Proteomic searches comparing two (*R*)-lacosamide affinity baits: An electrophilic arylisothiocyanate and a photoactivated arylazide group†

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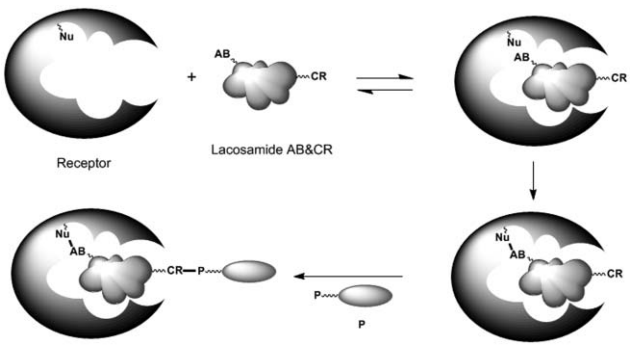
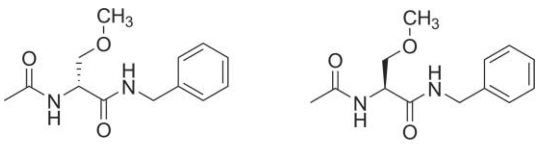
We have advanced a novel strategy to search for lacosamide ((*R*)-**1**) targets in the brain proteome where protein binding is expected to be modest. Our approach used lacosamide agents containing "affinity bait" (AB) and "chemical reporter" (CR) units. The affinity bait moiety is designed to irreversibly react with the target, and the CR group permits protein detection and capture. In this study, we report the preparation and evaluation of (*R*)-*N*-(4-azido)benzyl 2-acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-**3**) and show that this compound exhibits potent anticonvulsant activities in the MES seizure model in rodents. We compared the utility of (*R*)-**3** with its isostere, (*R*)-*N*-(4-isothiocyanato)benzyl 2-acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-**2**), in proteomic studies designed to identify potential (*R*)-**1** targets. We showed that despite the two-fold improved anticonvulsant activity of (*R*)-**3** compared with (*R*)-**2**, (*R*)-**2** was superior in revealing potential binding targets in the mouse brain soluble proteome. The difference in these agents' utility has been attributed to the reactivity of the affinity baits (*i.e.*, (*R*)-**2**: aryl isothiocyanate moiety; (*R*)-**3**: photoactivated aryl azide intermediates) in the irreversible protein modification step, and we conclude that this factor is a critical determinant of successful target detection where ligand (drug) binding is modest. The utility of (*R*)-**2** and (*R*)-**3** in *in situ* proteome studies is explored.

Introduction

Lacosamide[1] ((*R*)-**1**) is a first-in-class, antiepileptic drug (AED) that has been approved in the United States and Europe for the adjunctive treatment of partial-onset seizures in adults.[2] While recent reports indicate that (*R*)-**1** may modulate both the slow inactivation state of sodium channels[3,4] and the CRMP2 signaling pathway,[5,6] the full mechanism of action of this AED remains elusive. To gain additional information of (*R*)-**1** function, we advanced a strategy to identify drug targets where ligand binding is expected to be modest.[7] Our approach uses agents termed "affinity bait" (AB) and "chemical reporter" (CR), where these AB and CR units are incorporated within the drug framework (Scheme 1). The AB group permits irreversible adduction of the modified drug to the receptor, and the CR unit allows protein detection and capture upon subsequent attachment to a chemical probe (P). Key to this strategy is that neither the AB nor the CR group impedes drug binding to the cognate receptor(s).



Scheme 1 "Affinity bait" and "chemical reporter" (AB&CR) strategy to scan the brain proteome for (*R*)-lacosamide target(s).



(*R*)-**1** (*S*)-**1**

Our initial report used the lacosamide AB&CR agent (*R*)-**2** and (*S*)-**2**.[7] We showed the preferential enantiospecific adduction of CRMP2, a potential lacosamide target, by (*R*)-**2** compared with (*S*)-**2**. This stereochemical finding was in agreement with the observation that the principal anticonvulsant activity of **1** resided in the (*R*)-stereoisomer.[1] The AB unit in **2** is the electrophilic

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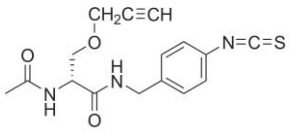
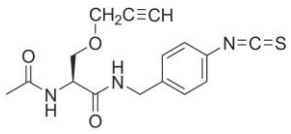
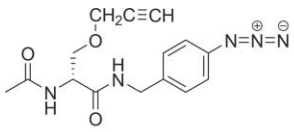
† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra of (*R*)-**3** and (*S*)-**3**, Table S1 providing the relative intensities of the CRMP2-containing band and other proteins bands *versus* an internal reference protein, Fig. S1 (effect of TCEP on azide reduction in **3**), Fig. S2 (treatment of GST-CRMP2 supplemented lysate with either (*R*)-**3** or (*S*)-**3**), Fig. S3 (treatment of GST-CRMP2 supplemented lysate with (*R*)-**3** without and with excess (*R*)-**1**), Fig. S4 (effect of GSH on Cu(I)-mediated cycloaddition reactions). See DOI: 10.1039/c000987c

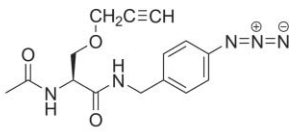
aryl isothiocyanate moiety.[8,9] Isothiocyanates display an excellent balance between stability and reactivity; they do not readily react with hydroxylic solvents (permitting their dissolution in aqueous solutions) but do react with nucleophiles (*e.g.*, thiols, amines) under appropriate conditions.[10]


(*R*)-**2**


(*S*)-**2**

The aryl azide group has been extensively utilized as photoaffinity agents in target adduction experiments.[11,12] Significantly, the aryl azide moiety is similar in size and shape to an aryl isothiocyanate group.[13] In this study, we report the synthesis and whole animal pharmacological data for the lacosamide AB&CR aryl azide agents, (*R*)-**3** and (*S*)-**3**. We compare the utility of both **2** and **3** for their ability to identify (*R*)-**1** targets in the mouse brain soluble proteome and for their use in *in situ* proteomic studies. Our findings show that while (*R*)-**3** exhibited enhanced anticonvulsant activity over (*R*)-**2**, (*R*)-**2** was superior in selectively modifying such potential targets of lacosamide as CRMP2. We document that the reactivity of the AB unit is a critical determinant in proteomic searches and further report the limitations of aryl azide photoprobes in *in situ* investigations.

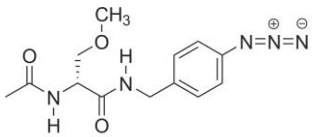

(*R*)-**3**

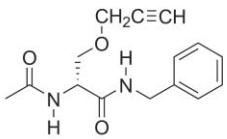

(*S*)-**3**

Results

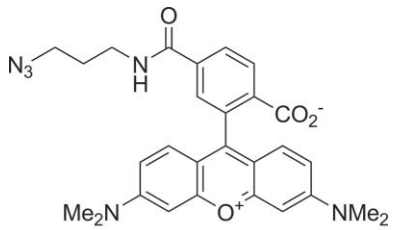
A. Choice of lacosamide AB&CR agents

Several factors led to our selection of lacosamide AB&CR agent **3**, which contained an aryl azide group as the AB moiety and an alkyne moiety as the CR group. First, the azide moiety in **3** is similar in size and shape to the isothiocyanate group in **2**.[13] We previously showed that (*R*)-**2** exhibited appreciable seizure protection in the maximal electroshock (MES) seizure test[14] in rodents (see Pharmacological Activity).[7] The MES test is a well-established animal model for identifying drug candidates that have potential human efficacy to treat generalized and partial seizures and that are secondarily generalized. Thus, we did not expect the replacement of the isothiocyanate moiety by the azide group to impede binding to the (*R*)-**1** receptor(s). Indeed, we recently reported that *N*-(4-azido)benzyl 3-methoxypropionamide ((*R*)-**4**) exhibited superb activity in the MES test (see Pharmacological Activity).[15] Second, aryl azides are among the most common photoaffinity groups wherein activation occurs under 254–360 nm UV light.[11,12] The photochemistry of the aryl azides is complex and leads to the generation of a short-lived singlet nitrene that can undergo bond insertion, conversion to the ground state triplet nitrene, and rearrangement to a strained dehydroazepine (a ketenimine) that is rapidly trapped by nucleophiles.[16–18] The lifetime of the dehydroazepine is ~1 ms, in the absence of nucleophiles.[17] Thus, photoactivated aryl azides can react with amino acid residues within a binding pocket either as a nitrene or as an electrophile and where protein adduction is expected to occur on a shorter time scale than an isothiocyanate group. Third, we used an alkyne as the CR group. We showed that replacing the *O*-methoxy group in (*R*)-**1** with an *O*-propargyl unit to give (*R*)-**5** led to little loss of anticonvulsant activity (see Pharmacological Activity).[7]


(*R*)-**4**


(*R*)-**5**

Finally, the bioorthogonal adduction of the CR alkyne group in **3** with the rhodamine azide probe **6**[19] under Cu(I)-mediated cycloaddition conditions led to little background protein adduction by **6**,[7] thus making the alkyne unit an ideal CR moiety for proteomic target studies.[19]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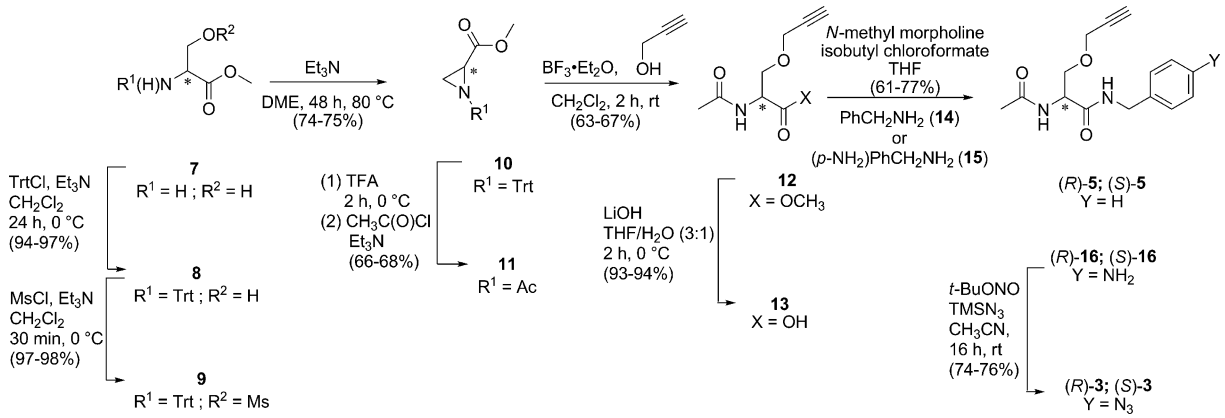
B. Synthesis of (*R*)-3 and (*S*)-3

Preparation of (*R*)-**3** and (*S*)-**3** followed the same general procedure developed for (*R*)-**2** and (*S*)-**2**,[7] except for the final step. In brief, we started with the enantiomerically pure serine methyl ester **7**, *N*-tritylation followed by sequential mesylation and treatment with a base gave the corresponding aziridine **10** (Scheme 2). Successive *N*-deprotection of the trityl group (TFA) and acetylation (acetyl chloride, Et_3N) gave *N*-acetyl aziridine **11** that was stereospecifically ring-opened[20–22] with propargyl alcohol and $BF_3{\cdot}Et_2O$ to afford **12**. Hydrolysis of the serine methyl ester **12** followed by mixed anhydride coupling[23] with 4-aminobenzylamine (**15**) gave **16**. Using the method of Moses and coworkers,[24] treatment of **16** with *tert*-butyl nitrite and $TMSN_3$ yielded **3**. We documented the enantiopurity of (*R*)-**3** and (*S*)-**3** using the chiral NMR resolving agent (*R*)-(–)-mandelic acid and ^{1}H NMR spectroscopy.[25]

C. Pharmacological activity

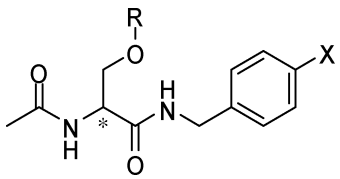
In Table 1, we list the anticonvulsant activities determined at the National Institute of Neurological Disorders and Stroke's (NINDS) Anticonvulsant Screening Program (ASP) for AB&CR **2** and **3**, AB **4**, and CR **5**, using the procedures described by Stables and Kupferberg.[26] Where possible, we provide the activities for these compounds in the MES,[14] 6 Hz psychomotor,[27] and neurological toxicity[28] models, and compare them with AEDs (*R*)-**1**,[1] phenytoin, phenobarbital, and valproate.[29] We observed that (*R*)-**3** displayed excellent seizure protection in the MES test in both mice (intraperitoneally, ip) (MES ED_{50} = 20 mg kg^{-1}) and



Scheme 2 Synthesis of lacosamide AB&CR **3**.

rats (orally, po) (MES ED_{50} = 12 mg kg^{-1}). When we compared **3** with **1**, we found that (*R*)-**3** exhibited lower anticonvulsant activity than (*R*)-**1** (mice (ip), MES ED_{50} = 4.5 mg kg^{-1}; rats (po), MES ED_{50} = 3.9 mg kg^{-1}),[1] but that for both **1**[1] and **3**, anticonvulsant activity principally resided in the (*R*)-stereoisomer. Similarly, (*R*)-**4** (mice (ip), MES ED_{50} = 8.4 mg kg^{-1}; rat (po), MES ED_{50} = 3.9 mg kg^{-1})[15] and (*R*)-**5** (mice (ip), MES ED_{50} = 16 mg kg^{-1}; rat (po), MES ED_{50} = 7.9 mg kg^{-1})[7] exhibited exceptional seizure protection while their (*S*)-counterparts, (*S*)-**4** and (*S*)-**5**, did not. When we compared (*R*)-**3** with (*R*)-**2** in mice (ip) ((*R*)-**2**: MES ED_{50} = 45 mg kg^{-1}), we found that (*R*)-**3** was about two-fold more potent. The superb activities of (*R*)-**3**, (*R*)-**4**, and (*R*)-**5** warranted further pharmacological testing. In the psychomotor 6 Hz (32 mA) seizure test,[27] (*R*)-**3** and (*R*)-**5** displayed ED_{50} values of 27 and 29 mg kg^{-1},[15] respectively. In the sensitive rat (ip) hippocampal kindled seizure test,[30] the ED_{50} values for (*R*)-**3**, (*R*)-**4**,[15] and (*R*)-**5** were 42, 6.0, and 14 mg kg^{-1}, respectively (Table 2). Finally, the metabolic stability of (*R*)-**3**, (*R*)-**4** and (*R*)-**5** were evaluated at the NINDS ASP against a panel of human cytochrome P450 enzymes.[31] Using a 500 μM concentration of (*R*)-**3**, no significant effects were observed for the cytochrome isozymes CYP 2A6, 2B6, 2C9, 2C19, and 2E1. For CYP 1A2 (K_i = 100 μM), 2D6 (K_i = 254 μM), and 3A4 (K_i = 131 μM), inhibition was observed. We saw less cytochrome isozyme inhibition with (*R*)-**4** and (*R*)-**5**. Using a 500 μM (*R*)-**4**, no significant effects were observed for the cytochrome isozymes CYP 1A2, 2A6, 2B6, 2C9, 2C19, 2D6 and 2E1. For CYP 3A4, a K_i of ~340 μM was determined. Similarly, when (*R*)-**5** was evaluated, no effects at 500 μM were found when we used CYP 1A2, 2A6, 2C9, 2C19, 2D6 and 2E1. For CYP 3A4, weak inhibition (K_i = 280 μM) was observed. We concluded that

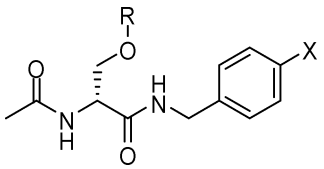
Table 1 Pharmacological activities of select AB&CR, AB, and CR agents in the maximal electroshock and 6 Hz seizure models[a]



Compd. No.	R	X	Stereo	Mice (ip)[b] MES, ED_{50}/ mg kg^{-1}[d]	Tox, TD_{50}/ mg kg^{-1}[e]	PI[f]	6 Hz, ED_{50}/ mg kg^{-1} (32 mA)	Rat (po)[c] MES, ED_{50}/ mg kg^{-1}[d]	Tox, TD_{50}/ mg kg^{-1}[g]	PI[f]
2[h]	$CH_2C{\equiv}CH$	NCS	(*R*)	45 [1] (41–48)	110 [0.25] (76–150)	2.4	53 [0.5] (42–68)	>30 [0.5]	>30 [0.5]	
			(*S*)	>300	>300		ND[i]	ND[i]	ND[i]	
3	$CH_2C{\equiv}CH$	N_3	(*R*)	20 [0.25] (18–22)	82 [0.25] (63–108)	4.1	27 [0.25] (19–37)	12 [1] (9.6–16)	>500	>41
			(*S*)	160 [0.25] (110–230)	> 300 [0.25]		ND[i]	> 30 [1]	> 30 [1]	
4[j]	CH_3	N_3	(*R*)	8.4 [0.25] (5.7–12)	46 [0.25] (41–52)	5.5	ND[i]	3.9 [0.5] (2.5–6.2)	>250 [0.5]	>64
			(*S*)	>100, <300 [0.5]	>300 [0.5]		>100 [0.5]	>30 [0.5]	>30 [0.5]	
5[h]	$CH_2C{\equiv}CH$	H	(*R*)	16 [0.25] (13–19)	59 [0.25] (55–66)	3.7	29 [0.5] (21–40)	7.9 [0.5] (4.7–11)	> 500 [0.5]	>63
			(*S*)	>300 [0.5]	>300 [0.5]		>100 [0.5]	>30 [0.5]	>30 [0.5]	
1[k]	CH_3	H	(*R*)	4.5 [0.5] (3.7–5.5)	27 [0.25] (38–47)	6.0	10	3.9 [0.5] (2.9–5.5)	>500 [0.5]	>130
			(*S*)	>100, <300	>300			>30	>30	
	Phenytoin[l]			9.5 [2] (8.1–10)	66 [2] (53–72)	6.9		30 [4] (22–39)	[m]	>100
	Phenobarbital[l]			22 [1] (15–23)	69 [0.5] (63–73)	3.2		9.1 [5] (7.6–12)	61 [0.5] (44–96)	6.7
	Valproate[l]			270 [0.25] (250–340)	430 [0.25] (370–450)	1.6		490 [0.5] (350–730)	280 [0.5] (190–350)	0.6

[a] The compounds were tested through the auspices of the NINDS ASP. [b] The compounds were administered intraperitoneally. Numbers in parentheses are 95% confidence intervals. The dose effect data was obtained at the "time of peak effect" (indicated in hours in the square brackets). [c] The compounds were administered orally. [d] MES = maximal electroshock seizure test. [e] Tox = neurologic toxicity determined from rotorod test. [f] PI = protective index (TD_{50}/ED_{50}). [g] Tox = behavioral toxicity. [h] Ref. 7. [i] ND = not determined. [j] Ref. 15. [k] Ref. 1. [l] Ref. 29. [m] No ataxia observed up to 3000 mg kg^{-1}.

Table 2 Advanced neurological test data for (*R*)-**3**, (*R*)-**4**, and (*R*)-**5**[a]



			Preliminary hippocampal kindling test (rats)				
			Seizure Score		After discharge duration/s		Hippocampal kindled rats (ip)
Compd. No.	R	X	Pre-drug	Drug	Pre-drug	Drug	ED_{50} (interval)/mg kg^{-1}
(*R*)-**3**	$CH_2C{\equiv}CH$	N_3	4–5	1	37–63	76	42 (27–63)
(*R*)-**4**	CH_3	N_3	4	0	29–65	0	6.0 (3–9)
(*R*)-**5**	$CH_2C{\equiv}CH$	H	4	0	32–50	35	14 (9–23)
(*R*)-**1**[b,c]	CH_3	H	5	0	33–53	0	14 (9.1–18)
Phenytoin[c]			4–5	3	42–72	51	34 (21–45)
Phenobarbital[c]							20 (14–28)
Valproate[c]							210 (150–280)

[a] The compounds were tested through the auspices of the NINDS ASP. [b] Ref. 50 [c] NINDS ASP internal control data.

the whole animal pharmacology for (*R*)-**3** mirrored that of (*R*)-**1** and that (*S*)-**3** served as a valued control compound.

D. Investigation of mouse brain soluble proteome adduction with 3 and comparison with 2

AB&CR agents **2** and **3** are structurally similar in size and shape.[13] Isothiocyanate **2** acts as an electrophilic, affinity-trapping agent, while in azide **3** photoactivation is required and protein modification occurs either by a nitrene insertion pathway or through electrophilic trapping of the rearranged azepine ketenimine.[16–18] Reports indicate that both nitrene insertion and ketenimine adduction are more rapid[17] than isothiocyanate addition reactions, which is consistent with the notion that photoactivated azide is a more reactive species than the corresponding isothiocyanate. Thus, the mechanism of protein adduction, the inherent reactivities of the AB unit, and the times required for adduction for AB&CRs **2** and **3** are all different.

We expected that the differences in isothiocyanate **2** and azide **3** would affect three diagnostic parameters that are useful in distinguishing selective from non-selective protein adduction (Scheme 1). These are the preferential adduction of (*R*)-**1**-specific binding targets *versus* nonspecific and abundant proteins; the enantioselectivity of (*R*)-AB&CR *versus* (*S*)-AB&CR protein adduction; and the affect of excessive (*R*)-**1** on the (*R*)-AB&CR protein adduction process. The first parameter is visualized in the in-gel fluorescence experiments by determining the relative intensities of the fluorescently-labeled bands and comparing them to the intensities of protein bands in the corresponding Coomassie blue-stained gels. The second parameter is determined by comparing the in-gel fluorescent bands for the (*R*)-AB&CR with the (*S*)-AB&CR reactions. A hallmark of (*R*)-**1** function is that the principal anticonvulsant activity resided in the D-enantiomer ((*R*)-stereoisomer) compared with the L-isomer ((*S*)-stereoisomer).[1] For **1** in the MES test in mice (ip), this difference exceeded 22-fold, for (*R*)-**2** it was >6.6-fold, and for (*R*)-**3**, it was 8-fold (Table 1). Indeed, using the mouse brain soluble proteome, we reported that (*R*)-**2** modified CRMP2 to a greater extent than (*S*)-**2**, but the extent of CRMP2 adduction by (*R*)-**2** compared with (*S*)-**2** was only 1.5–1.8 times higher.[7] We attributed the modest enantioselectivity differences for the *in vitro* CRMP2 adduction to several factors.[7] Among these were that the *in vivo* MES ED_{50} values are a composite of numerous pharmacological factors (*e.g.*, metabolism, transport, efflux) not present in the *in vitro* experiment and that different (*R*)-**1** targets may have different stereochemical preferences for the **2** enantiomers. The third protein selection parameter is assessed by determining if target modification by the AB&CR agent is competitively blocked by including excess (*R*)-**1** in the reaction solution.

In this study, we asked whether photoactivated AB&CR **3** would provide a protein adduction profile similar to that observed for AB&CR **2**, and whether the enhanced reactivity of the photoactivated species generated from **3** compared with **2** would markedly affect the parameters used to identify potential (*R*)-**1** targets. We asked these questions because reagents that are more reactive are generally less selective.[32] To test whether our protein selection criteria are affected by the reactivity of the AB moiety, we compared the CRMP2-containing band adduction that appears ~62 kDa (arrow, lower band)[7] with other proteins in the mouse brain soluble lysate. Two laboratories have reported that this protein is selectively adducted in the lysate by (*R*)-**1** analogues.[5–7] We expect that (*R*)-**1** will preferentially bind to several proteins in the brain but not all would be important for drug function. Accordingly, the physiological significance of the (*R*)-**1**–CRMP2 interaction has not been determined. Thus, if our notions are correct, AB&CR **3** may show decreased CRMP2 adduction levels compared with non-specific and abundant proteins, a decrease in preferential (*R*)-**3** *versus* (*S*)-**3** CRMP2 adduction, and diminished competitive inhibition for (*R*)-**3** adduction of target protein(s) by excess (*R*)-**1** when compared with (*R*)-**2**.

In Fig. 1A, we provide the in-gel fluorescence results for mouse brain soluble proteome adduction using (*R*)-**2**, (*S*)-**2**, (*R*)-**3**, and (*S*)-**3**. The lysate samples were passed through a Nap-10 column prior to use. For compound **3**, we activated the photoprobe using 320 nm light (2 min, 4 °C), while protein adduction by **2** was promoted by incubation at 22 °C (30 min). The modified

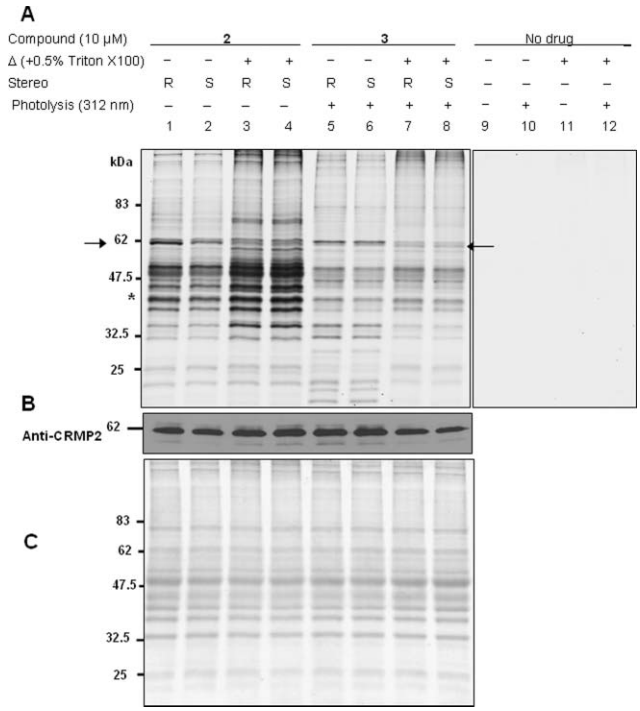


Fig. 1 Proteome reactivity profiles of lacosamide AB&CR isothiocyanate (NCS) agent **2** and lacosamide AB&CR azide (N_3) agent **3**. A: Probe-labeled proteins were detected by in-gel fluorescence scanning after Cu(I)-mediated cycloaddition to a rhodamine-azide reporter probe **6**. An arrow marks selectively labeled protein with **2** identified as CRMP2[7] and an asterisk marks an internal reference protein band. Proteins observed using heat-denatured lysate (△) were considered non-specifically labeled. B: CRMP2 protein was detected by western blot using anti-CRMP2. C: Proteins in lysate visualized by Coomassie blue staining after in-gel fluorescence scanning. (All images shown in grayscale).

lysates were treated with **6** using Cu(I)-mediated cycloaddition conditions ($CuSO_4$, TCEP, 60 min).[33–35] We independently showed that the TCEP concentrations (1 mM) used for cycloaddition reactions rapidly reduced the azide group in **3** to the corresponding 4-amino adduct **16** (<30 min) (Fig. S1 in the ESI†),[36] while the isothiocyanate moiety in **2** was largely unaffected by this reaction (data not shown). Thus, protein modification by **3** presumably occurred within minutes at 4 °C, while modification by **2** may have occurred over 90 min. We observed preferential modification of the ~62 kDa band for (*R*)-**2** compared with (*S*)-**2** (Fig. 1A, lanes 1, 2; arrow), as previously reported.[7] Using a protein band (~40 kDa, asterisk) as an internal standard, we observed that the in-gel fluorescence for the ~62 kDa was ~82% of the ~40 kDa band for the (*R*)-**2** reaction and ~45% for the (*S*)-**2** reaction after normalization. The relative increase in the fluorescence intensity has been attributed to the preferential adduction of CRMP2 by (*R*)-**2** *versus* (*S*)-**2**, and CRMP2 modification is supported by western blot analysis with anti-CRMP2 (Fig. 1B). Significantly, when the lysate sample was denatured (75 °C, 5 min, 0.5% Triton X100) prior to use, diminished amounts of the ~62 kDa band adduction, *versus* other protein bands (Fig. 1A, lanes 3, 4), were detected, and no difference was observed in the levels of (*R*)-**2** *versus* (*S*)-**2** adduction, further confirming that CRMP2 adduction was specific. For both (*R*)-**2** and (*S*)-**2**, the ~62 kDa band was 28–29% the level of the ~40 kDa band after normalization. Cravatt and coworkers have shown that protein denaturation leads to a reduction in selective AB protein modification.[37] These collective findings are consistent with the preferential adduction of CRMP2 by (*R*)-**2**.

Using (*R*)-**3** and (*S*)-**3**, in place of (*R*)-**2** and (*S*)-**2**, we observed lower levels of CRMP2 modification compared with other adducted proteins in the lysate, and found no measurable differences in the (*R*)-**3** compared with the (*S*)-**3** CRMP2 adduction levels (Fig. 1A, lanes 5, 6). Little differences in the in-gel fluorescence gel patterns were observed for (*R*)-**3** and (*S*)-**3** in the denatured lysate reactions (Fig. 1A, lanes 7, 8), except for diminished levels of the labeled protein bands observed at ~30 and 32 kDa. Since the extent of CRMP2 modification by (*R*)-**3** was small, we added to the mouse brain soluble lysate GST-CRMP2[7] (2.4 μM) and then either (*R*)-**3** or (*S*)-**3** (Fig. S2 in the ESI†). Again, we did not see any preferential modification of GST-CRMP2 by (*R*)-**3** compared with (*S*)-**3**. In contrast, we observed higher levels of GST-CRMP2 adduction by (*R*)-**2** compared with (*S*)-**2** in the GST-CRMP2 supplemented lysate, as we previously reported.[7] We attributed the differences between the **2** and the **3** adduction profiles in Fig. 1 to the enhanced reactivity of the photoprobe intermediates generated from **3**, compared with **2**. Thus, our results are in agreement with the general finding that more reactive agents are less selective than less reactive agents.[32]

Next, we investigated whether potential (*R*)-**1** targets could be detected by conducting a competition experiment using excess (*R*)-**1**. We reasoned that if a target is selectively adducted by a lacosamide AB&CR agent then co-administration of (*R*)-**1** could reduce the extent of the adduction observed for the AB&CR agent in the in-gel fluorescence gel. Accordingly, we added either 700 or 3500 equivalents of (*R*)-**1** to the (*R*)-**2** and (*R*)-**3** adduction experiments that used the mouse brain soluble proteome. We observed a dose-dependent reduction of the ~62 kDa band (arrow, corresponding to CRMP2) labeled by (*R*)-**2** as the concentration of (*R*)-**1** was increased from 0 to 700 to 3500 equiv. (Fig. 2A, lanes 1–3). After normalization of the ~62 kDa band *versus* the internal ~40 kDa reference protein band (asterisk), we found a 33% intensity decrease of the ~62 kDa band with 700 equiv. of (*R*)-**1**, and a 61% decrease with 3500 equiv. (Table S1 in the ESI†). By comparison, we observed little changes in the level of modification for other protein bands (*i.e.*, ~34 kDa, ~37 kDa) when increasing amounts of (*R*)-**1** were added to the reaction. When (*S*)-**1** was used in place of (*R*)-**1**, the extent of competition dropped significantly (Fig. 2A, lanes 5–7). We estimated only a 4% decrease in the ~62 kDa band intensity with 700 equiv. of (*S*)-**1** and a 19% decrease with 3500 equiv. of (*S*)-**1**, and again other proteins bands were largely unaffected (Table S1 in the ESI†).

The competition experiments were repeated using (*R*)-**3**. We detected no significant intensity loss of the weak band at ~62 kDa in the (*R*)-**3** reactions, as the amount of either (*R*)-**1** (Fig. 2B, lanes 1–3) or (*S*)-**1** (Fig. 2B, lanes 5–7) in the reaction was increased from 0 to 3500 equiv. (Table S1 in the ESI†). A similar finding was observed for the GST-CRMP2 (3.6 μM) supplemented lysate upon addition of (*R*)-**1** (700, 3500 equiv.) (Fig. S3 in the ESI†). Thus, the (*R*)-**1** competition experiments with (*R*)-**2** and (*R*)-**3** provided different signatures for the selective CRMP2 adduction process. For the photoactivated agent (*R*)-**3**, which generates reactive intermediates, no apparent competition by (*R*)-**1** was detected, but it was seen in the less-reactive isothiocyanate agent (*R*)-**2**, albeit

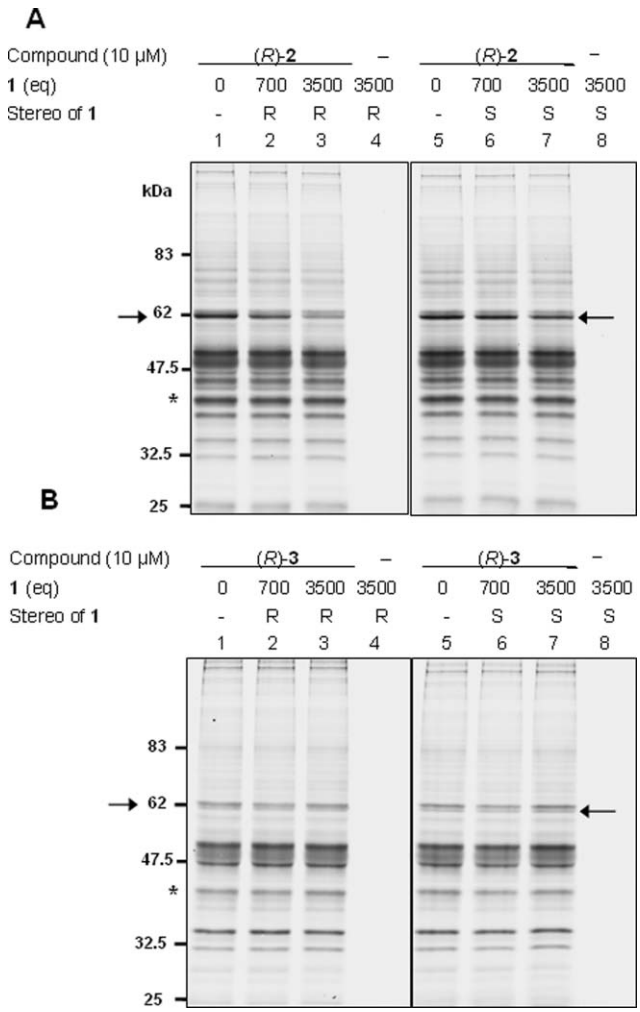


Fig. 2 Competition experiments using lacosamide AB&CR isothiocyanate (NCS) agent (*R*)-**2** and lacosamide AB&CR azide (N_3) agent (*R*)-**3** with either excess (*R*)-**1** or (*S*)-**1**. A: Proteins detected by in-gel fluorescence scanning after adduction with (*R*)-**2** in the presence of excess **1** (room temperature, 20 min) followed by Cu(I)-mediated cycloaddition with **6**. An arrow marks selectively labeled protein with **2** identified as CRMP2[7] and an asterisk marks an internal reference protein band. B: Proteins detected by in-gel fluorescence scanning after adduction with (*R*)-**3** in the presence of excess **1** (312 nm, 2 min, 4 °C) followed by Cu(I)-mediated cycloaddition with **6**. An arrow marks selectively labeled protein with **2** identified as CRMP2[7] and an asterisk marks an internal reference protein band.

at high levels of (*R*)-**1** (700–3500 equiv.). The high levels of (*R*)-**1** needed in these experiments could be attributed to the irreversible adduction process that follows the initial binding event (Scheme 1). We expect that covalent modification of the target protein by (*R*)-**1** AB&CR drives the reaction to completion, thus minimizing the effect of competing amounts of (*R*)-**1** during the initial binding event.

In proteomic search for drug targets, it is important to identify the proteins of interest within the cell. The significant anticonvulsant activities of (*R*)-**2** and (*R*)-**3** in rodents (Table 1) demonstrated that both compounds cross into the central nervous system (CNS) upon administration (ip, po) and likely enter neuronal cells. We asked whether the AB&CR agents (*R*)-**2** and (*R*)-**3** were suitable for *in situ* proteome experiments using readily accessible human 293T cells. Accordingly, we incubated cultured human 293T cells with either (*R*)-**2** or (*R*)-**3** (37 °C in the dark, 2 h) and then the (*R*)-**3**-treated cells were irradiated with 312 nm light (4 °C, 10 min). The (*R*)-**2**-treated cells were lysed, centrifuged to remove cellular debris, and the supernatant treated with **6** under Cu(I)-mediated cycloaddition conditions. The (*R*)-**3** cells were similarly treated, except that the recovered supernatant was equally divided and then one of the two aliquots was re-irradiated at 312 nm (4 °C, 5 min) before both aliquots were treated with **6**. We observed extensive protein modification for (*R*)-**2** by in-gel fluorescence (Fig. 3A, lane 1) but essentially none for (*R*)-**3** (Fig. 3A, lanes 2, 3), even though the corresponding Coomassie blue-stained gels for total proteins were similar (Fig. 3B).

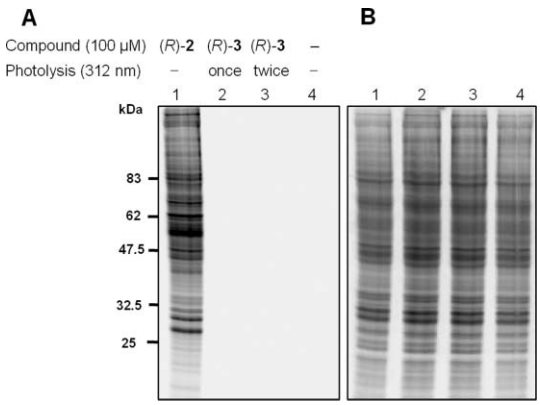


Fig. 3 Proteome reactivity profiles of lacosamide AB&CR (*R*)-**2** and (*R*)-**3** using 293T cells. A: Probe-labeled proteins were detected by in-gel fluorescence scanning after Cu(I)-mediated cycloaddition to a rhodamine-azide reporter probe **6**. B: Proteins in lysate visualized by Coomassie blue staining after in-gel fluorescence scanning. (All images shown in grayscale).

We briefly explored the difference between (*R*)-**2** and (*R*)-**3** results using 293T cells. Aryl azides are known to be reduced by thiols,[38] and glutathione (GSH) is a major constituent in the cell (low mM).[39] In addition, photolysis of aryl azides generates intermediates that can react with intracellular thiols.[17] Accordingly, we added GSH (0.1 mM, 0.5 mM) to the Nap-10-treated mouse brain soluble proteome used in Fig. 1 and determined the relative levels of protein adduction for 10 μM (*R*)-**2** and 10 μM (*R*)-**3**. For (*R*)-**2**, we observed no significant effect of 0.1 and 0.5 mM GSH on the protein adduction profiles (Fig. 4, lanes 5–7). For (*R*)-**3**, however, when we included 0.1 mM GSH in the lysate we found a dramatic reduction in the protein band intensities, which were further diminished with 0.5 mM GSH (Fig. 4, lanes 1–3). We tested whether GSH reacted with aryl azides. Using 0.5–10 mM GSH, we observed no appreciable reduction of (*R*)-**4** (TLC analysis, data not shown). Next, we determined whether GSH affected the Cu(I)-mediated cycloaddition reaction with **6**. Our finding that (*R*)-**2** provided appreciable levels of protein adduction in 293T cells (Fig. 4, lane 1) suggested that this was not likely. Nonetheless, we added GSH (0.1 M, 0.5 mM) to the mouse brain soluble lysate reactions *after* AB&CR **3** adduction. There was no noticeable intensity loss for the in-gel fluorescence bands upon reaction with **6** (Fig. S4, lanes 1–3 in the ESI†). However, when the GSH concentration was raised to 2.5 mM a significant intensity reduction of the in-gel fluorescence bands was observed (Fig. S4,

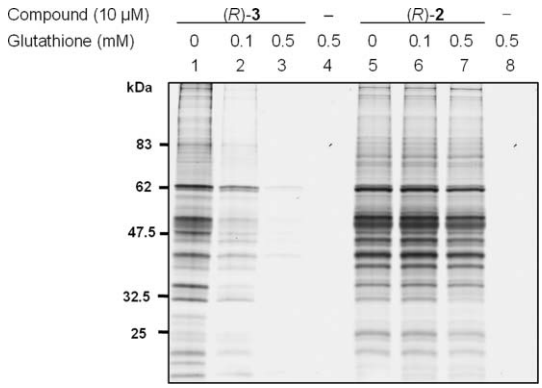


Fig. 4 Glutathione (GSH) effect on *in vitro* labeling with lacosamide AB&CR (*R*)-**2** and (*R*)-**3** in the mouse brain soluble proteome.

lane 4†). We concluded that the GSH levels in the 293T cells did not appreciably affect the Cu(I)-mediated cycloaddition reaction and that this step did not account for the near total absence of protein bands in the (*R*)-**3** experiments. Rather, our collective findings suggested that intracellular nucleophiles inactivated the photoactivated **3** aryl azide intermediates, thereby preventing them from reacting with 293T cellular proteins.

Discussion

We have advanced a general approach to investigating the proteome for ligands (drugs) whose binding is expected to be modest (Scheme 1).[7] Key to the implementation of this approach is selecting an AB unit that irreversibly modifies the target site. The AB group should not perturb drug binding, be sufficiently stable to permit dissolution and protein binding, yet sufficiently reactive to irreversibly modify the target. While the importance of these factors for selective protein adduction is recognized, little is known how AB reactivity can affect the protein selection process.

Aryl isothiocyanates[8,9] and aryl azides[11,12] are established affinity baits used in receptor tagging and identification studies. While structurally similar,[13] their methods of activation, pathways of protein adduction, and inherent reactivity may impact their use in proteomic searches. As an electrophilic affinity bait, the isothiocyanate group displays an excellent balance between stability and reactivity, thus allowing dissolution in aqueous solutions but permitting reaction with nucleophilic amino acids (*e.g.*, cysteine, lysine).[10] Accordingly, isothiocyanates have proven effective in selectively modifying target proteins within brain membrane fractions[8,9] and have been used in *in vivo* protein adduction studies after intracerebroventricular (icv) administration.[40] Similarly, aryl azides have been extensively used in protein labeling studies.[11,12] Indeed, an aryl azide analog of (*S*)-levetiracetam was employed to identify the synaptic vesicle protein SV2A as the binding site for this AED.[41,42]

Using the AB&CR strategy, we showed that the lacosamide AB&CR agent (*R*)-**2**, containing the electrophilic isothiocyanate group, preferentially modified CRMP2, a protein previously reported to bind (*R*)-**1**.[5–7] In this study, we explored the utility of **3**, an isostere of **2**, to identify lacosamide binding targets in the mouse brain soluble proteome fraction, and we compared **3** with **2**.

We first showed that (*R*)-**3** exhibited excellent seizure protection in both mice (ip) and rats (po) and that similar to **1** and **2**,[1,7] anticonvulsant activity principally resided in the (*R*)-stereiosomer, compared with the corresponding (*S*)-isomer (mice (ip): 8-fold). (*R*)-**3** displayed a ~2-fold increase in potency compared with (*R*)-**2** in the MES test (mice, ip). *In vitro* liver enzyme assays using (*R*)-**3** and the corresponding AB agent (*R*)-**4** showed that the aryl azide moiety was not readily metabolized. These collective findings suggested that (*R*)-**3** may serve as an excellent AB&CR agent in proteomic searches for (*R*)-**1** binding targets.

Using **3**, we found low adduction levels for the ~62 kDa band (Fig. 1A, lanes 5, 6) containing CRMP2 (Fig. 1B), as determined by in-gel fluorescence gel, and that there was little difference between the (*R*)-**3** and the (*S*)-**3** experiments. Correspondingly, higher levels of adduction of the ~62 kDa band were observed for **2**, and (*R*)-**2** preferentially modified this band compared with (*S*)-**2** (Fig. 1A, lanes 1, 2). Protein denaturation of the soluble lysate prior to AB&CR modification led to diminished levels of ~62 kDa protein(s) adduction by (*R*)-**2** and a loss in the preferential selectivity of (*R*)-**2** *versus* (*S*)-**2** protein modification (Fig. 1, lanes 3, 4). In contrast, no significant changes were observed for the (*R*)-**3** and (*S*)-**3** adduction processes with protein denaturation (Fig. 1, lanes 7, 8).

When the (*R*)-**2** experiments were repeated in the presence of excess (*R*)-**1**, we observed a significant loss in the in-gel fluorescence intensity of the ~62 kDa band (Fig. 2A, lanes 1–3). Diminished competition was observed when (*S*)-**1** was substituted for (*R*)-**1** (Fig. 2A, lanes 5–7). These findings are consistent with the notion that (*R*)-**1** binds to the same binding pocket in CRMP2 as (*R*)-**2** does and that (*R*)-**1** can block protein modification by (*R*)-**2**. When the competition experiment was conducted using (*R*)-**3**, no intensity loss of the ~62 kDa band by excess (*R*)-**1** was observed (Fig. 2B, lanes 1–3). Similar results were obtained when GST-CRMP2 was added to the lysate (Fig. S3, ESI†). Thus, the less-reactive AB&CR agent (*R*)-**2** was more sensitive to (*R*)-**1** competition than its more reactive counterpart (*R*)-**3**.

Together, these results provided valuable information on the AB properties needed to identify protein binding targets for (*R*)-**1**, a ligand for which protein binding is expected to be modest. We found that the less reactive aryl isothiocyanate AB moiety exhibited better enantioselectivity for protein modification and proved to be more sensitive in competition experiments with excess (*R*)-**1** than the more reactive aryl azide AB group.

We compared the potential use of AB&CR agents **2** and **3** for *in situ* proteomic search experiments. Using human 293T cells, we observed significant labeling with (*R*)-**2** but not with (*R*)-**3** (Fig. 3A, lanes 1–3). Whole animal pharmacological studies demonstrated that both compounds exhibited significant seizure protection (Table 1), and therefore, we have assumed both agents can enter 293T cells. We briefly explored why (*R*)-**3** was ineffective in labeling proteins under the irradiation conditions we used. The mouse brain soluble proteome shown in Fig. 1 and 2 was passed through a Nap-10 column prior to use. This size exclusion column preferentially removes small molecules from the lysate. One such molecule is likely to be GSH. Thiols are known to react with aryl azides,[37] and they are also expected to react with the aryl azide intermediates generated by photolysis.[17] We showed that progressively adding GSH (0.1–0.5 mM) to the Nap-10-treated lysate steadily decreased the amounts of protein adduction

observed upon (*R*)-**3** photoactivation (Fig. 4, lanes 1–3). Control experiments indicated that GSH did not noticeably reduce the azide group in the (*R*)-**3**. Similar results for other aryl azides and GSH have been reported.[38a] In addition, moderate levels of GSH did not markedly affect the Cu(I)-cycloaddition reaction with **6** under the employed conditions (Fig. S4, ESI†). These findings indicated that (*R*)-**3** photoactivated intermediates are likely destroyed by intracellular constituents, thus making this AB&CR a poor choice for identifying drug binding targets in intact cells.

The findings for our (*R*)-**3** proteomic labeling studies need to be placed in the context of the anticipated (*R*)-**3**-protein interaction. Lacosamide is a low molecular weight drug (MW = 250) whose protein(s) binding is anticipated to be modest. Using (*R*)-**3**, we observed no preferential protein labeling in the mouse brain soluble lysate. We have attributed this result to the modest binding strength of the (*R*)-**3**–protein binding interaction and to the reactivity of the (*R*)-**3** photoprobe intermediates. Correspondingly, we expect that aryl azide-containing ligands[43] that strongly bind to proteins will lead to selective adduction, provided that nucleophiles in the lysate or in the cell do not destroy the photoactivated species. We anticipate that lysate preparations that have been passed through size-exclusion columns, extensively washed, or dialyzed will provide higher yields of photoactivated aryl azide protein adducts than those that have not been pretreated. It should be noted that the selective adduction of CRMP2 by (*R*)-**2** in the mouse brain soluble lysate does not, by itself, document that this protein is a physiological target for (*R*)-**1**. We suspect that several proteins can bind (*R*)-**1**[44] and that target validation will require detailed functional and pharmacological studies.

Conclusions

Our findings for **2** and **3** have provided insights into the utility and limitations of the aryl isothiocyanate and aryl azide affinity baits, two functional groups commonly employed in target identification studies.[8–12] In our studies, we have incorporated either an electrophilic isothiocyanate ((*R*)-**2**) or a photoactivated ((*R*)-**3**) affinity bait on the (*R*)-**1** framework. The two affinity baits are structurally similar in size and shape,[13] but differ in their reactivity and where photoactivation of the aryl azide group in (*R*)-**3** leads to a more reactive agent than the isothiocyanate group in (*R*)-**2**. (*R*)-**1** is a low molecular weight drug that is expected to bind to its receptors (not necessary at the active site) in the brain with only modest affinity. Accordingly, our investigation differs from activity-based protein profiling (ABPP) methodologies,[33b,c,37,46–48] that utilize mechanism-based small molecules to identify active enzymes within the proteome, and from studies that incorporate affinity baits on ligands that bind tightly to their cognate receptors.[49] In these investigations, protein adduction is expected to be efficient. We showed that despite the two-fold improved anticonvulsant activity of (*R*)-**3** compared with (*R*)-**2**, (*R*)-**2** was superior in revealing potential binding targets in the mouse brain soluble proteome. Moreover, the incorporation of an isothiocyanate group in **2** allowed us to document the enantioselective adduction of CRMP2 by (*R*)-**2** compared with (*S*)-**2** within the proteome, and that (*R*)-**2** adduction was competitively blocked by excess amount of (*R*)-**1**. Thus, our findings demonstrated that in a case where ligand binding is modest, the reactivity of the AB unit within the AB&CR agent, along with the agent's pharmacological properties and structural size, are important determinants for successful identification of potential ligand binding partners in the proteome. Finally, we showed that the utility of the aryl azide affinity bait for *in situ* experiments can be affected by the ease with which the photoactivated aryl azide intermediate(s) is quenched by intracellular thiols.

Experimental section

General methods

Melting points were determined in open capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra (IR) were run on an ATI Mattson Genesis FT-IR spectrometer. Absorption values are expressed in wavenumbers (cm^{-1}). Optical rotations were obtained on a Jasco P-1030 polarimeter at the sodium D line (589 nm) using a 1 dm path length cell and are given in units of deg cm^3 g^{-1} dm^{-1}. NMR spectra were obtained at 300 MHz (^1H) and 75 MHz (^{13}C) using TMS as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm) from tetramethylsilane. Low-resolution mass spectra were obtained with a BioToF-II-Bruker Daltonics spectrometer by Drs Matt Crowe and S. Habibi at the University of North Carolina Department of Chemistry. The high-resolution mass spectrum was performed on a Bruker Apex-Q 12 Telsa FTICR spectrometer by Drs Matt Crowe and S. Habibi. Microanalyses were performed by Atlantic Microlab, Inc. (Norcross, GA). Reactions were monitored by analytical thin-layer chromatography (TLC) plates (Aldrich, Cat # Z12272-6) and analyzed with 254 nm light. The reaction mixtures were purified by flash column chromatography using silica gel (Dynamic Adsorbents Inc., Cat #02826-25). All chemicals and solvents were reagent grade and used as obtained from commercial sources without further purification. THF was distilled from blue sodium benzophenone ketyl. Yields reported are for purified products and were not optimized. All compounds were checked by TLC, ^1H and ^{13}C NMR, MS, and elemental analyses. The TLC, NMR and the analytical data confirmed the purity of the products was ≥95%.

(*R*)-*N*-(4-Azidobenzyl) 2-acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-3)

To a cooled (0 °C) CH_3CN solution (20 mL) of (*R*)-**16**[7] (1.04 g, 3.60 mmol) maintained under Ar, *t*-BuONO (1.28 mL, 10.80 mmol) followed by $TMSN_3$ (1.14 mL, 8.64 mmol) were slowly added. The resulting solution was allowed to stir at room temperature (16 h). The solvent was removed *in vacuo*, and the product was purified by column chromatography (SiO_2; 1 : 9 acetone–EtOAc) to give 0.87 g (76%) of (*R*)-**3** as a yellowish white solid: mp 135–136 °C; $[\alpha]_D^{24}$ −15.7 (*c* 1.0, $CHCl_3$); R_f 0.40 (1 : 9 acetone–EtOAc); IR (nujol mull) 3277, 2927, 2110, 1635, 1553, 1459 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.04 (s, C**H**$_3$C(O)), 2.46 (t, J = 2.5 Hz, CH_2CCH), 3.64 (dd, J = 7.1, 9.3 Hz, C**H**H′OCH$_2$), 3.93 (dd, J = 4.2, 9.3 Hz, CH**H**′OCH$_2$), 4.15 (1/2**H**H′$_q$, J = 2.5, 16.0 Hz, OCH**H**′C), 4.23 (1/2H**H**′$_q$, J = 2.5, 16.0 Hz, OCH**H**′C), 4.37-4.51 (m, C**H**$_2$Ar), 4.57-4.63 (m, C**H**), 6.52 (br d, J = 6.3 Hz, N**H**CH), 6.75-6.80 (br m, N**H**CH$_2$), 6.96-7.01 (m, 2 Ar**H**),

7.25-7.27 (m, 2 Ar**H**), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**3** gave only a single signal for the acetyl methyl protons and the alkyne proton, addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**3** and (*S*)-**3** (1 : 2 ratio) gave two signals for the acetyl methyl protons (δ 2.006 (*R*) and 2.021 (*S*) (Δppm = 0.015)), and two signals for the alkyne proton (δ 2.402 (*S*) and 2.448 (*R*) (Δppm = 0.046)) in a ~1 : 2 ratio; ^{13}C NMR ($CDCl_3$) δ 23.4 (**C**H_3C(O)), 43.2 (**C**H_2Ar), 52.7 (**C**H), 58.9 (**C**H_2CCH), 69.2 (**C**H_2OCH_2), 75.6 (CH_2**C**CH), 79.0 (CH_2**C**CH), 119.5, 129.2, 134.8, 139.5 (**C**$_6$H$_4$), 169.9, 170.6 (2 **C**(O)); HRMS (ESI) Calcd. for $C_{15}H_{18}N_5O_3$ (MH^+) 316.1410. Found 316.1407. Found: C, 57.30; H, 5.46; N, 22.10. $C_{15}H_{18}N_5O_3$ requires C, 57.13; H, 5.43; N, 22.21%.

(*S*)-*N*-(4-Azidobenzyl) 2-acetamido-3-(prop-2-ynyloxy)propionamide ((*S*)-3)

Utilizing the preceding procedure, and using (*S*)-**16**[7] (1.10 g, 3.81 mmol), *t*-BuONO (1.36 mL, 11.42 mmol), $TMSN_3$ (1.20 mL, 9.14 mmol) gave 0.89 g (74%) of (*S*)-**3** as a yellowish white solid: mp 135-136.5 °C; $[\alpha]_D^{24}$ +15.4 (*c* 1.0, $CHCl_3$); R_f 0.40 (1 : 9 acetone–EtOAc); IR (nujol mull) 3279, 2924, 2111, 1638, 1551, 1458 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.03 (s, C**H**$_3$C(O)), 2.46 (t, J = 2.4 Hz, CH_2C**CH**), 3.65 (dd, J = 7.2, 9.1 Hz, C**H**H′OCH_2), 3.92 (dd, J = 4.4, 9.1 Hz, CH**H**′OCH_2), 4.15 (1/2**H**H′$_q$, J = 2.4, 15.9 Hz, OC**H**H′C), 4.23 (1/2H**H**′$_q$, J = 2.4, 15.9 Hz, OCH**H**′C), 4.36-4.50 (m, C**H**$_2$Ar), 4.58-4.64 (m, C**H**), 6.48 (br d, J = 6.9 Hz, N**H**CH), 6.80-6.84 (br m, N**H**CH_2), 6.96-7.00 (m, 2 Ar**H**), 7.24-7.28 (m, 2 Ar**H**), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*S*)-**3** gave only a single signal for the acetyl methyl protons and the alkyne proton, addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**3** and (*S*)-**3** (1 : 2 ratio) gave two signals for the acetyl methyl protons (δ 2.006 (*R*) and 2.021 (*S*) (Δppm = 0.015)), and two signals for the alkyne proton (δ 2.402 (*S*) and 2.448 (*R*) (Δppm = 0.046)) in a ~1 : 2 ratio; ^{13}C NMR ($CDCl_3$) δ 23.4 (**C**H_3C(O)), 43.2 (**C**H_2Ar), 52.7 (**C**H), 58.9 (**C**H_2CCH), 69.3 (**C**H_2OCH_2), 75.6 (CH_2**C**CH), 79.1 (CH_2**C**CH), 119.5, 129.2, 134.8, 139.5 (**C**$_6$H$_4$), 169.9, 170.6 (2 **C**(O)); HRMS (ESI) Calcd. for $C_{15}H_{18}N_5O_3$ (MH^+) 316.1410. Found 316.1406. Found: C, 56.99; H, 5.45; N, 22.10. $C_{15}H_{17}N_5O_3$ requires C, 57.13; H, 5.43; N, 22.21%.

Preparation of mouse brain soluble proteomes

Mouse brains (male, 2 months, ICR mice [Rockland Immunochemicals, Gilbertsville, PA]) were Dounce-homogenized in 50 mM HEPES buffer (pH 7.4). The lysate was centrifuged at slow speed (1200 × g for 12 min at 4 °C) to remove debris. The supernatant was then centrifuged at high speed (100 000 × g for 1 h at 4 °C). The resulting supernatant was collected and stored at –80 °C until use. The total protein concentration was determined by using the Bradford assay.

AB&CR 2 labeling, cycloaddition reaction, and in-gel fluorescence scanning

Mouse brain lysate (1 mL, 50 mM HEPES buffer (pH 7.4)) was passed through a Nap-10 column (GE Healthcare) to exchange buffer to an aqueous 50 mM HEPES buffer (pH 7.8). Lysate aliquots (50 µL of 2.0 mg mL^{-1} protein in 50 mM HEPES buffer (pH 7.8)) were treated with either (*R*)-**2** or (*S*)-**2** (10 µM) without or with competing ligands ((*R*)-**1**, (*S*)-**1**) at room temperature (20 min). The modified lysates were sequentially treated with rhodamine reporter tag **6** (50 µM), TCEP (1 mM), TBTA (100 µM) and $CuSO_4$ (1 mM). Samples were shaken and allowed to rotate using Roto-shake (8 rpm, Scientific Industries Inc., Model No. SI-1100, Bohemia, NY) at room temperature (1 h). Proteins were separated by SDS-PAGE after addition of 4× SDS-PAGE loading buffer and visualized by in-gel fluorescence using a typhoon 9400 scanner (GE Healthcare/Amersham Bioscience) with excitation at 555 nm and detection at 580 nm.

AB&CR 3 labeling, cycloaddition reaction, and in-gel fluorescence scanning

Utilizing the preceding procedure, lysate aliquots (50 µL of 2.0 mg mL^{-1} protein in 50 mM HEPES buffer (pH 7.8)) were treated with either (*R*)-**3** or (*S*)-**3** (10 µM) without or with competing ligands ((*R*)-**1**, (*S*)-**1**) at 4 °C (10 min) and irradiated with 312 nm light (8 W, Spectroline EB-280C, Spectronics Corp., New York, USA) at 4 °C (2 min). The reaction mixtures were quenched with TCEP (1 mM). The modified lysates were sequentially treated with rhodamine reporter tag **6** (rhodamine-azide (Rho-N_3) (50 µM)), TBTA (100 µM) and $CuSO_4$ (1 mM). Samples were shaken and allowed to rotate using Roto-shake (8 rpm, Scientific Industries Inc., Model No. SI-1100, Bohemia, NY) at room temperature (1 h). Proteins were separated by SDS-PAGE after addition of 4× SDS-PAGE loading buffer and visualized by in-gel fluorescence using a typhoon 9400 scanner (Amersham Bioscience) with excitation at 532 nm and detection at 580 nm.

In situ proteome reactivity profiling

293T cells were grown to ~50% confluency in DMEM supplemented with 10% fetal bovine serum in T75 flask. Medium was changed, and DMSO solution (200 µL, 5 mM) of either (*R*)-**2** or (*R*)-**3** was added directly to 10 mL medium in the flask. After incubating (37 °C in the dark, 2 h), the (*R*)-**3** treated cells were irradiated with 312 nm light (4 °C, 10 min). Both (*R*)-**2** and (*R*)-**3** treated cells were washed with PBS (×3) and harvested by scraping. Soluble proteome extracts were prepared by needle sonication of cell pellets in 50 mM HEPES buffer (pH 7.8) and centrifugation (100 000 × g for 1 h at 4 °C). The supernatant of (*R*)-**2** treated cells was treated with **6** under Cu(I)-mediated cycloaddition conditions. The supernatant of (*R*)-**3** treated cells was first equally divided, and then one of the two aliquots was re-irradiated at 312 nm (4 °C, 5 min) before both aliquots were treated with **6**. Proteins were separated by SDS-PAGE after addition of 4× SDS-PAGE loading buffer and visualized by in-gel fluorescence using a typhoon 9400 scanner (Amersham Bioscience) with excitation at 532 nm and detection at 580 nm.

Pharmacology

Compounds were screened under the auspices of the National Institutes of Health's Anticonvulsant Screening Program. Experiments were performed in male rodents [albino Carworth Farms No. 1 mice (intraperitoneal route, ip), albino Spague-Dawley rats (oral route, po)]. Housing, handling, and feeding were in accordance with recommendations contained in the 'Guide for

the Care and Use of Laboratory Animals'. Anticonvulsant activity was established using the MES test,[14] 6 Hz,[26] hippocampal kindled seizure,[29] and the scMet test,[25] according to previously reported methods.[45]

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