with glutathione. *N*-α-acetyl-L-lysine is considered as a hard nucleophile while glutathione is a soft nucleophile. The β-vinyl carbon of acrylamides and the carbon in nitriles are soft electrophilic centers. Hence the soft nucleophile glutathione should react faster than the harder nucleophile *N*-α-acetyl-L-lysine -amine[26]. In sulfones and sulfonamides, the presence of two electronegative oxygen atoms make the vinyl group more polarizable because the net atomic negative charge in sulfone analogs resides in sulfone group (SOO).[27] Hence the softness of vinyl group may be decreased significantly. The polar nature of S-O bond in sulfone and sulfonamides has been discussed previously in the literature. Phillips *et al*.[28] proposed double bond characteristics of the S-O bond based on small dipole moments (2.2 – 2.9 D) of S-O linkage, a short bond length and greater bond strength. However Cumper and Walker[29] recalculated the dipole moments and found that the dipole moment is in the range of 2.8 to 4.3 D. Similarly Toshiyasu *et al*.[30] determined the dipole moment of the diaryl sulfone as high as 4.5 D. These larger dipole moments suggest that the S-O linkage as charge separated. This indicates that the sulfones and sulfonamides act as strong electron withdrawing groups and could decrease the electron density significantly in the vinyl group resulting in a harder terminal β-carbon electrophile.

<Insert Table 5>

To test experimental error and the reproducibility of the results, a small set of selected electrophiles over a wide range of reactivity were assessed by running experiments for five electrophiles four times. The average half-lives and standard deviations are presented in Table 6. Excellent reproducibility of the measured half-lives for fast to moderately reactive electrophiles (**51**, **12**, **55**, **16**) is seen but reproducibility decreased for a less reactive electrophile (**21**). The percent relative standard

deviation for the reactions having half-lives of less than 30 hours was found to be less than 10%. For a slow reacting electrophilic moieties, reactions were monitored only for 21 hours. Hence experimental errors for the slow reacting electrophilic moieties (half-lives higher 40 hours) can be expected to be higher. As expected, the percent relative standard deviation of compound **21** which had a 71 hour half-life was found to be 22%.

<Insert Table 6>

<Insert Figure 3>

In conclusion, the intrinsic reactivity of *N*-α-acetyl-L-lysine with electrophilic covalent reactive groups (acrylamides, vinyl sulfones, cyanamides and nitriles) was assessed. Pseudo first order rate constants were used to calculate electrophile half-lives and compounds were then ranked ordered on that basis. The general reactivity trends are presented in Figure 3. Unsubstituted vinyl acrylamides had half-lives starting from less than an hour to 26 h. The *N*-aryl acrylamide reacted faster than compared to the *N*-alkyl acrylamide. Vinyl substituted acrylamides were found to be less reactive. Aza-aromatic nitriles have half-lives from 4 to 10 hours whereas benzonitriles were unreactive. Ring strain was an important factor in reactivity of cyanamides with *N*-α-acetyl-L-lysine. The reactivity of electrophiles was influenced by electronic effects, inductive effects and ring strain of substituents distal to the reacting group. Most interestingly we discovered the partial selectivity in intrinsic reactivity of vinyl sulfones and sulfonamides with *N*-α-acetyl-L-lysine over glutathione. These findings can potentially be useful in the design of covalent inhibitors for the therapeutic targets containing activated lysines in the active site.

Experimental

Materials and instruments: *N*-α-acetyl-L-lysine was purchased from Acros Organics. All the electrophiles were obtained from commercial sources or Pfizer's compound library. Reactivity assessments were carried out using a Gilson 215SW liquid handler system coupled with an Agilent HPLC

system and MS detector. NMR assays were carried out using a Bruker 600 MHz NMR (Bruker BioSpin Corporation, Billerica, MA) controlled with Topspin V3.1 equipped with a 5mm SEF probe.

ReactArray method to determine kinetic parameters for *N*-α-acetyl-L-lysine reactivity: Reactions were carried out with a ReactArray Workstation, which consists of a reactivity rack with individual temperature and stirring controlled reaction vessels, a solvent rack, a reagent rack and an analytical rack; a Gilson 215SW liquid handler with a 402 dual syringe dilutor, an injection module for automatic sample injection into an Agilent 1100 HPLC/MS for online analysis and ReactArray control software to automatically set up, sample and quench the reactions, and to prepare analytical samples for LCMS analysis. Reactions were run at 37 °C. Stock solutions and reactions were stored and run under nitrogen. A 250 µl aliquot of 20.0 mM solution of electrophile in *N,N*-dimethylacetamide was manually transferred to a reaction vial. A 250 µl aliquot of a 2.0 mM solution of indoprofen (used as internal standard in MS analysis) in *N,N*-dimethylacetamide was automatically transferred to the vial using the liquid handler system of the ReactArray. *N*-α-acetyl-L-lysine (4.5 ml of 55.5 mM solution in 100 mM borate buffer pH 10.2) was automatically transferred to the vial using the ReactArray's liquid handling system to begin each reaction (t = 0). Reactions were run for 7 h with aliquots being removed every hour by taking out 100 µL of the reaction mixture and diluting with 900 µL of water. For slow reacting electrophiles, samples were taken every 2 hours for 14 hours or every 3 hours for 21 hours. Control reactions were run in absence of *N*-α-acetyl-L-lysine in 100 mM borate buffer at pH 10.2. Reactants, products and the internal standard were separated by liquid chromatography (LC) using an Atlantis T3 column (3.0 x 75 mm, 3 µm) and quantified using the MS detector. The LC mobile phase consist of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol). The liquid chromatography was initiated with 95% mobile phase A and was held for 2 minutes followed by linear increase to make the solvent B to 95% in 6 min. The 95% of solvent B was held for 2 minutes, and then rapidly and linearly decreased to 5% in 0.1 minutes. At the end the 95% of solvent A was held for 2.9 min making the overall run-time 13 minutes.

ReactArray method to determine kinetic parameters for glutathione reactivity: Experiments were carried out using the method described above for *N*-α-acetyl-L-lysine with some minor changes. Briefly, all the stock solutions and solvents were bubbled with nitrogen for at least 6 hours and stored under nitrogen during reactions. All reactions were run under nitrogen. Electrophile (250 µl of 20.0 mM solution in *N,N*-dimethylacetamide) was manually transferred to a reaction vial. Indoprofen (250 µl of 2.0 mM *N,N*-dimethylacetamide solution; used as internal standard in MS analysis) was automatically transferred to the vial using the ReactArray liquid handler system. Glutathione (4.5 ml of 11.1 mM solution in 100 mM potassium phosphate buffer pH 7.4) was automatically transferred to the vial to initiate the reaction. Reactions were run for 7 h with aliquots being removed every hour by taking out 100 µL of the reaction mixture and diluting with 900 µL of water.

NMR method to determine kinetic parameters for *N*-α-acetyl-L-lysine reactivity: Stock solutions of electrophile (2.0 mM) and *N*-α-acetyl-L-lysine (100 mM) were prepared in 100 mM borate buffer (pH = 10.2) containing 10% D_2O. A 5 mm (ID) NMR tube containing 0.5 mL aliquot of the 2.0 mM electrophile solution was placed in a Bruker 600 MHz NMR spectrometer. The probe temperature was set to 37 °C and the sample was spun in the spectrometer probe. The NMR signal was locked on D_2O and then the instrument was tuned and matched prior to shimming the magnet. The tube was then removed from the spectrometer and diluted 1 to 1 using 0.5 mL of a 100 mM solution of *N*-α-acetyl-L-lysine. The NMR tube was capped and inverted several times for mixing prior to being placed back in the spectrometer. Typically, 1D spectra were recorded using a pre-saturation pulse sequence with a sweep width of 7500 Hz and a total recycle time of 7 s. Each acquisition consisted of 2 dummy scans followed by 32 scans for an elapsed time of 5.75 minutes. A typical analysis consisted of 100 successive acquisitions for a total elapsed time of 9.5 hours. Control reactions of electrophiles were run in absence of *N*-α-acetyl-L-lysine in 100 mM borate buffer at pH 10.2.

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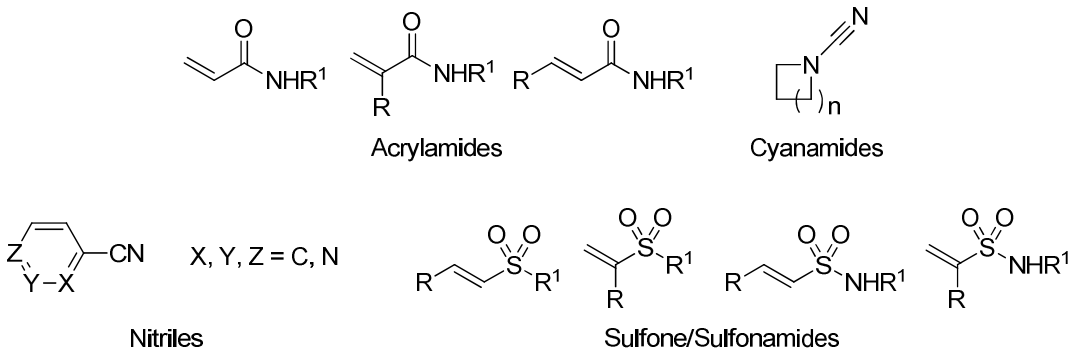


Figure 1. Classes of electrophilic moieties selected for the reactivity profiling with *N*-α-acetyl-L-lysine.

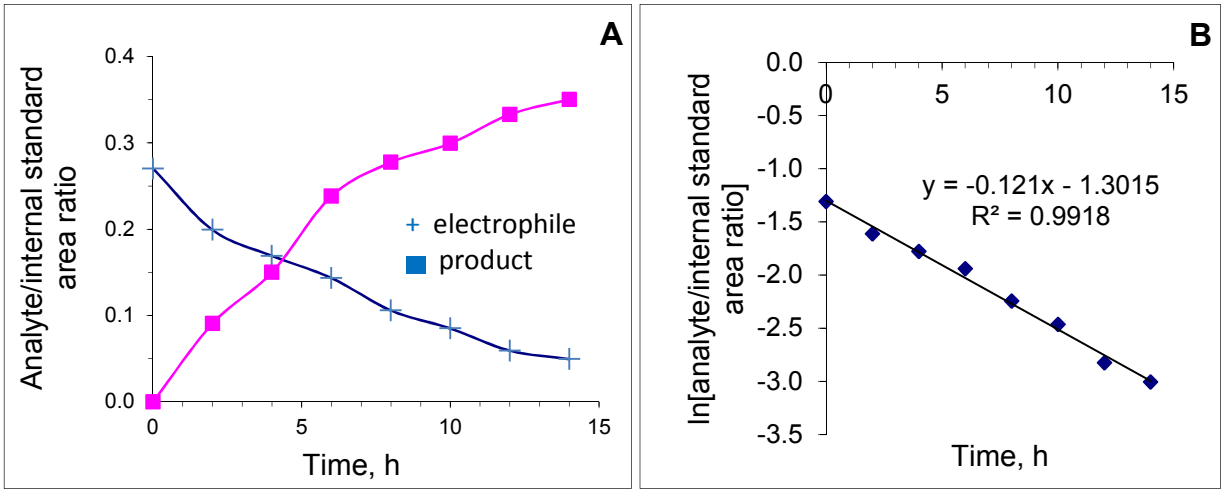


Figure 2. A) Plot of analyte/internal standard area ratio of electrophile **11** and product against time to show disappearance of reactant and formation of product, and B) Plot of natural log of analyte/internal standard area ratio of electrophile **11** against time to get the pseudo-first order rate constant as a negative slope.

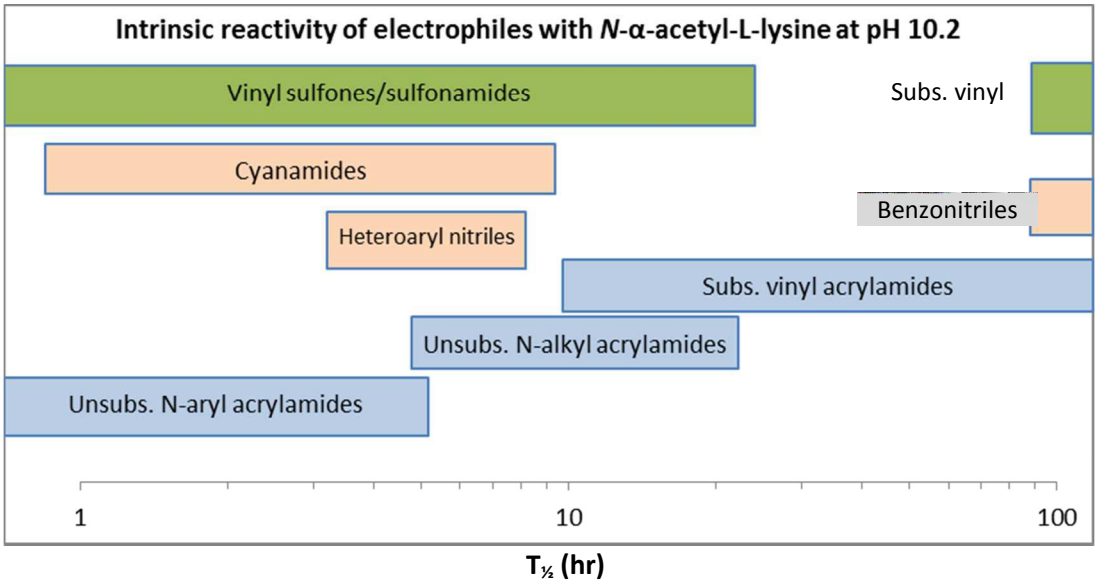
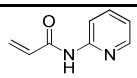
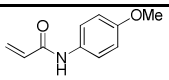
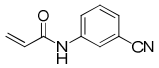
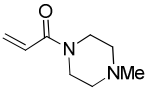
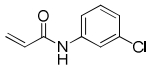
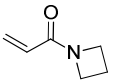
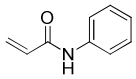
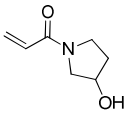
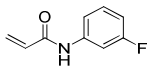
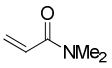
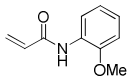
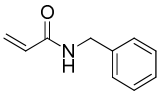
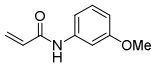
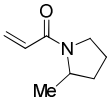
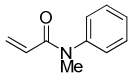
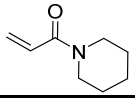


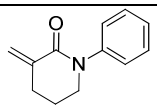
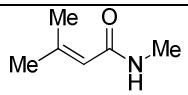
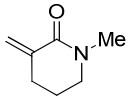
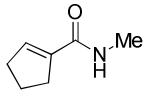
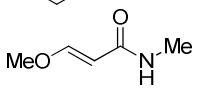
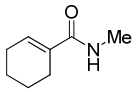
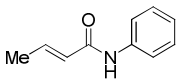
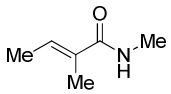
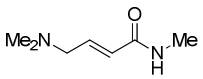
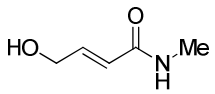
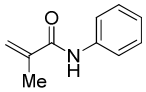
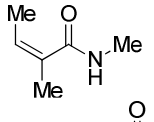
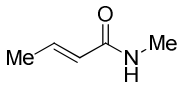
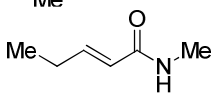
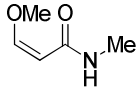
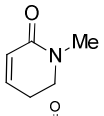
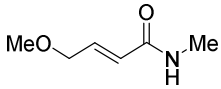
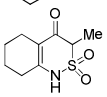
Figure 3. Half-life ranges of different classes of electrophiles with *N*-α-acetyl-L-lysine at 37 °C using 50 equivalent *N*-α-acetyl-L-lysine in 100 mM borate buffer pH 10.2.

Table 1. Reactivity of Unsubstituted-Vinyl Acrylamides with *N*-α-Acetyl-L-Lysine.

Compound number	Structure	[a]Half-life, h	Compound number	Structure	[a]Half-life, h
1		0.67	**9**		5.94
2		1.13	**10**		3.53
3		1.88	**11**		5.71
4		2.06	**12**		6.17
5		2.24	**13**		11.2
6		2.85	**14**		14.4
7		3.58	**15**		16.9
8		3.76	**16**		26.1

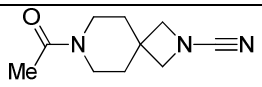
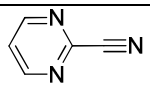
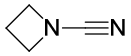
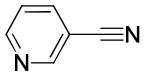
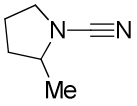
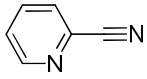
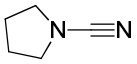
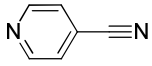
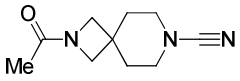
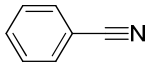
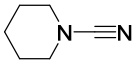
[a]Half-life values are from single assay. Reproducibility of the results was tested using a small library of the compounds which is presented in Table 6.

Table 2. Reactivity of Substituted Vinyl Acrylamides with *N*-α-Acetyl-L-Lysine.

Compound number	Structure	[a]Half-life, h	Compound number	Structure	[a]Half-life, h
17		5.51	**27**		>100
18		45.9	**28**		>100
19		47.5	**29**		>100
20		68.8	**30**		>100
21		71.3	**31**		>100
22		77.0	**32**		>100
23		>100	**33**		>100
24		>100	**34**		>100
25		>100	**35**		>100
26		>100			

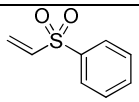
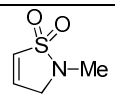
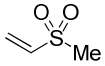
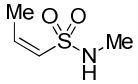
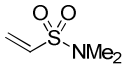
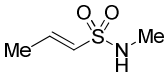
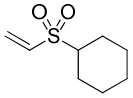
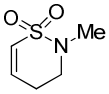
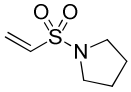
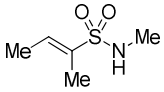
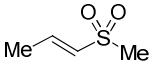
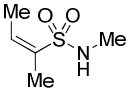
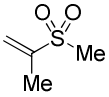
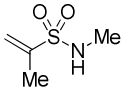
[a]Half-life values are from single assay. Reproducibility of the results was tested using a small library of the compounds which is presented in Table 6.

Table 3. Reactivity of Cyanamides and Nitriles with *N*-α-Acetyl-L-Lysine.

Compound number	Structure	Half-life, h	Compound number	Structure	Half-life, h
36		1.39	**42**		4.78
37		2.59	**43**		7.21
38		7.27	**44**		8.01
39		7.70	**45**		10.1
40		9.33	**46**		>100
41		11.1			

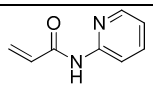
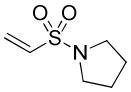
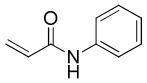
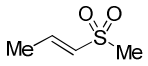
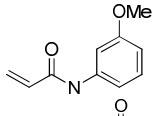
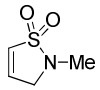
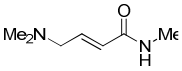
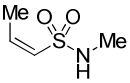
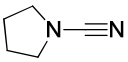
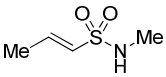
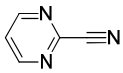
[a]Half-life values are from single assay. Reproducibility of the results was tested using a small library of the compounds which is presented in Table 6.

Table 4. Reactivity of Vinyl Sulfones/Sulfonamides with *N*-α-Acetyl-L-Lysine.

Compound number	Structure	[a]Half-life, h	Compound number	Structure	[a]Half-life, h
47		0.11 [b, c]	**54**		7.85
48		0.17 [b, c]	**55**		11.4
49		0.22	**56**		15.6
50		0.28 [b]	**57**		29.8
51		0.33	**58**		100
52		3.08	**59**		100
53		5.21 [b]	**60**		100

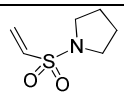
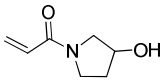
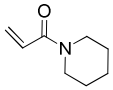
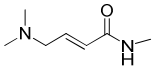
[a]Half-life values are from single assay. Reproducibility of the results was tested using a small library of the compounds which is presented in Table 6.
[b]^{1}H NMR was used to monitor reaction, [c]Only 10 eq. of *N*-α-acetyl-L-lysine was used

Table 5. Comparison of Intrinsic Reactivities of *N*-α-Acetyl-L-Lysine and Glutathione with Electrophilic Moieties.

Compound	Structure	$^{a}T_{1/2}$ N-α-acetyl-L-Lys, h	$^{a}T_{1/2}$ GSH, h	Compound	Structure	$^{a}T_{1/2}$ with Lys, h	$^{a}T_{1/2}$ with GSH, h
1		0.67	0.13	**51**		0.33	0.53
4		2.06	0.66	**52**		3.08	28.0
7		3.58	1.55	**54**		7.85	31.0
21		71.4	59.0	**55**		11.4	23.0
39		7.70	2.91	**56**		15.6	60.0
42		4.78	0.5	**57**		29.8	48.7

[a]Half-life values are from single assay.

Table 6. Average Half-Life (n= 4) of Selected Electrophilic Moieties with *N*-α-Acetyl-L-Lysine at pH 10.2 to Test Reproducibility.

Compound	Structure	Average half-life with *N*-α-acetyl-L-Lys, h	Standard deviation	%RSD[a]
51		0.34	0.01	2.9
12		7.14	0.66	9.2
55		9.67	0.96	9.9
16		24.0	1.5	6.2
21		71.1	15.4	22

[a]%RSD, percent relative standard deviation.