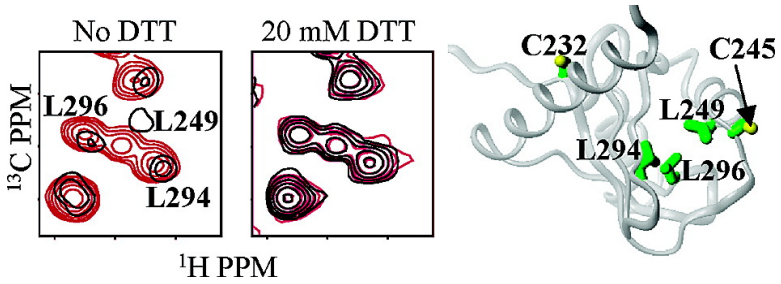
Article

ALARM NMR: A Rapid and Robust Experimental Method To Detect Reactive False Positives in Biochemical Screens

Jeffrey R. Huth, Renaldo Mendoza, Edward T. Olejniczak, Robert W. Johnson, Darlene A. Cothron, Yaya Liu, Claude G. Lerner, Jun Chen, and Philip J. Hajduk

J. Am. Chem. Soc., **2005**, 127 (1), 217-224• DOI: 10.1021/ja0455547 • Publication Date (Web): 02 December 2004



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 5 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 12/02/2004

ALARM NMR: A Rapid and Robust Experimental Method To Detect Reactive False Positives in Biochemical Screens

Jeffrey R. Huth, Renaldo Mendoza, Edward T. Olejniczak, Robert W. Johnson, Darlene A. Cothron, Yaya Liu, Claude G. Lerner, Jun Chen, and Philip J. Hajduk*

Contribution from Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, Illinois 60064

Received July 23, 2004; E-mail: philip.hajduk@abbott.com

Abstract: High-throughput screening (HTS) of large compound collections typically results in numerous small molecule hits that must be carefully evaluated to identify valid drug leads. Although several filtering mechanisms and other tools exist that can assist the chemist in this process, it is often the case that costly synthetic resources are expended in pursuing false positives. We report here a rapid and reliable NMR-based method for identifying reactive false positives including those that oxidize or alkylate a protein target. Importantly, the reactive species need not be the parent compound, as both reactive impurities and breakdown products can be detected. The assay is called ALARM NMR (a La assay to detect reactive molecules by nuclear magnetic resonance) and is based on monitoring DTT-dependent ^{13}C chemical shift changes of the human La antigen in the presence of a test compound or mixture. Extensive validation has been performed to demonstrate the reliability and utility of using ALARM NMR to assess thiol reactivity. This included comparing ALARM NMR to a glutathione-based fluorescence assay, as well as testing a collection of more than 3500 compounds containing HTS hits from 23 drug targets. The data show that current in silico filtering tools fail to identify more than half of the compounds that can act via reactive mechanisms. Significantly, we show how ALARM NMR data has been critical in identifying reactive compounds that would otherwise have been prioritized for lead optimization. In addition, a new filtering tool has been developed on the basis of the ALARM NMR data that can augment current in silico programs for identifying nuisance compounds and improving the process of hit triage.

Introduction

The cost of drug discovery continues to escalate and is due in large part to financing the discovery and development of compounds that fail to become drugs. The explosion of new biological data brought about by genomics and other new technologies has greatly increased the number of targets pursued in preclinical drug development, and efficient evaluation of these targets necessitates the rapid and reliable identification of high-quality drug leads that act via the desired mechanism of action. Leads against proteins are generally obtained by high-throughput screening (HTS) of large chemical libraries. Unfortunately, HTS assays can be plagued by false positives that can confound the triage process.[1] While many sources of false positive results can be directly assessed (e.g., assay interference from fluorescent compounds), others can be more subtle. For example, compound aggregation has recently been shown to be a significant factor in the generation of false positives.[2] Both fluorescent and highly aggregated false positives can be triaged using NMR techniques to validate binding when the target is amenable to this technique.[3,4] Compound reactivity is another significant concern.[5] To address this issue, informatics departments at pharmaceutical companies typically identify many reactive compounds in silico by searching for known reactive groups such as epoxides, anhydrides, and Michael acceptors.[6] However, the continued prevalence of reactive false positives highlights the need to experimentally assess large numbers of screening hits for their propensity for reactivity toward thiol groups.

Recently, an experimental method has been proposed to identify reactive screening hits.[7] In this method, a fluorescence-based assay was used to measure the reactivity of compounds toward glutathione (GSH) by quenching the reaction of GSH with the fluorogenic reagent, fluorescein-5-maleimide. While this assay can be very useful for the careful analysis of particular screening hits, it has several disadvantages that limit its applications to the rapid analysis of large numbers of structurally diverse compounds. First, fluorescein is only moderately red-shifted and many compounds can interfere with the assay and yield ambiguous results. Second, reaction of a test compound

(1) Rishton, G. M. *Drug Discov. Today* **2003**, *8*, 86−96.
(2) McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. *J. Med. Chem.* **2002**, *45*, 1712−1722.
(3) Hajduk, P. J.; Burns, D. J. *Comb. Chem. High Throughput Screen.* **2002**, *5*, 613−622.
(4) Jahnke, W.; Widmer, H. *Cell. Mol. Life Sci.* **2004**, *61*, 580−599.
(5) Rishton, G. M. *Drug Discov. Today* **1997**, *2*, 382−384.
(6) Roche, O.; Schneider, P.; Zuegge, J.; Guba, W.; Kansy, M.; Alanine, A.; Bleicher, K.; Danel, F.; Gutknecht, E. M.; Rogers-Evans, M.; Neidhart, W.; Stalder, H.; Dillon, M.; Sjogren, E.; Fotouhi, N.; Gillespie, P.; Goodnow, R.; Harris, W.; Jones, P.; Taniguchi, M.; Tsujii, S.; von der Saal, W.; Zimmermann, G.; Schneider, G. *J. Med. Chem.* **2002**, *45*, 137−142.
(7) Epps, D. E.; Taylor, B. M. *Anal. Biochem.* **2001**, *295*, 101−106.

10.1021/ja0455547 CCC: $30.25

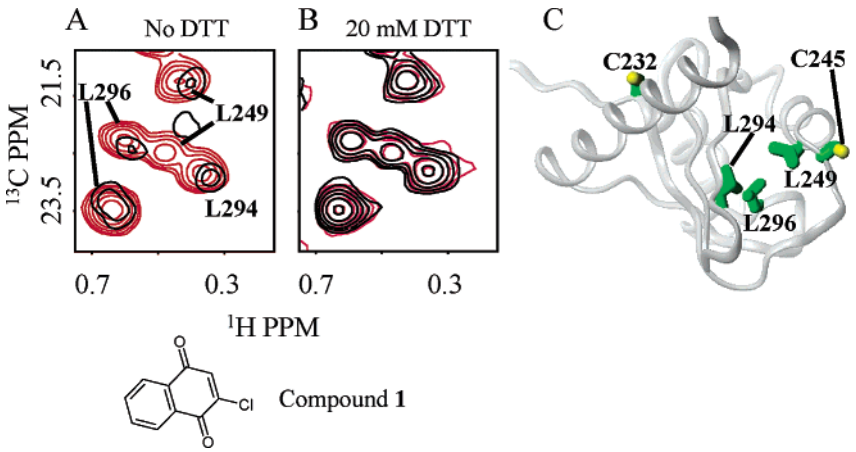


Figure 1. ALARM NMR data. (A) Expanded region of 2D ^{1}H-^{13}C HSQC spectra showing cross-peaks for four methyl groups of the human La antigen in the absence (red) and presence (black) of 0.4 mM compound **1** and no DTT. The complete spectra are shown in Figure S1. (B) Same spectra for samples to which 20 mM DTT had been added. (C) Structure of the C-terminal RNA recognition motif (RRM) domain from the human La antigen protein.[9] Cysteine residues and leucine residues whose methyl chemical shifts are shown in A and B are indicated.

with fluorescein-5-maleimide can hinder the ability to assess reactivity with GSH.[7] Third, cysteines in a proteinaceous environment can have a significantly different reactivity profile than a small molecule,[8] raising doubt about the reliability of using GSH as a surrogate for protein targets. Fourth, GSH is unstable and can rapidly oxidize, particularly at low concentrations (Huth, J.; Johnson, R., unpublished observations). This requires careful handling to minimize false positive results. In addition, the highly efficient reaction of GSH with fluorescein-5-maleimide can preclude the detection of compounds that react more slowly.[7] All of these considerations highlight the continued need for rapid and reliable assays that are capable of evaluating a wide diversity of screening hits for thiol reactivity.

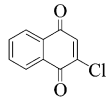
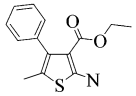
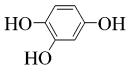
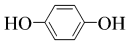
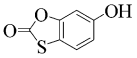
Here, we report the characterization of a proteinaceous probe for measuring thiol reactivity that is stable to air oxidation and that can be used to rapidly analyze large numbers of screening hits. The method is called ALARM NMR and is based on monitoring chemical shift or mass changes that occur in the human La antigen protein[9] upon cysteine modification by a test compound. The discovery, implementation, and utility of ALARM NMR in impacting drug discovery projects will be described, as will a comparison of this method to a glutathione-based fluorescence assay. On the basis of the compound reactivity profiles that have been observed, we have identified chemical substructures that are prone to being thiol-reactive. These substructures can be included in filtering protocols to identify potential thiol-reactive compounds in silico.

Results and Discussion

Discovery of the La Antigen as a Reactivity Probe. In the search for new antiviral agents, we performed an NMR-based screen[10] of the human La antigen. This protein normally stabilizes human RNA transcripts against exonucleolytic digestion,[11] but several infectious viruses also utilize the human La antigen during the RNA processing of their viral genomes.[12] A clone containing amino acids 100−324 yielded high-resolution HSQC spectra and was suitable for NMR-based screening. Unexpectedly, the screen yielded a very high hit rate, with more than 7% of our 10 000 compound fragment library inducing spectral perturbations. Some of these hits caused extensive line broadening suggesting disruption of the protein fold. Because this clone of the La antigen contains two cysteines, it was thought that some of these effects may be due to covalent modification of cysteine sulfur atoms and that this could be prevented by the addition of DTT to the sample buffer. This did in fact prevent the spectral perturbations caused by hits from the screen. As shown in Figure 1A, incubation of the La protein with **1**, which is a known Michael acceptor, caused shift changes and line broadening of L249, L294, and L296. The complete spectrum showing additional spectral changes are provided in the Supporting Information (Figure S1A). However, inclusion of 20 mM DTT in the buffer completely prevented these spectral perturbations (Figure 1B) suggesting that modification of a cysteine was involved. This is supported by titration experiments in which chemical shifts were measured as a function of the concentration of **1**. The largest chemical shift change at low compound concentration occurred for L249, which is in close proximity to C245[9] (Figure 1C). Additional evidence came from mass spectrometry of the La protein after exposure to several compounds that triggered the NMR assay (Table 1). In some cases, the cysteines were simply oxidized, resulting in a mass increase of 32 or 48 daltons (e.g., **2**). In other cases, direct covalent adducts of the compounds could be observed (e.g., **1**, **3**, and **4**). Mass increases were also observed that were not consistent with the putative structure of the compound (e.g., **5**), suggesting the presence of a reactive impurity or a breakdown product. To our surprise, NMR studies of all La antigen screening hits in the presence and absence of reducing agent failed to yield a valid lead. In fact, a rescreen of 46 000 compounds in the presence of DTT did not yield a single valid hit. These results indicated that the La protein lacks a pocket that is suitable for small molecule inhibitors[13] but contains a cysteine that is very sensitive to modification by electrophilic compounds. However, despite its sensitivity to thiol-reactive

(8) Wilson, J. M.; Wu, D.; Motiu-DeGrood, R.; Hupe, D. J. *J. Am. Chem. Soc.* **1980**, *102*, 359−363.
(9) Jacks, A.; Babon, J.; Kelly, G.; Manolaridis, I.; Cary, P. D.; Curry, S.; Conte, M. R. *Structure* **2003**, *11*, 833−843.
(10) Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1997**, *278*, 497−499.
(11) Maraia, R. J.; Intine, R. V. *Mol. Cell. Biol.* **2001**, *21*, 367−379.
(12) Ali, N.; Siddiqui, A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2249−2254.
(13) Hajduk, P. J.; Mack, J. C.; Olejniczak, E. T.; Park, C.; Dandliker, P. J.; Beutel, B. A. *J. Am. Chem. Soc.* **2004**, *126*, 2390−2398.

Table 1. Comparison of ALARM NMR and ALARM MS[a]

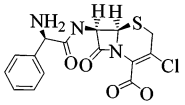
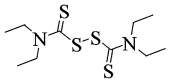
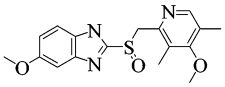
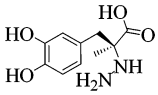
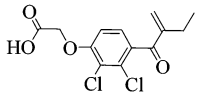
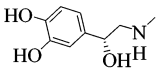
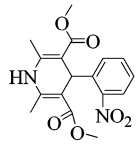
Comp. No.	Structure	MW	Increase in La Mass by MS	Covalent Modification
1		192	157, 314	Direct Adduct
2		261	32, 48	Oxidation
3		126	244	Direct Adduct
4		110	108, 219	Direct Adduct
5		168	219, 281	Unknown Adducts

[a] An additional 10 compounds that were negative in the ALARM NMR assay were chosen at random and submitted for LC/MS analyses. None showed an increase in mass of the La protein.

compounds, the NMR spectra of the La protein were remarkably stable even in the absence of reducing agent, with no deterioration in spectral quality even after several days at room temperature. These characteristics of the La protein prompted us to consider using it as a protein probe to detect reactive screening hits during hit triage. This La-based thiol reactivity assay was named ALARM NMR (A La Assay to detect Reactive Molecules by Nuclear Magnetic Resonance) to reflect its ability to flag nuisance compounds missed by other methods.

Sensitivity of the ALARM NMR Assay. Because the La antigen protein contains an exceptionally reactive cysteine, a question that needed to be addressed was whether the ALARM NMR assay was too sensitive and may yield a large number of false positive results. To this end, we chose a panel of 219 commercial drugs to serve as a control compound set (see Supporting Information, Table S1) and tested them against La in the presence and absence of DTT. Only seven of the compounds (cefaclor, disulfiram, omeprazole, carbidopa, epinephrine, nifedipine, and ethacrynic acid) were positive in the ALARM NMR assay (Table 2). Significantly, except for nifedipine and epinephrine, all of these compounds exert their physiological effects via reactive mechanisms.[14−18] Thus, the ALARM NMR assay is sensitive enough to detect the inherent reactivity of these structurally diverse molecules. The observed reactivity of nifedipine and epinephrine is also not surprising. Nifedipine is photoreactive, with the aromatic nitro group being converted to a reactive nitroso group,[19] while epinephrine contains an *o*-catechol moiety, which is prone to oxidation. Overall, these analyses demonstrate that the ALARM NMR assay is remarkably sensitive to a wide range of chemically reactive molecules, while also achieving a very low incidence of false positive results.

Table 2. Drugs Positive in the ALARM NMR Assay

Drug Name	Structure	Documented Reactivity
cefaclor		Protein-penicillenate Conjugate[14]
disulfiram		Glutathione Oxidation[15]
omeprazole		Covalent Inhibition of K-ATPase[16]
carbidopa		Reacts with Cysteine[17]
ethacrynic acid		Reacts with Cysteine[18]
epinephrine		
nifedipine		

Comparison of ALARM NMR to a Glutathione-Based Method of Detecting Reactive False Positives. Glutathione is routinely used to detect reactive compounds, particularly in metabolism assays.[20] However, it is not clear that glutathione can serve as a universal probe for assessing the thiol reactivity of large and diverse compound sets. One important distinction is that the reactivity of a cysteine sulfur varies depending on the protein environment and that this is not mimicked by a short, unfolded peptide such as glutathione. Indeed, our results of screening hits for 23 drug targets, all of which contain cysteines, clearly demonstrate a highly variable rate of reactivity with electrophilic compounds. Another issue is the stability of a sulfur-containing probe to air oxidation, as this may increase the false-positive hit rate if test compounds, solvents, or other assay variables affect this reaction. At the same time, slowly reacting compounds may be missed. Cysteines in peptides are notoriously reactive. Consistent with this, the half-life of glutathione in the fluorescein-5-maleimide competition assay (see Experimental Section) is ∼2 h (data not shown).

To compare the types of reactive compounds that are identified by ALARM NMR to those identified using glutathione, 28 drugs that were negative in the ALARM NMR assay were selected for testing in a fluorescence competition assay that uses glutathione as a probe (see Supporting Information, Table S1).[7] In addition, 34 positive control compounds were randomly selected from a database of compounds that were reactive in the ALARM NMR assay but were not flagged by internal nuisance alerts, which included published[5,21−24] as well

(14) Hewitt, W. L. *J. Infect. Dis.* **1973**, *128*, S312−319.
(15) Nobel, C. I.; Kimland, M.; Lind, B.; Orrenius, S.; Slater, A. F. *J. Biol. Chem.* **1995**, *270*, 26202−26208.
(16) Lambrecht, N.; Corbett, Z.; Bayle, D.; Karlish, S. J.; Sachs, G. *J. Biol. Chem.* **1998**, *273*, 13719−13728.
(17) Hisaka, A.; Kasamatsu, S.; Takenaga, N.; Ohtawa, M. *J. Chromatogr.* **1989**, *494*, 183−189.
(18) Tingey, D. P.; Schroeder, A.; Epstein, M. P.; Epstein, D. L. *Arch. Ophthalmol.* **1992**, *110*, 699−702.
(19) de Vries, H.; Beijersbergen van Henegouwen, G. M. *J. Photochem. Photobiol. B* **1998**, *43*, 217−221.
(20) Evans, D. C.; Watt, A. P.; Nicoll-Griffith, D. A.; Baillie, T. A. *Chem. Res. Toxicol.* **2004**, *17*, 3−16.
(21) Hann, M.; Hudson, B.; Lewell, X.; Lifely, R.; Miller, L.; Ramsden, N. *J. Chem. Inf. Comput. Sci.* **1999**, *39*, 897−902.

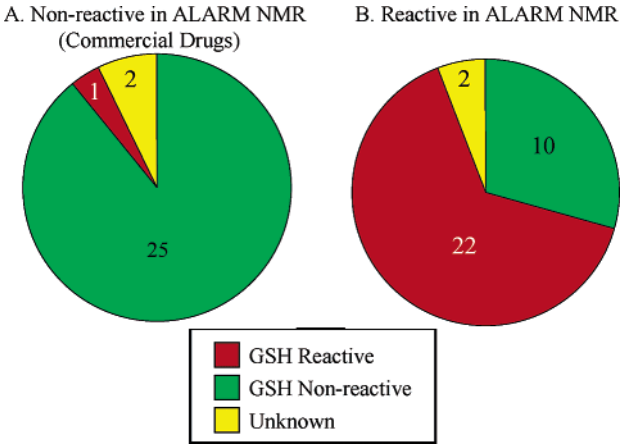


Figure 2. Analyses of control compounds in a glutathione-based fluorescence assay.[7] (A) 28 negative control compounds (commercial drugs that are negative in ALARM NMR). (B) 34 positive control compounds (positive in ALARM NMR). "Unknown" indicates that the reactivity could not be determined because either the test compound was fluorescent or it reacted directly with fluorescein-5-maleimide.

as proprietary structures (Martin, Y., personal communication). Compounds were tested at 0.2 mM from 10 mM DMSO stocks, which is typical for screening HTS hits. In the negative control set of 28 drugs, only isotretinoin was reactive, consistent with a report noting an interaction between isotretinoin and thiol-containing compounds.[25] Two other compounds (hydroxyzine and amoxapine) were either fluorescent or reacted with the fluorescent probe, such that their reactivity with GSH could not be determined. Thus, although the false positive rate seems to be low, compound interference in the fluorescence-based assay may limit the ability to rapidly test large numbers of structurally diverse screening hits. Greater discrepancies between the GSH and ALARM NMR assays were observed for the positive control set of known reactive compounds. In this set, only 22 of 34 ALARM-reactive compounds (65%) were identified as reactive and the reactivity of two others could not be determined (Figure 2). One possible explanation for this result is that slow-reacting compounds require a longer incubation with glutathione prior to addition of the fluorescent probe. However, repeating the experiment using a 1 h incubation at 37 °C did not improve the results (data not shown). Alternatively, glutathione may simply not be a suitable probe molecule for certain reactive chemotypes. In all, these data demonstrate that the use of the La protein in an NMR assay captures a greater number and type of chemically reactive moieties.

Application of ALARM NMR to HTS Hit Triage. ALARM NMR can be routinely applied during hit triage to identify potentially reactive false positives. Table 3 shows results for three hits against different targets that had passed internal nuisance alerts and were being considered for hit to lead synthetic programs. Compound **6** exhibited low micromolar potency in a screen against MurA, a possible antibacterial target. However, binding to labeled MurA could not be confirmed by NMR spectroscopy, nor could X-ray structures of a complex

Table 3. Impact of ALARM NMR on Candidate Selection for Lead Optimization

target	compd no.	ALARM NMR result	fold loss in IC_{50}	other result
Mur A	**6**	reactive	19[a]	
HCV polymerase	**7**	reactive	19[b]	
PLK1 polobox	**8**	reactive	3[c]	↑ mass polobox by mass spec

[a] Assay conducted with 0.5 mM and 5 mM DTT. [b] Assay conducted with 0 mM and 5 mM DTT. A 5-fold loss in potency was observed when the [DTT] was raised from 0.5 mM to 5 mM DTT. [c] Assay conducted with 0 mM and 10 mM DTT.

Table 4. Propensity of Drug Targets To Be Inhibited by Thiol-Reactive Compounds

propensity for inhibition by electrophilic compounds	no. targets	no. leads tested	ALARM positive	nuisance positive	% false positives detected by ALARM
high[a]	6	725	263	90	36
low[b]	17	1623	213	66	12

[a] High propensity: 25−50% of hits found to be reactive false positives (ave. hit rate: 36%). [b] Low propensity: Less than 25% of hits found to be reactive false positives (ave. hit rate: 12%).

be determined (Sun, C., and Stamper, G., personal communications). The behavior of **6** was in distinct contrast to Fosfomycin, a known covalent inhibitor of MurA, for which binding could be observed by NMR (unpublished observations) and a crystal structure obtained.[26] As a result, the reactivity of **6** in the ALARM NMR assay prompted additional studies in which inhibition of MurA activity was measured as a function of the concentration of DTT in the buffer. A 19-fold loss in potency was observed when the DTT concentration was increased from 0.5 mM to 20 mM, in contrast to no change in potency with a control inhibitor. All of these studies indicated that **6** inhibits MurA via a nonspecific, reactive mechanism and the series was dropped before chemistry was initiated.

Over the past several years, more than 2000 screening hits from 23 drug targets have been tested in the ALARM NMR assay. The results of these studies (Table 4) reveal that, for the majority of targets, the percent of reactive false positives is approximately 12%. However, HTS hits for several targets exhibited an exceptionally high percentage of reactive compounds (ranging from 27 to 60%). Such a result suggests that the assay or the target itself is highly susceptible to reactive compounds, increasing the likelihood that a reactive false positive could be chosen for lead optimization programs. For instance, oxidation or alkylation of active site amino acids or alkylation-induced denaturation of the protein target would result in false positives. The latter was observed for the La protein when it was screened. Two examples of other targets whose HTS hits contained a large percentage of reactive compounds are HCV polymerase and the Polo box domain of Plk-1. Twenty-seven percent of the hits for HCV polymerase assayed by ALARM NMR were reactive. The chemical reactivity of these hits against HCV polymerase was confirmed in biochemical studies where inhibition of enzymatic activity depended on the DTT concentration (e.g., **7** in Table 3). For the PoloBox, 47% of the hits assayed by ALARM NMR were reactive. Several of

(22) Wang, J.; Ramnarayan, K. *J. Comb. Chem.* **1999**, *1*, 524−533.
(23) Martin, E. J.; Critchlow, R. E. *J. Comb. Chem.* **1999**, *1*, 32−45.
(24) Baurin, N.; Baker, R.; Richardson, C.; Chen, I.; Foloppe, N.; Potter, A.; Jordan, A.; Roughley, S.; Parratt, M.; Greaney, P.; Morley, D.; Hubbard, R. E. *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 643−651.
(25) Shih, T. W.; Lin, T. H.; Shealy, Y. F.; Hill, D. L. *Drug Metab. Dispos.* **1997**, *25*, 27−32.
(26) Schonbrunn, E.; Eschenburg, S.; Krekel, F.; Luger, K.; Amrhein, N. *Biochemistry* **2000**, *39*, 2164−2173.

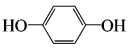
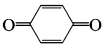
these screening hits potently inhibited substrate binding to the polobox ($IC_{50} < 1\ \mu M$) and NMR studies using labeled polobox confirmed an interaction with the protein. However, the observed ALARM NMR reactivity instigated mass spectrometry studies of the polo-box protein following incubation with the hits. The mass of the protein increased by an amount consistent with adduct formation and the series was dropped (e.g., **8** in Table 3).

In these three very different examples, screening hits were found that were chemically reasonable but in each case they inactivated the target protein by an undesirable mechanism. A lead validation process using ALARM NMR identified these compounds as potential false positives in the HTS of the target protein. These data prompted further studies that convincingly showed the screening hits to be invalid, and the three series were dropped before chemistry was initiated. Although **6**−**8** are reactive in the ALARM NMR assay, they were not identified as hits for all of the target proteins. This highlights the sensitivity of the La protein to different reactive chemotypes as well as the highly variable reactivity of thiol groups in different environments.

Effects of pH. The ALARM NMR assay is conducted at pH 7.0 to mimic the conditions of most high-throughput screening assays and to be compatible with sensitive detection of amide protons in $^{15}N-^{1}H$ HSQC NMR experiments. However, in some instances it may be desirable to assay for reactivity at a lower or higher pH to mimic the conditions of a particular biological screening assay. In pH titration experiments, we have found that the La(100−324) protein is stable between pH 6.0 and 9.5 on the basis of 2D $^{13}C-^{1}H$ NMR HSQC experiments (data not shown). However, it is not known whether one would detect the same set of reactive screening hits across this pH range. Protein thiol groups can have a wide range of reactivity depending on their pK_a and surface accessibility.[8] As a result, it is typical for reactions, such as disulfide bond formation, to be accelerated at a more basic pH where the sulfur exists as a thiolate anion.[27] To investigate the effect of pH on the ALARM NMR assay, the five compounds in Table 1 were tested for reactivity at pH 8.0 and pH 6.0. In all cases, perturbations of the La $^{13}C-^{1}H$ NMR HSQC spectra were observed in the presence of compound, and these effects were reversed by DTT. These results mimic those obtained at pH 7.0 indicating that compound reactivity can be assessed at both acidic and basic pH levels.

Predicting Thiol-Reactive False Positives. On the basis of our observations for more than 3500 compounds, it was clear that certain structural elements consistently contributed to thiol reactivity. Interestingly, many of these recurring structural elements were not captured by in silico filtering programs for detecting reactive compounds.[28] For example, of the 476 screening hits identified as reactive in the ALARM NMR assay, only 156 (33%) were flagged as nuisance compounds (Table 4). To rigorously capture and quantify these observations, rules for thiol reactivity were derived from the ALARM NMR data. First, each compound in the dataset was fragmented into structural descriptors using a modification of the RECAP algorithm.[29] This allowed us to calculate the frequency (F) with which compounds containing these substructures were reactive (see Table 5). As expected, groups known to be reactive with protein thiol groups (e.g., *p*-catechols, quinones, free sulfhydryls, and alkylhalides) occurred in reactive compounds at high frequencies ($F > 30\%$), while more stable groups (e.g., phenyl groups) occurred only at low frequencies ($F < 10\%$). Surprisingly, many groups that have the potential for thiol reactivity were not represented at high frequencies. For example, only 6 and 8% of compounds containing an aldehyde and formaldehyde group, respectively, were reactive. This may be due to a low rate of reaction that was not detected under the experimental conditions of the ALARM NMR assay. Studies with peptides have shown that reactions of formaldehyde with amino acids including cysteine are incomplete (3−22% conversion) even after a 48 h incubation at 35 °C with 50 times excess of formaldehyde.[30] Furthermore, the reaction is sequence specific and some lysine-containing peptides do not react with formaldehyde even after these long incubations. In contrast to the aldehyde functionality, compounds containing several other structural groups (e.g., 2-oxo-1,3-oxathiolanes, aminothiophenes, thiadiazoles, cyclic thioamides, and benzofurazans) were frequently found to be reactive.

Table 5. Subset of Structural Descriptors Used To Predict Thiol Reactivity[a]

Name	Substructure	N[b]	F (%)[c]	TRI[d]
p-Catechol		35	60	0.3
Quinone		16	100	0.3
Sulhydryl		37	34	0.3
Alkylhalide		62	33	0.3
Phenyl		3768	10	0.0
Aldehyde		125	6	0.06
Formaldehyde		39	8	0.07
2-oxo-1,3-oxathiolane		20	85	0.3
Aminothiophene		30	44	0.3
Thiadiazole		60	34	0.3
Cyclic thioamide		74	31	0.3
Benzofurazan		27	48	0.3
Pyrrole-2,5-dione		46	37	0.1
Naphthylamine		66	31	0.1

[a] A full listing is given in the Supporting Information. [b] Number of tests. [c] Percent of compounds containing this substructure that were reactive. [d] Thiol reactivity index as described in the text.

A statistical analysis was then performed to quantitate the contribution of each substructure to the observed thiol reactivity which may be different from the observed frequency of

(27) Huth, J. R.; Feng, W.; Ruddon, R. W. *Biotechnol. Bioeng.* **1994**, *44*, 66−72.
(28) Walters, W. P.; Stahl, M. T.; Murcko, M. A. *Drug Discov. Today* **1998**, *3*, 160−178.
(29) Lewell, X. Q.; Judd, D. B.; Watson, S. P.; Hann, M. M. *J. Chem. Inf. Comput. Sci.* **1998**, *38*, 511−522.
(30) Metz, B.; Kersten, G. F.; Hoogerhout, P.; Brugghe, H. F.; Timmermans, H. A.; de Jong, A.; Meiring, H.; ten Hove, J.; Hennink, W. E.; Crommelin, D. J.; Jiskoot, W. *J. Biol. Chem.* **2004**, *279*, 6235−6243.

Table 6. Reactive Chemical Moieties Identified by ALARM NMR

	observed by ALARM NMR	
predicted by TRI	reactive	nonreactive
reactive	486	990
nonreactive	23	2005

occurrence. To accomplish this, the probability that a given compound was reactive was represented as a linear combination of its corresponding substructures weighted by a thiol reactivity index (TRI) for each substructure. As known reactive groups all exhibited values of $F > 30\%$, a reactivity probability (P_R) of 0.30 was used as the cutoff for classifying compounds as reactive or nonreactive. The TRIs for each substructure were then adjusted in a nonlinear regression analysis to maximize the correspondence between the observed and predicted reactivity (see Methods). Thus, the resulting thiol reactivity indices are quantitative estimates of the contribution of each group to the probability that a given compound will be reactive. For most compounds, the TRI was close to the observed frequency of occurrence (see Table 5 and Supporting Information, Table S2). However, for some compounds (e.g., pyrrole-2,5-diones and naphthylamines, Table 5), the TRI was substantially reduced relative to the observed frequency, suggesting that other structural descriptors were adequate to explain the reactivity of compounds containing these groups. A total of 75 structural descriptors had nonzero TRI values, and a full listing is given in the Supporting Information (Table S2).

A comparison of the in silico predictions and the actual ALARM NMR results is given in Table 6. Of the 509 reactive compounds in the dataset, 486 (95%) were predicted to be reactive on the basis of the TRI values, while 2005 of the 2995 nonreactive compounds (67%) were correctly identified. The observation that 1476 compounds were predicted to be reactive while only 486 compounds (33%) were experimentally reactive is perfectly in line with a P_R cutoff value of 0.3, which dictates that only 30% of the compounds predicted to be reactive are expected to trigger the ALARM NMR assay. This is a useful alert, as the main utility of the in silico predictions is to triage compounds for reactivity testing, such as by ALARM NMR, or in an assay with varying concentrations of DTT (e.g., Table 3), without predicting false negative results. For this test set of 3504 compounds, triaging with TRI calculations would have eliminated 57% of the compounds from experimental testing while capturing 95% of the reactive compounds.

Using ALARM Data in Hit Evaluation and Library Design. Compounds that appear as hits in a biochemical screen and are determined to be reactive using the ALARM assay should be carefully evaluated. It is significant to note that chemical reactivity could cause artifacts in biochemical screens through a variety of means. Direct inactivation of the target protein is one mechanism, but covalent modification of a substrate, a fluorogenic probe, or some other assay or buffer component (including, for example, DTT) may also lead to a false positive result. Performing the biochemical assay in the presence of varying amounts of reducing reagent can determine whether chemical reactivity is the likely source of the positive assay result (Table 3). If covalent modification of the target is considered to be an unacceptable characteristic of a lead, then all compounds that trigger a biochemical assay via a reactive mechanism should be ignored. However, covalent modification of the protein may be acceptable for some targets (e.g., serine proteases). In these cases, the mechanism of inactivation (e.g., adduct formation vs oxidation) as well as the chemical matter causing the observed reactivity (e.g., parent compound, breakdown product, or an impurity) must be determined. The mechanism of inactivation can be determined by collecting mass spectroscopy of the target protein of interest to look for adduct formation or oxidation in the presence of the test compound (Table 3). Identifying the chemical matter causing the observed reactivity can be more difficult, but an ALARM negative result (i.e., nonreactive) using repurified compound or material from a different lot (preferably from powder) can give evidence for a breakdown product or impurity causing the reactivity. We have found in many cases that compounds stored for long periods of time in DMSO can yield ALARM positive results, whereas these same compounds tested from fresh powder are nonreactive. In cases where a breakdown product or impurity is the likely source of the positive assay result, the compounds should be abandoned.

The thiol reactivity indices (TRIs) reported in Tables 4 and S2 can also be useful in evaluating compounds for acquisition. The TRIs can be used in conjunction with other filters (analogous to the REOS filters described by Vertex)[28] to remove or deprioritize compounds that are highly likely to be reactive with protein thiols. However, it is important to stress that use of the TRI values only results in a probability that a given compound will be reactive. As we have observed with our ALARM data, reactivity can be highly context dependent. For example, as shown in Tables 4 and S2, very few substructures are reactive 100% of the time. While 100% of the *p*-quinones were reactive, only 60% of the *p*-catechols were reactive. This has to do in part with the substitution pattern off of the various structures, which can radically change the electronics of the system or simply inhibit chemical reactivity through steric effects. In addition, as indicated above, compound storage conditions can affect breakdown rates and therefore alter the likelihood of covalent interference with biochemical screens. All of these considerations must be carefully weighed when implementing a new filtering tool for compound acquisition.

ALARM MS. We have shown here that either NMR or mass spectrometry can be used to detect modified La protein (Table 1). NMR spectroscopy was used to validate the ALARM technique because of the ready availability of reagents (>200 mg of ^{13}C-labeled La(100−324) can be obtained from 10 L of bacterial growth), its relative speed (~100 tests can be performed per day), and its inherent insensitivity to false positive or negative results in detecting ligand binding. In addition, the data processing and analysis is straightforward and easily interpretable. On the other hand, ALARM MS affords potentially higher throughput with much less protein consumption. While our preliminary data has shown generally good agreement between the two detection methods, there are some differences. This highlights the need to evaluate more compounds to understand what types of substructures are detected using MS before using it as a filtering tool. A comparison of the two methods is currently in progress with an emphasis on detecting low concentrations of reactive compounds using ALARM MS. It is anticipated that ALARM MS will join ALARM NMR as a routine tool for detecting reactive compounds.

Conclusions

In summary, we have described assays using a novel protein probe, the La antigen, to experimentally detect reactive compounds in sets of screening hits. In particular, the ALARM NMR assay has been validated by testing hundreds of negative control compounds as well as thousands of hits from actual drug screens. The data from ALARM NMR have already been critical for both validating and invalidating HTS hits in numerous internal programs. For the three targets described in this manuscript, the ALARM NMR data ultimately resulted in abandoning lead optimization candidates prior to initiating organic synthesis. The savings in resources for these projects have encouraged the formation of a highly integrated lead validation process, where HTS hits are critically evaluated by ALARM NMR, NMR-based screening, centrifugal-enhanced affinity selection,[31] optical spectroscopy, X-ray crystallography, and other biochemical and biophysical techniques. Finally, the identified substructures that frequently contribute to thiol reactivity have been implemented in computational tools that will not only improve the triage of hits from HTS but also improve the quality of compounds added to our corporate repository from internal and external sources and thus increasing the probability of identifying quality drug leads that have high potential for development into therapeutic agents.

Experimental Section

Preparation of La Proteins. Guided by limited proteolysis experiments of full length human La antigen, the gene encoding amino acids 100−324 was cloned into a modified version of pET15b (Novagen) such that the amino acids LEHHHHHH were appended to E324. A second construct encoding amino acids 223−324 with the C-terminal tag LEHHHHHH was prepared after preliminary NMR structural studies showed that only the second RRM domain, contained within this sequence, adopted a stable fold. The 223−324 construct was used for ALARM MS experiments since much better signal-to-noise ratio was obtained with this lower MW probe. Both the 100−324 and 223−324 contructs were used for ALARM NMR with no difference in reactivity profile observed. However, the 100−324 construct was preferred since the protein seemed to be more stable during storage in the absence of DTT. Both proteins were expressed in *Escherichia coli* BL21(DE3) cells (Novagen) and labeled with ^{13}C at the δ-methyl groups of leucine, δ-methyl group of isoleucine, and γ-methyl groups of valine by including α-ketobutyrate and α-ketoisovalerate in the medium 30 min prior to induction with IPTG.[32] Samples for ^{15}N−HSQC experiments were grown in the presence of [^{15}N]ammonium chloride. The protein was purified as described using Ni^{2+} affinity chromatography and dialyzed into 25 mM sodium phosphate, pH 7.0, mM DTT. Protein was dialyzed against two changes of 4 L of 25 mM sodium phosphate, pH 7.0, over 24 h to generate reduced protein in the absence of DTT for the ALARM NMR experiments. In the absence of DTT, protein was used within 1−2 weeks and then rereduced with 20 mM DTT before further use.

ALARM NMR Experiments. ^{1}H/^{13}C−HSQC and ^{1}H/^{15}N−HSQC spectra were acquired on protein samples in 25 mM sodium phosphate buffer, pH 7.0, 90:10 H_2O:D_2O, with and without 200−400 μM compound, and in the presence and absence of 20 mM DTT. Data were recorded at either 310 K (La(100−324)) or 303 K (La(223−324)) on DRX500 spectrometers equipped with a cryoprobe (Bruker) or a DRX600 spectrometer equipped with a standard xyz gradient probe (Bruker). Using a cryoprobe and ^{13}C-methyl labeled protein, ^{1}H/^{13}C−HSQC spectra were collected on 25 μM protein samples using 16 scans, 1024 complex points in F2, and 38 points in F1. Using a cryoprobe and ^{15}N-labeled protein, ^{1}H/^{15}N−HSQC spectra were collected on 100 μM protein samples using 32 scans, 1024 complex points in F2, and 48 points in F1. On a DRX600 with a standard probe, 80 and 16 scans were signal-averaged per experiment using ^{13}C-methyl labeled protein samples of 25 and 75 μM, respectively. Reactive compounds were identified when 20 mM DTT modulated the line broadening and chemical shift effects of compounds. Using NMR, compounds can be screened as mixtures,[33] including in the ALARM NMR assay. However, because of the potential for compound−compound reactivity, HTS hits were all tested individually to robustly identify reactive false positives.

Interpretation of ALARM NMR Results. Compounds fall into several categories on the basis of ALARM NMR results. Nonreactive compounds are easily identified by the absence of chemical shifts (^{13}C-methyl or ^{15}N-amide) in the presence and absence of 20 mM DTT. One class of reactive compounds induce chemical shifts in the absence of DTT. The chemical shifts predominately involve amino acids near C245 and C232, such as L296, L249, and L294, consistent with cysteine modification. See Figure S1A for complete ^{13}C−HSQC spectra with and without a reactive false positive and Figure S1B for complete ^{15}N−HSQC spectra. Chemical shifts for La(223−324) are available online at www.bmrb.wisc.edu (accession number 5235). Titration experiments in which the concentration of a reactive compound was increased from subequivalent to a 2-fold excess over the protein probe showed that chemical shifts of amino acids near C245 occur first, followed by chemical shifts of amino acids near C232. This pattern was observed whether the ^{13}C-methyl or ^{15}N-amide chemical shifts were measured. Thus, C245 seems to be more reactive than C232, but both are observed to be modified by reactive compounds on the basis of NMR (Figure S1) and LC MS data (Table 1). For some reactive false positives, spectral effects are severe and only random coil shifts for methyl groups are observed in the presence of compound. This effect is reversed by 20 mM DTT. A second type of reactive compound causes chemical shifts or line broadening of the La protein in the presence of 20 mM DTT but not in its absence. Only about 10% of false positives show this profile. The mechanism likely involves DTT-catalyzed formation of oxygen radicals or hydrogen peroxide that oxidize the cysteine thiol groups of La.[34−36]

Competitive Fluorescence Assay.[7] The suitability of glutathione to trap thiol-reactive compounds was assessed by preparing in a 96-well plate 100-μL reactions containing 200 μM of test compound, 0.3 μM GSH, 25 mM degassed sodium phosphate buffer, pH 7.0, 2% DMSO, and 0.15 μM fluorescein-5-maleimide (Molecular Probes Inc., Eugene, OR). Under these conditions, a 2.5-fold increase in fluorescence for glutathione versus samples lacking glutathione was observed after a 12-min incubation at room temperature. To detect compound reactivity with the probe, control reactions where glutathione was omitted were also prepared for each compound. After 12 min at room temperature, fluorescence intensities were measured on a 96-well plate using a SpectraMax GeminiXS (Molecular Devices, Sunnyvale, CA) with excitation at 485 nM and emission at 538 nM. The percent changes in fluorescence compared to a 2% DMSO control for samples with and without glutathione were calculated. Compounds were identified as reactive when a 50% or greater decrease in fluorescence was observed in the presence of glutathione, corresponding to a decrease in fluorescent

(31) Harlan, J. E.; Egan, D. A.; Ladror, U. S.; Snyder, S.; Tang, M. I.; Buko, A.; Holzman, T. F. *Assay Drug Dev. Technol.* **2003**, *1*, 507−519.
(32) Hajduk, P. J.; Augeri, D. J.; Mack, J.; Mendoza, R.; Yang, J. G.; Betz, S. F.; Fesik, S. W. *J. Am. Chem. Soc.* **2000**, *122*, 7898−7904.
(33) Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Q. Rev. Biophys.* **1999**, *32*, 211−240.
(34) Guertin, K. R.; Setti, L.; Qi, L.; Dunsdon, R. M.; Dymock, B. W.; Jones, P. S.; Overton, H.; Taylor, M.; Williams, G.; Sergi, J. A.; Wang, K.; Peng, Y.; Renzetti, M.; Boyce, R.; Falcioni, F.; Garippa, R.; Olivier, A. R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2895−2898.
(35) Tjernberg, A.; Hallen, D.; Schultz, J.; James, S.; Benkestock, K.; Bystrom, S.; Weigelt, J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 891−895.
(36) Wang, Q.; Dube, D.; Friesen, R. W.; LeRiche, T. G.; Bateman, K. P.; Trimble, L.; Sanghara, J.; Pollex, R.; Ramachandran, C.; Gresser, M. J.; Huang, Z. *Biochemistry* **2004**, *43*, 4294−4303.

units of 89 or more. Fluorescent compounds and those that react directly with fluorescein-5-maleimide caused an increase in fluorescence in the absence of GSH. For these, the reactivity with GSH could not be determined without additional studies[7] and were considered ambiguous.

MS Measurements. LC-MS experiments were performed on a Q-Tof I mass spectrometer fitted with a Z-spray ion source (Micromass, Milford, MA). Spectra were collected in the positive ion mode with the capillary voltage set at 3.1 kV, the sample cone voltage at 30 V, and the extraction cone voltage at 4 V for all experiments. The source and desolvation temperatures were set at 110 °C and 220 °C, respectively. N_2 was used as the nebulizing gas and the desolvation and nebulizer gas flow rates were kept at 250 L/h and 15 L/h, respectively. The mass spectrometer was controlled using MassLynx software (version 3.3 build 004). Deconvolution of *m*/*z* spectra, to mass spectra, was performed with the MaxEnt 1 software program. Chromatography was performed with an Ultra-Plus micro-LC system (MicroTech Scientific, Vista, CA) that was interfaced to the mass spectrometer. Reversed-phase chromatography was performed at a flow rate of 50 μL/min utilizing a Jupiter C_4 column, 1 × 50 mm, 5 μm, 300 Å pore size (Phonemenex, Torrence, CA), and gradient elution (water/acetonitrile, 0.04% TFA, gradient from 5 to 95% acetonitrile).

Enzymatic Activity Assays for HCV Polymerase. The activity of hepatitis C virus (HCV) polymerase in the presence of compound was measured by detecting the incorporation of [^{3}H]UTP into RNA transcripts. The 80 nM polymerase from the 1b HCV patient isolate (where the last 55 amino acids were truncated) was mixed with 40 μM each of ATP, CTP, and GTP, 0.61 μM (0.5 μCi) [^{3}H]UTP, 6 ng/μL RNA template from the 651 nucleotides of the HCV 3′-NTR, 20 U Rnase inhibitor, in 50 μL of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, and 0, 0.5, or 5 mM DTT. Reactions in the presence of titrating amounts of compound were incubated for 2 h at 25 °C. Polymerase activity was then stopped with 50 μL of 500 mM EDTA. Ninety microliters of the reaction mixture was transferred onto a DEAE filter plate (Millipore), washed three times with 200 μL of 0.3 M $CHOONH_4$, washed three times with 100 μL of ethanol, and air-dried for 30 min before addition of 30 μL of Supermix scintillant. The amount of incorporated [^{3}H]UTP was measured using a Wallac 1450 liquid scintillation counter.

Compound Binding to Plk-1 Polo-Box Domain. Polo-box inhibition was measured by fluorescence polarization. A 100 nM recombinant polo-box protein[37] and 15 nM evoblue-labeled peptide probe (QSpTPLNGKK) were incubated for 30 min in the presence and absence of compound in 20 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 0.05% PF-60, 50 mM NaCl, and dithiothreitol as indicated in the text. Polarization measurements were obtained with the Analyst AD spectrometer (LJL Biosystem).

Enzymatic Activity Assays for MurA. Compound inhibition of MurA activity was measured by incubating compounds in 150 μL of 50 mM Tris-HCl, pH 8.0, 20 mM KCl, 4% DMSO, 1.2 μg of *E. coli* MurA, with and without DTT. After a 30-min incubation at room temperature, 50 μL of substrate mix in the same buffer was added to achieve 0.5 mM uridine 5′-diphospho-*N*-acetylglucosamine and 0.6 mM phospho(enol)pyruvate in the reaction. After 2−3 h at room temperature, 10 μL of the reaction was added to 160 μL of color reagent and 20 μL of 34% sodium citrate. The color reagent consisted of a 3:1 mix of 0.045% malachite green and 4.2% ammonium molybdate in 4 N HCl. The percent enzyme inhibition was calculated relative to an enzyme control reaction that lacked experimental compound.

Statistical Analyses of ALARM NMR Results. The set of 3504 compounds, which included 2348 screening hits and 1156 additional compounds from a random screen of the La protein, was broken into molecular fragments (structural descriptors) using a modification of the RECAP procedure,[29] in which the bonds of the molecules were recursively cleaved until only the desired types of fragments remained. In addition to the bond cleavage rules employed in RECAP, carbon−halogen bonds and nonring bonds between aromatic carbon and aliphatic carbon, nitrogen, oxygen, and sulfur atoms were also broken. This process resulted in a set of 131 structural descriptors that were used in the analysis (each descriptor was represented at least 10 times in the data set). The probability that a given compound will be thiol reactive (P_R) was expressed as a weighted linear combination of the descriptors,

$$P_R = \sum_{i=0}^{N} \mathrm{TRI}_i x_i$$

where N is the number of structural descriptors, TRI_i is the weighting coefficient (referred to as the thiol reactivity index) for the ith descriptor, and x_i is the number of times that the ith descriptor occurs in the test molecule. A compound was predicted to be reactive if its P_R value exceeded 0.3. A scoring function was then calculated according to the following equation:

$$\mathrm{Score} = \sum_{i=0}^{M} C(O,P_R)_i$$

where M is the number of compounds and $C(O,P_R)_i$ is the result of the comparison between the observed (O) and predicted (P_R) thiol reactivity for the ith compound, where

$$C(\text{ALARM NMR reactive}, P_R \geq 0.3) = 1$$

$$C(\text{ALARM NMR nonreactive}, P_R \geq 0.3) = -1$$

$$C(\text{ALARM NMR nonreactive}, P_R < 0.3) = 0.3$$

$$C(\text{ALARM NMR reactive}, P_R < 0.3) = -0.3$$

Thiol reactivity indices (TRI_i) that maximized the scoring function were generated using the nonlinear regression package Solver available within Microsoft Excel 2000. Structural descriptors not generated in the initial RECAP analysis were added during the procedure until essentially all reactive elements were captured.

Acknowledgment. The authors wish to thank Kerry Swift for assistance in the fluorescence spectroscopy experiments and Dr. Vincent Stoll and Dr. Yvonne Martin for helpful discussions.

Supporting Information Available: Figure depicting the complete ^{15}N− and ^{13}C−HSQC spectra for the La protein in the presence of reactive false positives, a table listing the commercial drugs testing in the ALARM NMR assay, and a table listing 75 structural descriptors used to predict thiol reactivity. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0455547

(37) Elia, A. E.; Rellos, P.; Haire, L. F.; Chao, J. W.; Ivins, F. J.; Hoepker, K.; Mohammad, D.; Cantley, L. C.; Smerdon, S. J.; Yaffe, M. B. *Cell* **2003**, *115*, 83−95.