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Original Article

Development of novel 4-aminopyridine derivatives as potential treatments for neurological injury and disease

Daniel T. Smith [a,*], Riyi Shi [b], Richard B. Borgens [b], Jennifer M. McBride [b], Kevin Jackson [c], Stephen R. Byrn [a]

[a] *Department of Industrial and Physical Pharmacy, Purdue University, 575 Stadium Mall Drive, West Lafayette, IN 47907, USA*
[b] *Center for Paralysis Research, School of Veterinary Medicine, School of Engineering, Purdue University, West Lafayette, IN 47907, USA*
[c] *Section of Neurosurgery, Wishard Memorial Hospital, Indiana University Medical Center, 1001 West 10th Street, Indianapolis, IN 46202, USA*

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Abstract

The amine position of the K^+ channel blocker 4-aminopyridine was functionalized to form amide, carbamate and urea derivatives in an attempt to identify novel compounds which restore conduction in injured spinal cord. Eight derivatives were tested in vitro, using a double sucrose gap chamber, for the ability to restore conduction in isolated, injured guinea pig spinal cord. The methyl, ethyl and *t*-butyl carbamates of 4-aminopyridine induced an increase in the post injury compound action potential. The methyl and ethyl carbamates were further tested in an in vivo model of spinal cord injury. These results represent the first time that 4-aminopyridine has been derivatized without losing its ability to restore function in injured spinal cord tissue.
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1. Introduction

The majority of clinical spinal cord injuries (SCI) do not involve severing the spinal cord but instead result from the compression or contusion of the cord, leaving variable amounts of the spinal cord intact post injury [1]. In the surviving spinal cord, the mechanical insult leads to the destruction of the nerve's myelin sheath at the site of injury, exposing paranodal K^+ channels. The exposed K^+ channels provide a pathway for the exodus of K^+ ions during neuronal activity, which effectively quenches the nerve impulse as it reaches the section of damaged spinal cord. The overall effect is essentially the same as if the nerve were severed: no conduction occurs through the site of injury [1,2].

Fast voltage-gated (A current) potassium channel blockers have been found to be useful tools in counteracting the effects of demyelination in SCI. By blocking the potassium ions' route of exodus from the cell, it is possible to permit nerve impulse conduction to effectively bridge the area of myelin damage. Unfortunately, there are relatively few compounds that are known to block voltage-gated potassium channels. The most commonly cited examples are the aminopyridines (Fig. 1), tetraethylammonium (TEA) salts and a family of polypeptide toxins isolated from the venoms of scorpions, snakes, bees and anemones [3–8]. Of these compounds, 4-aminopyridine (4-AP) has shown the greatest potential as a treatment of SCI.

Study of 4-AP as a potential treatment for SCI was initiated in the late 1980s [9] and, more recently, it has moved into human clinical trials [10–16]. Behavioral improvements attributed to 4-AP treatment include: voluntary motor control, ambulation, continence, respiratory control along with decreases in spasticity and idiopathic pain [10–15,17–21]. However, the positive improvements induced by 4-AP are variable in their expression and may evoke serious side effects such as mood swings along with a heightened state of anxiety and seizures, both mild and severe [11,12,20,22,23]. It has become clear that the maximal safe dose is insufficient to produce the maximal beneficial functional recovery in many cases [24].

* Corresponding author. Tel.: +1 765 494 0513; fax: +1 765 494 6545.
E-mail address: dtsmith@pharmacy.purdue.edu (D.T. Smith).

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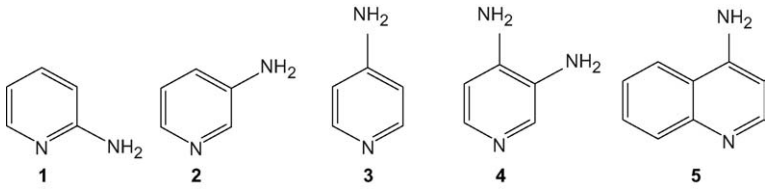

Fig. 1. The aminopyridines. 2-Aminopyridine (2-AP, **1**), 3-aminopyridine (3-AP, **2**), 4-aminopyridine (4-AP, **3**), 3,4-diaminopyridine (3,4-DAP, **4**), and 4-aminoquinoline (4-AQ, **5**).

This behavioral "cap", in a addition to the fact that very few 4-AP derivatives have been tested as alternatives (all unsuccessfully [25–30]), led us to investigate the chemical derivatization of 4-AP as a means of generating novel compounds that restore conduction in injured spinal cord, possibly leading to an alternative therapy for treating conditions that result from nerve demyelination.

2. Chemistry

At physiological pH, the basic nature of 4-AP ($pK_a = 9.4$) [31] leads to an equilibrium between the neutral and protonated forms that strongly favors the pyridinium ion (~99%). It has been shown that the neutral from is responsible for crossing the cell's membrane while the protonated form blocks the channel from the cytoplasmic side of the cell [3,26–28,32–37]. We feel that the hydrogen bonding ability of 4-AP's protonated form, which is strengthened by its ability to delocalize the charge throughout the molecule, is responsible for the drug–channel interaction. This assertion is supported by the theoretical work performed by Niño et al. [38,39].

In identifying derivatives of 4-AP to synthesize, functional groups where chosen that would not completely eliminate the pyridine–pyridinium equilibrium responsible for both transport and biological activity. The addition of functionality that was capable of some amount of hydrogen bonding, either as a donor or acceptor, was also considered to be important. In that way, the two features most pertinent to 4-AP's biological activity would be maintained.

Fig. 2 illustrates the range of compounds tested in this study. 4-(Dimethylamino)pyridine (DMAP, **6**) was chosen as an extension of the previous work using 4-aminopyrinine methiodide (4-AMPI) [26–28,30,32]. The amide, carbamate and urea functional groups were seen as a means of increasing the available hydrogen bonding groups while maintaining at least some of the pyridine–pyridinium equilibrium.

Compounds **7**–**11** and **13** were synthesized from 4-AP via standard synthetic methodology. Cyclic urea **12** was synthesized from 3,4-diaminopyridine. DMAP (**6**) was purchased (Aldrich, Milwaukee, WI.) and used as received.

3. Bioevaluation

Each of the derivatives was tested in vitro to determine its ability to restore conduction in isolated, injured guinea pig spinal cord. This was done using a double sucrose gap isolation and recording chamber [40,41]. Strips of spinal cord white matter were placed in the chamber then allowed to stabilize for 1 h in order to identify a characteristic response to electrical stimulation. A stretch injury caused the loss of conduction, which improved slightly with time. Once a stable "recovery" compound action potential (CAP) was achieved (the baseline), the injured cord was exposed to a solution of the test compound. The amount of restored conduction was determined by noting the percentage increase of CAP amplitude over the baseline.

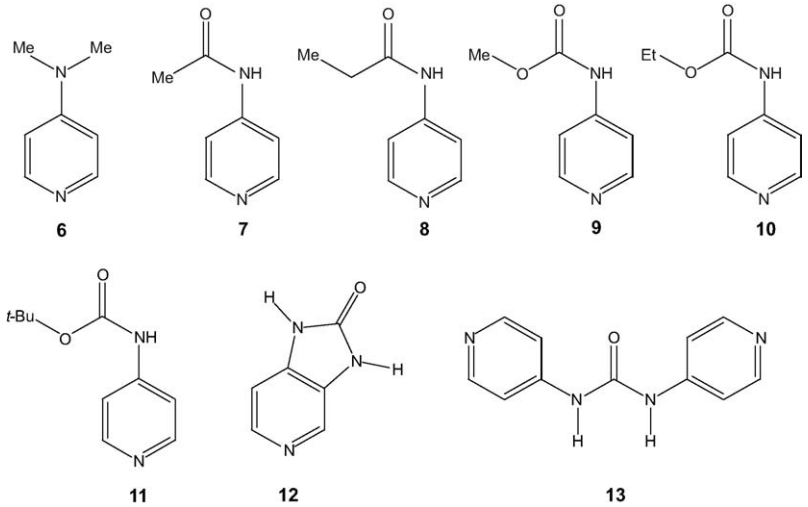

Fig. 2. 4-AP derivatives tested in this study.

For determining activity in vivo, a normal regimen of somatosensory evoked potential (SSEP) testing was carried out in the intact animal. A standardized injury to the guinea pig spinal cord was induced surgically. Upon recovery from surgery and stabilization of the injury (~1 week), a baseline ability of the spinal cord to conduct an impulse from the tibal nerve of the hind limb to the contralateral cortex was determined. The animal was dosed with the test compound by gavage and the amount of increase in the evoked potential (EP) over baseline was determined.

4. Results

4.1. In vitro testing of conduction through guinea pig spinal cord

A total of eight 4-AP derivatives have been tested in vitro in order to determine their ability to restore conduction in stretched spinal cord. DMAP (**6**), the amides (**7** and **8**) and the ureas (**12** and **13**) all failed to produce any enhancement in the recovery CAP amplitude at the same concentration that is most effective for 4-AP (100 μM). The negative results are not shown here.

Three compounds, *N*-(4-pyridyl) methyl carbamate (**9**); *N*-(4-pyridyl) ethyl carbamate (**10**) and *N*-(4-pyridyl) *t*-butyl carbamate (**11**), demonstrated the ability to restore conduction through injured spinal cord. Pilot trials with these three carbamates established that working concentrations equal to or below that of 4-AP were capable of inducing a reproducible recovery of conduction in the double sucrose gap recording chamber. In this type of experiment, 4-AP has been shown to induce a recovery of about 25% at 100 μM [42]. For methyl carbamate **9**, a concentration of 100 μM produced an increase in the amplitude of the recovered CAP averaging 30.6 ± 11.7% ($n = 4$). For ethyl carbamate **10**, the increase was 18.6 ± 8.7% ($n = 8$) at the same concentration. The increases in amplitude were statistically significant ($P \leq 0.04$). *t*-Butyl carbamate **11**, on the other hand, was found to be toxic to white matter at 100 μM when added to the bathing solution. Toxicity in these in vitro trials was defined as the significant reduction of the CAP after exposure to the test compound. It was found, however, that at a concentration of 1 μM, carbamate **11** reproducibly increased the recovery CAP amplitude by 15.4 ± 3.4% ($n = 5$) in the absence of toxicity. This increase was very significant relative to the pre-drug CAPs ($P \leq 0.002$).

For all three carbamates, a 1 h wash returned the enhanced CAP amplitude to pre-drug levels. Fig. 3 provides examples of the enhanced CAP electrical records for each of these three drugs and graphically displays the data. Based on the encouraging results seen in the in vitro studies, it was decided to move the three carbamates to in vivo testing.

4.2. In vivo testing in guinea pig

Post surgery, SSEPs were eliminated in all animals by the time electrical records could be accomplished (< 1 h). In all but two animals these "flat-line" recordings were characteristic of the 1 week recordings as well, indicating there was little evidence for spontaneous recovery of conduction in any of the guinea pigs 1 week after surgery. In the two exceptions, a small and very early arriving EP (approximately 25 ms latency) was noted, however, the magnitude of this peak was barely detectable above baseline and it was not dependably evoked. Therefore, gavage and further recordings were carried out on these animals and their data are pooled with the rest. Gavage of 4-AP and carbamates **9** and **10** was uneventful; however, we were unable to carry out this procedure using the *t*-butyl carbamate **11**. Aqueous solubility proved to be problematic with this carbamate and the initial attempts at producing a solution of known concentration for oral insertions were unsuccessful. As a result, we can provide only the preliminary in vitro data below for comparison purposes.

4-AP produced a strong and dependable enhancement of EPs within 30–60 min after insertion as observed by a return of an early arriving EP (over baseline). Two of the 10 animals failed to respond to 4-AP by 4 h after gavage. There were no failures to respond when using carbamates **9** and **10**.

Even though the sample size was small, we were encouraged by an apparent enhancement of EP recovery by methyl carbamate **9** (Fig. 4). Thus, some statistical comparison between these groups is provided, however, we caution that these data should be considered as preliminary since placement and replacement of either stimulating or recording electrodes does affect the size and duration of EP measured over the sensory cortex. All data were normalized by dividing the area (expressed as pixels) beneath recovered EP by the same pre-injury data.

Given these precautions, it is likely that carbamate **9** significantly enhanced the recovery of EP compared to 4-AP at 1 week post injury. Both two-way ANOVA and a straight forward Student's *t*-test confirmed a significant increase (ANOVA = 0.02; two-tailed Student's $T = 0.47$. Given that the latter test is more conservative relative to small sample size we only provide this comparison in Table 1). Even more striking was the enhancement of EPs by methyl carbamate **9** compared to the structurally similar ethyl carbamate **10** (Table 1). The latter carbamate did not meet significance compared to 4-AP data.

5. Discussion

5.1. Derivative design

It was felt that any potential analogue designed would have to account for two features salient to 4-AP. First, the acid–base equilibrium inherent to 4-AP would have to be maintained to some extent as it is directly responsible for both the transported species and the active species. Second, at least some of 4-AP's hydrogen bonding ability would need to be maintained in order to bind with the channel. Chemically, functionalizing the amine nitrogen was the simplest course of action that would meet the requirements.

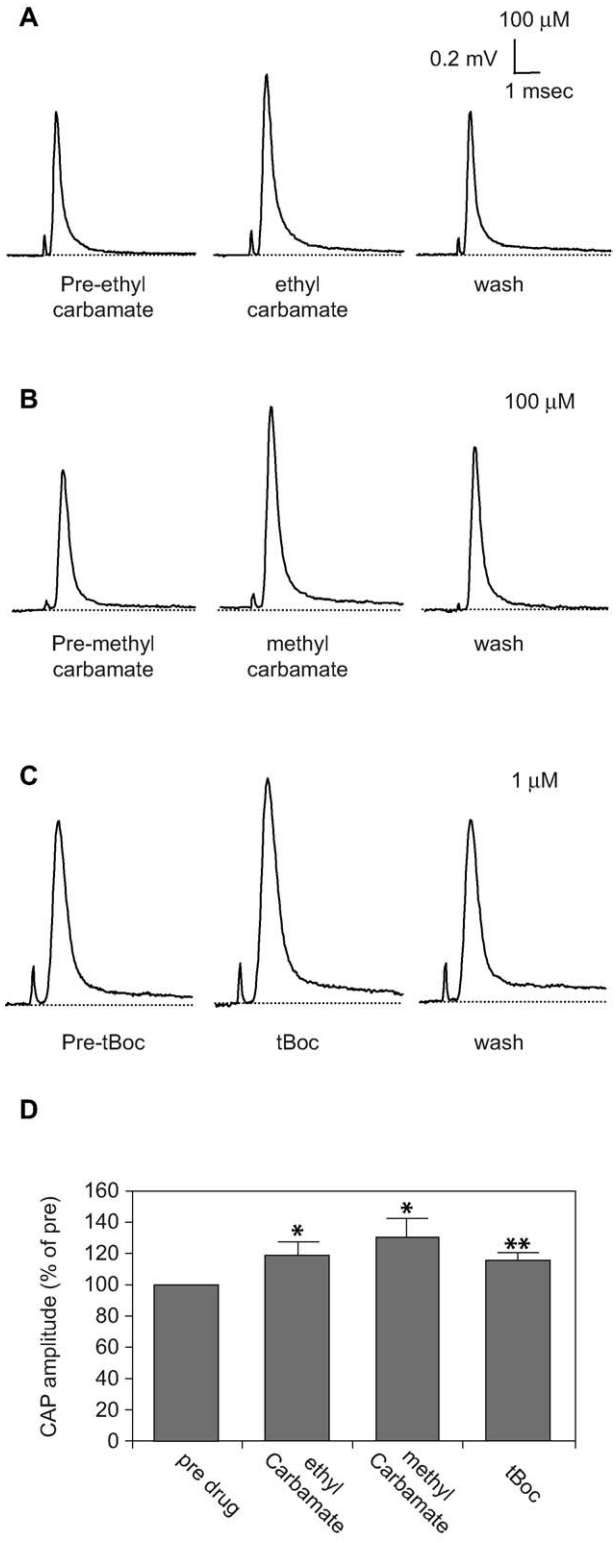

Fig. 3. Responses of recovered compound nerve impulses to three 4-AP derivatives. In **A**, the first electrical record shows the recovered CAP prior to the addition of ethyl carbamate **10** (the initial small wave form is the stimulus artifact). The second record was taken about 1/2 h after the addition of carbamate **10**. Note the ~ 20% increase in the amplitude of the CAP. The third record shows that the amplitude of this enhanced CAP has fallen back to pre-drug levels after removal of the drug and a 30 min wash. **B** shows a similar set of electrical records obtained after introduction of methyl carbamate **9**. Again, the increase in amplitude in the presence of the drug is obvious. This enhancement was only sustained during the administration of the drug and approximate pre-drug levels were measured after a washing out of the drug. **C** depicts a similar set of records to **A** and **B**, using *t*-butyl carbamate **11**. **D** provides a histogram of all data. Carbamates **9** and **10** statistically enhanced CAP amplitudes at 100 μM concentration ($P \leq 0.04$; one asterisk), while carbamate **11** statistically improved the CAP at 1 μM compared to pre-drug amplitudes ($P \leq 0.002$; two asterisks).

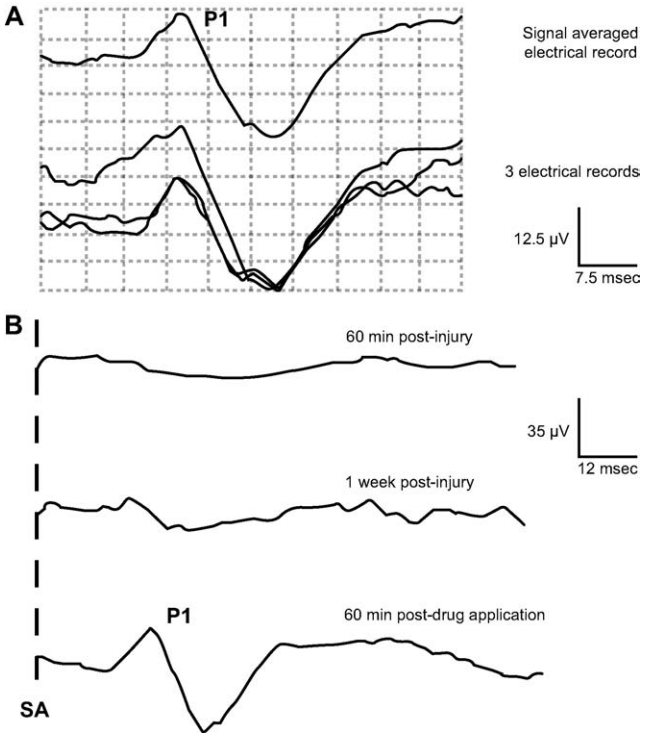

Fig. 4. Recovery of spinal cord conduction subsequent to oral administration of *N*-(4-pyridyl) methyl carbamate (**9**). An unedited electrical record of a normal SSEP is shown in **A**. The waveforms were recorded by electrodes located over the sedated guinea pig's sensory cortex after electrical stimulation of the tibial nerve of the hind paw. The bottom three records are individual traces of 200 repetitive stimulations of the nerve as discussed in Section 7. The dotted line (SA) marks the stimulus artifact. An early arriving cortical potential is shown, approximately 30 ms after stimulation (marked P 1) in the uninjured guinea pig (**A**). In **B**, responses to the oral ingestion of the test compound are shown in the same animal providing the record in **A**. The top record was made 1 h after a crush injury to the midthoracic spinal cord as described in the text. Note the complete elimination of the cortical potential after stimulation of the tibial nerve of the hind paw. One week later (second trace) this failure to conduct impulses through the SCI has changed little. The bottom record was made 1 h after gastric tube administration of *N*-(4-pyridyl) methyl carbamate (**9**). Note the rapid establishment of a nearly normal cortical potential (P 1).

Table 1
Summary of in vivo results

Test drug	*n*	Mean	S.D.	S.E.M.	Range	Statistic
4-AP	10	40.0	31.0	9.8	0–100	4-AP vs. **9**, $P = 0.47$
Methyl carbamate (**9**)	5	78.0	14.2	5.8	58–100	**9** vs. **10**, $P = 0.003$
Ethyl carbamate (**10**)	4	41.5	13.2	6.6	27–55	4-AP vs. **10**, $P = 0.93$

DMAP (**6**) was seen as an ideal a probe to explore the importance of the amine hydrogens to 4-AP's biological activity. A number of published reports indicate that methylation of the pyridine nitrogen (4-AMPI) [26–30] effectively removes 4-AP's channel blocking ability. While "severe" steric constraints at the active site were used to explain the lack of activity [26], the replacement of a hydrogen bonding donor with a non-polar, non-hydrogen bonding methyl group could also explain the lack of activity.

Similar to 4-AMPI, DMAP demonstrated no ability to restore conduction in injured spinal cord, indicating that complete methylation of either "hemisphere" of 4-AP is detrimental to its activity. Unfortunately, these results do not help to clarify the causes of the reduced biological activity, as either the steric argument or the hydrogen bonding argument could be invoked. However, in light of the restorative effects observed with several sterically larger 4-AP derivatives, we are inclined to believe that changes in hydrogen bonding ability is of greater importance than the increase in steric size of either methylated derivative vs. 4-AP.

In addition to alkylating 4-AP, we were also interested in testing several acylated derivatives. Acylation of 4-AP's amine group would remove a hydrogen bond donor, however, the introduced oxygen atom(s) might serve as a hydrogen bond acceptor, interacting with the channels amino acid backbone and offsetting the loss of the amine proton. This course of derivatization was expected to have a significant effect on the molecule's pK_a and the pyridine–pyridinium equilibrium. For example, the pK_as of the carbamates have been calculated to be between 6.0 and 6.4 [43]. This reduction in pK_a would shift the equilibrium in favor of the neutral pyridine, decreasing the amount of pyridinium ion in solution from 99% to between 4% and 9%.

Easily prepared amides **7** and **8**, along with ureas **12** and **13** were chosen as representative members of their respective classes of 4-AP derivatives. We also chose to synthesize the three carbamates **9**–**11**. While the carbamates represented a third class of 4-AP derivative, they also offered the ability to vary the steric bulk of the carbamate alkyl group, providing the ability to probe the nature, if any, of the steric requirements within the active site.

The amides (**7** and **8**) and ureas (**12** and **13**) induced no improvement of the recovery CAPs in isolated spinal cords and, as a result, no in vivo testing was performed with these compounds. In light of the positive results obtained with the carbamates, the lack of activity from these derivatives is puzzling, as they would seem to fit the structural requirements set forth equally as well as the carbamates. In particular, the inability of ureas **12** and **13** to restore conduction is curious. These compounds represent only slight modifications of two well known K^+ channel blockers (3,4-DAP and 4-AP, respectively) with only a carbonyl group inserted between amine groups. At this time, there is no clear explanation as to why the carbamates exhibit a restorative effect while the amides and ureas do not.

The most promising results were obtained with the three carbamate derivatives of 4-AP (**9**–**11**). All three compounds induced an increase in CAP above the pre-drug level and did so, at the appropriate concentration, without any indication of toxicity. These three compounds are the first derivatives of 4-AP to demonstrate the ability to restore conduction in injured spinal cord. Methyl and ethyl carbamates **9** and **10**

both produced similar recoveries to those previously observed with 4-AP and did so at the same concentration (100 μM). The toxicity observed at 100 μM with *t*-butyl carbamate **11** was unexpected considering its structural similarity with the methyl and ethyl derivatives. Equally surprising was the level of restored conduction once the concentration was reduced 100-fold, which was equal to ethyl carbamate **10** and almost as much as 4-AP itself.

The source of the *t*-butyl carbamate's lower effective concentration has not yet been determined. The carbamate derivatives were designed to mimic and possibly improve upon 4-AP' binding. While improved drug–channel interaction certainly could explain the lower effective concentration of **11**, other explanations can not be ruled out at this time. These include: a lower pK_a than 4-AP, increased lipophilicity or a slightly different binding site within the channel. The reduction in pK_a shifts the pyridine–pyridinium equilibrium towards the neutral pyridine form, which is likely to increase the rate of transport and lead to higher intracellular concentrations of the drug. Similarly, increasing lipophilicity vs. 4-AP should also lead to an increase in the intracellular concentration. Finally, carbamate **11** might bind to a different set of amino acids, altering its activity vs. 4-AP.

A quick stability study was used to determine whether the carbamate derivatives might be "decomposing" back to 4-AP under the experimental conditions, leading to false positive results. A solution was made of each carbamate in both Krebs solution (the in vitro dosing medium) and simulated human gastric fluid. The solutions were analyzed by TLC after 24 h at r.t. to determine whether any decomposition had occurred. No changes were apparent in any of the samples. While these qualitative results are not unequivocal proof that the carbamates are the active species, they do suggest that the carbamates are stable to the dosing conditions and are likely to be the active species, particularly in the in vitro experiments.

For all three carbamates, it is proposed that they restore conduction in injured spinal cord through a K^+ channel blockade mechanism. The structural similarity between these derivatives and 4-AP along with the similarity in restoration of CAPs suggests to us that a similar mechanism is in effect. True "proof" of a channel blockade mechanism will need to be determined through the use of patch clamp electrophysiology. Efforts to further understand the mechanisms by which these derivatives restore conduction are currently underway and will be reported in due course.

5.2. In vivo testing

In vivo recording of SSEPs provide data supportive of the of test compounds' ability to restore conduction through the spinal cord lesion. As mentioned, one should be cautious to not over interpret the quantitative data. For example, a recovery to 100% of the normal pre-injury recording *should not* be interpreted as evidence of an absolute recovery of normal EPs conducted through the lesion since the amplitude and duration of these waveforms are sensitive to electrode placement. On the other hand, such a recovery over the flat-line within minutes after drug ingestion does suggest a marked recovery of conductance. Even given the small sample size used to screen these first compounds as a proof of concept, it is very likely that methyl carbamate **9** will outperform 4-AP in live animal testing, and even more certain that this compound will be more effective than its close analog, ethyl carbamate **10**.

While the results detailed herein are an effective proof of concept that derivatized 4-AP can restore conduction in injured spinal cord, a number of practical question remain to be answered. More exploratory work with *t*-butyl carbamate **11** is required. Early attempts to dose guinea pigs with this compound suffered from this compound's relatively low water solubility. Efforts are continuing to develop an acceptable dosage regimen in order to determine whether this derivative is as active in the animal as it is in vitro. As demonstrated by carbamate **11**, the dose–response relationships of the carbamates needs to be developed in order to determine their optimal dosage so these derivatives' effectiveness as a treatment of SCI can be more closely compared with each other and 4-AP. Work is ongoing in order to provide answers to these questions and the results of will be reported in due course.

6. Conclusion

In summary, several derivatives of 4-AP have been synthesized and tested in isolated spinal cord and whole animal models of SCI in order to determine their ability to restore conduction in injured nerves. The effects of alkylation and acylation of 4-AP's amine nitrogen were explored. The alkylated compound did not exhibit any detectable ability to restore conduction in vitro, similar to the results previously reported for 4-AMPI. Somewhat surprisingly, the amide and urea derivatives did not demonstrate the ability to restore conduction to isolated, injured spinal cord. However, the methyl, ethyl and *t*-butyl carbamates **9**–**11** all allowed recovery of conduction through injured spinal cord at or below 4-AP's optimal concentration, with the *t*-butyl carbamate exhibiting a similar level of restoration at 1% of the concentration required for 4-AP. These results represent the first successful attempt at synthesizing a 4-AP derivative that maintains 4-AP's ability to restore conduction in injured spinal cord and suggests the possibility of developing additional molecules that could be useful in circumventing the behavioral cap seen when using 4-AP as a treatment of conditions that result from demyelination.

7. Experimental protocols

7.1. Chemistry

DMAP (**6**) was purchased from Sigma-Aldrich, Milwaukee, WI. Amides **7** [44] and **8** [45], along with ureas **12** [46] and **13** [47], were synthesized using published methods. 4-AP

was purchased from Richman Chemical Co., Lower Gwynedd, PA. All reagents were used as received without further purification. Melting points were determined in capillary tubes using a Thomas Hover melting point apparatus and are uncorrected. NMR spectra were obtained on a Bruker ARX-300 instrument using the indicated solvent.

*7.1.1. Synthesis of N-(4-pyridyl) methyl carbamate (**9**)*

To a solution of 4-aminopyridine (15.0 g, 160 mmol) in CH_2Cl_2 (200 ml) at 0 °C was added triethylamine (30.0 ml, 212 mmol) then methyl chloroformate (15.0 ml, 192 mmol). The resulting mixture was allowed to warm to r.t. overnight then was concentrated. The solid products were slurried with saturated aqueous $NaHCO_3$, stirred for 1 h, concentrated and dried under vacuum. The crude product was slurried with hot MeOH (500 ml) for 1 h, filtered and the filtrate was concentrated. The crude carbamate was recrystallized from water to give 15.9 g (66% yield) of pure methyl carbamate **9**. M.p. = 168–170 °C. ^{1}H NMR (300 MHz, MeOH-d_4) δ 8.49 (d, J = 6.5 Hz, 2H, Ar); 7.67 (d, J = 6.5 Hz, 2H, Ar); 5.22 (br s, 1H, N–H); 3.94 (s, 3H, OMe). ^{13}C NMR (75 MHz, MeOH-d_4) δ 156.0 (C=O); 151.0 (Ar); 149.2 (Ar); 114.3 (Ar); 53.3 (OMe). Anal. $C_7H_8N_2O_2$ (MW = 152.15).

*7.1.2. Synthesis of N-(4-pyridyl) ethyl carbamate (**10**)*

To a solution of 4-aminopyridine (20.0 g, 212 mmol) in CH_2Cl_2 (200 ml) at 0 °C was added triethylamine (30.0 ml, 212 mmol) and ethyl chloroformate (20.3 ml, 212 mmol). The resulting mixture was allowed to warm to r.t. overnight then was concentrated. The solid products were slurried with saturated aqueous $NaHCO_3$, stirred for 1 h, concentrated and dried under vacuum. The crude product was slurried with hot MeOH (500 ml) for 1 h, filtered and the filtrate was concentrated. The crude carbamate was recrystallized from toluene/hexanes to give 31.8 g (90% yield) of pure ethyl carbamate **10**. M.p. = 127–128 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.02 (s, 1H, N–H); 8.56 (d, J = 6.2 Hz, 2H, Ar); 7.63 (d, J = 6.2 Hz, 2H, Ar); 4.33 (q, J = 7.1 Hz, 2H, OCH_2); 1.38 (t, J = 7.1 Hz, 3H, Me). ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.3 (C=O); 150.2 (Ar); 147.3 (Ar); 113.3 (Ar); 61.8 (OCH_2); 14.8 (Me). Anal. $C_8H_{10}N_2O_2$ (MW = 166.18).

*7.1.3. Synthesis of N-(4-pyridyl) t-butyl carbamate (**11**)*

To a solution of 4-aminopyridine (50.0 g, 531 mmol) in triethylamine/CH_2Cl_2 (1:1, 200 ml) at 0 °C was slowly added a solution of di-*t*-butyl-dicarbonate (116 g, 531 mmol) in CH_2Cl_2 (150 ml). The resulting mixture was allowed to warm to r.t. overnight then was concentrated. The crude product was taken up in hot EtOAc, filtered and precipitated with hexanes. The precipitate was collected by filtration, washed with hexanes and dried under vacuum to give 91.0 g (88% yield) of pure *t*-butyl carbamate **11**. M.p. = 147–148 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.97 (s, 1H, N–H); 8.39 (d, J = 5.2 Hz, 2H, Ar); 7.41 (d, J = 5.2 Hz, 2H, Ar); 1.46 (s, 9H, *t*-Bu). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.1 (C=O); 150.3 (Ar); 147.3 (Ar); 113.0 (Ar); 81.5 (O–C–R_3); 28.6 (Me). Anal. $C_{10}H_{14}N_2O_2$ (MW = 194.23).

7.2. Biology

In vitro and in vivo testing in guinea pigs with SCI was used to test for a given compound's ability to restore conduction in injured spinal cord white matter. 4-AP was used as a comparator compound. These procedures, along with construction of the double sucrose gap chamber, have been thoroughly described previously [40–42,48–50] and were carried out in accordance with policies set forth by the United States' National Institutes of Health. Herein are brief but specific details of the double sucrose gap recording, SSEP and surgery regimes.

7.2.1. In vitro testing: double sucrose gap recording

Fig. 5 shows the double sucrose gap recording chamber. The entire spinal cord of a guinea pig is quickly dissected from the deeply anesthetized animal, cut into 40–45 mm strips of predominately white matter and placed in aerated Krebs solution. There are three large compartments which contain different bathing media: a central one in which oxygenated Krebs solution is continuously pumped and withdrawn (by aspiration) and two end chambers filled with isotonic KCl. The length of the spinal cord spans all three chambers along with two small reservoirs filled with isotonic sucrose which is continuously perfused at a rate of 1 ml/min. Stimulation at one end of the spinal cord produces CAPs that are conducted through the white matter to be recorded at the other end of the chamber.

Typically, the spinal cord is allowed to stabilize in the recording chamber for about 1 h in order to produce a characteristic response to electrical stimulation. Subsequently the

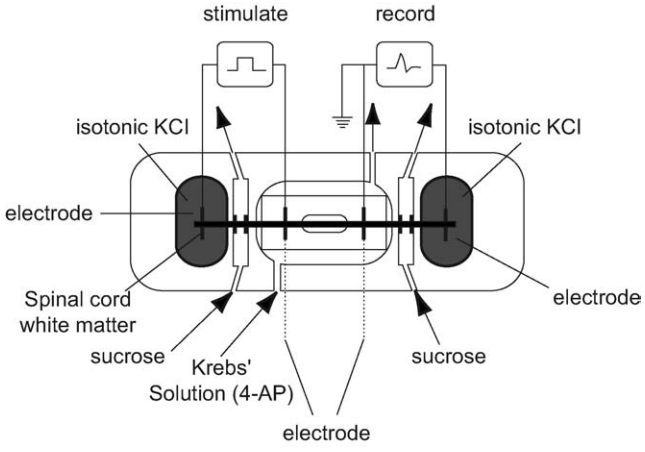

Fig. 5. Double sucrose gap recording and isolation chamber. The isolated spinal cord (stippled band) is shown mounted in the chamber, with the central well continuously perfused with oxygenated Krebs solution (similar to extracellular fluid). The test drugs were added to this chamber. The two ends of the spinal cord lie in separate wells filled with isotonic KCl (similar to intracellular fluid) divided from the central well by narrow gaps filled with isotonic sucrose solution. Electrodes were formed from Ag/AgCl wires. Action potentials were generated through a pair of electrodes at the left hand sucrose gap, conducted through the central part of the spinal cord and recorded by another pair of electrodes in the right-hand gap by conventional bridge amplification techniques. Injury to the cord is performed at its approximate mid-point, within the central chamber.

cord was injured by stretching which is described in detail in previous publications [41,51]. The injury induces a transitory loss in CAP propagation across the lesion, which generally improves with time. Once spontaneous recovery produces a stable "recovery CAP", drug is added to the medium bathing the central chamber. Recording of CAPs is continuous, however about 0.5 h is required for the drug induced changes in amplitude to stabilize. The response to the drug is reported as an increase (or decrease) in the "pre-drug" recovered potential (which is normalized to 100%). Subsequently the drug is washed out of the chamber, and the cords lesion exposed to fresh aerated Krebs solution for approximately 1 h prior to obtaining "post-drug" electrical recordings.

7.2.2. In vivo testing

Large (>350 g) adult female guinea pigs were sedated (see Section 7.2.3 for details) and a normal regimen of SSEP testing was carried out in the intact animal. This included recording EPs from the sensorimotor cortex in response to stimulation of the median nerve of the contralateral fore limb and the median nerve of the contralateral hind limb. After these baseline records were obtained, animals were deeply anesthetized, the spinal cord surgically exposed and crushed by a standardized technique. Within an hour, a second set of electrical records was obtained, demonstrating the loss of conduction of EP conduction through the fresh lesion. It is important to first establish that the neural circuit above the level of the crush injury was intact and that normal SSEPs could be recorded subsequent to medial nerve stimulation in this animal, which constitutes an internal control on SSEP recording procedures (Fig. 6A).

After these records were obtained, the animals recovered from anesthesia and were maintained for 1 week. Another set of EPs were recorded to establish the extent of the remaining conduction deficit ~ 1 week post injury (Fig. 6D). After these records were taken, animals were administered either 4-AP, which was used as a standard, or one of the derivatives by gavage. The effect that the drugs had on conduction was then monitored at $t = 30$ min, 1 h, and then hourly up to 4 h. After the period of recording was concluded, the animals were allowed to recover and then were returned to their cages. A final set of records was obtained the next day (~18 h later) then the animal was euthanized.

7.2.3. Surgery

The same method of producing a standardized compression lesion to the exposed guinea pig spinal cord has been employed in these labs for over a decade. A constant displacement injury is favored over a constant impact as a means to reduce the variability of behavioral and anatomical consequences of the injury. For details, the interested reader is referred to previously published procedures (see [24,49]).

Briefly: Adult guinea pigs (> ~ 350 g; Hartley Strain) were anesthetized by an intramuscular injection of 100 mg/kg ketamine HCl and 20 mg/kg xylazine. A dorsal laminectomy procedure exposed the spinal cord at about the 12th thoracic vertebral level (T12) to the first lumbar level (L1). The exposed cord was crushed using a specially modified forceps possessing a détente. To immobilize animals for electrical records, a more gentle sedation was produced by intramus-

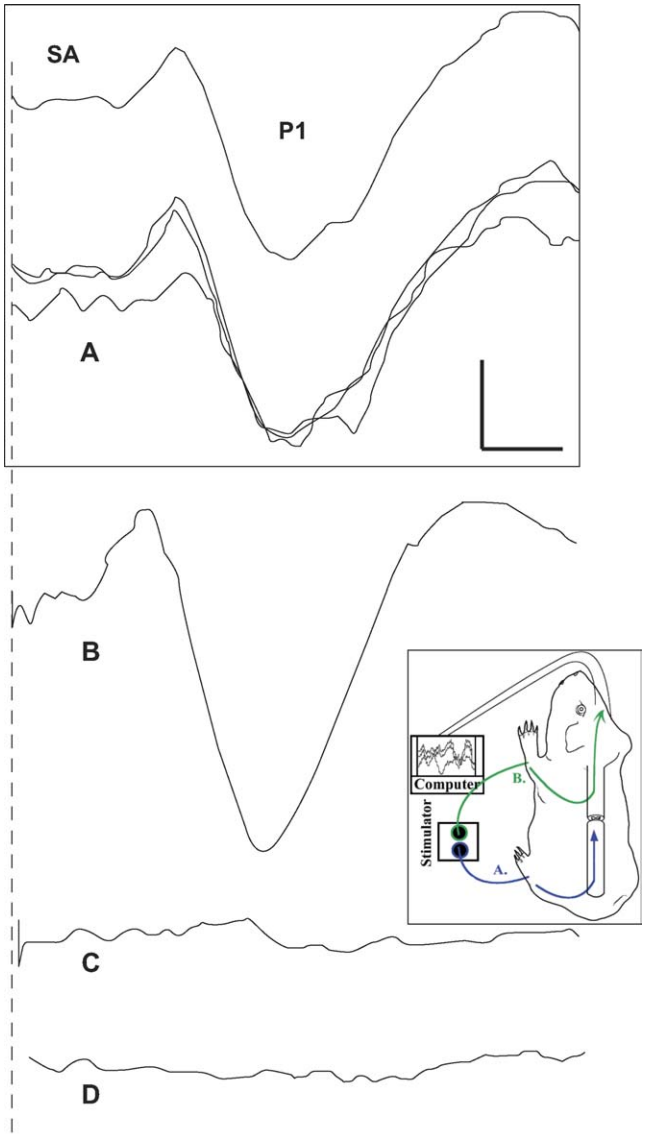

Fig. 6. Measurement of SSEPs in the sedated guinea pig. Record A is an unedited electrical record of early arriving EPs ascending the spinal cord in response to stimulation of the tibial nerve (peak 1; P 1). The bottom three traces are individual trains of the 200 stimulations. The single trace above, along with records B–D, is the averaged waveform. This record was obtained from a sedated guinea pig prior to spinal cord compression. In B, the waveform, taken 1 h after spinal cord compression, shows a control stimulation of the medial nerve of the forelimb in the same animal. Note the large amplitude of the early arriving EP. In C, the complete loss of SSEP conductance through the spinal cord lesion is revealed subsequent to stimulation of the tibial nerve of the hind limb at the same time and in the same animal. This complete loss in the ability of ascending volleys of CAPs to be conducted through damaged spinal cord white matter is still obvious l week later in this animal (D). Such records are characteristic of all animals used in this report. The drawing of the guinea pig shows the control circuit activated by stimulation of the medial nerve (in green). The neural circuit (in blue) activated by stimulation of the tibial nerve of the hind limb is shown to be interrupted by damage to the spinal cord.

cular injection (0.1 cm^3 sodium pentobarbital, 50 mg/ml). At the end of the study, while the animals were under anesthesia, the guinea pigs were euthanized by increasing the anesthetic dose significantly, followed by perfusion/fixation (glutaraldehyde in phosphate buffered Ringer's solution).

7.2.4. SSEPs

It is the loss of nerve impulse conduction through the white matter of the spinal cord lesion that is associated with the catastrophic deficits in behavior observed in SCI [1]. These volleys of compound nerve impulses ascending and descending the spinal cord are associated with numerous axons and synapses, and are referred to as EPs when stimulated synchronously by electrical activation of a compound nerve of the lower or upper limbs (in the SSEP) or activation of the cortex (during motor EP recording, or MEPs, not performed here). This form of stimulation of largely ascending impulses and then recording them at the contralateral sensorimotor cortex of the brain is referred to as SSEP testing.

Stimulation of the tibial nerve of the hind limb was accomplished using a pair of needle electrodes inserted near the nerve at the popliteal notch of the knee. Similar SSEPs were evoked by stimulation of the median nerve of the forelimb with pair of stimulation electrodes. Recording electrodes were located in the scalp covering the contralateral cortex region, with an indifferent electrode usually located in the pinna of the ear.

A complete electrical record for one period of investigation involved stimulating the nerve 200 times in a train (2 mA square wave, 200 μs duration at 3 Hz). Three to four sets of these records were then averaged to produce a single waveform for quantitative study. Recording and stimulation used a Nihon Kohden Neuropak 4 and a Power Mac G-3 computer.

Quantitative evaluation involved measurements of latency from the initiation of stimulation (noted by the stimulus artifact) to the initiation of the EP, however, the most reliable and informative comparative data involved a determination of the area beneath the EP in pixels using IPLab™ spectrum software (Scanalytics, Farifax, VA). These areas beneath the peak waveform (that is above baseline) were normalized by dividing the post injury (or post treatment) EP by the area of the initial EP recorded in the normal animal. If all (100%) nerve fibers normally fired in synchrony by the stimulation regimen before injury were theoretically recruited into conduction after a treatment, the average SSEPs should thus reach unity (1.0).

7.2.5. Drug administration

Drugs were administered by gavage using the following methods. All tested drugs were introduced, in solution form, into the guinea pig stomach using a round tipped feeding needle (either 18 gauge, 55 mm length or 16 gauge, 75 mm length; Fine Science Tools). Gavage is only carried out on the sedated animal. In guinea pigs, the soft palate is continuous with the base of the tongue with only a small opening to pass the tube. The feeding needle is advanced between the incisors and the beginning of the molars, which initiates a swallowing or gag reflex, facilitating advancement of the feeding needle to the stomach. Prior to this operation, the correct needle (sized relative to the size of the guinea pig) is marked by placing its end adjacent the animal's last rib and marking the proximal portion of the needle near the tip of the nose. This provides an estimate of the required length to advance to the stomach during gavage. The needle can be connected to either a syringe or an aspirator allowing material to be introduced into, or withdrawn from, the stomach.

A starting dosage for guinea pigs was estimated by working within the range of 4-AP given in clinical cases of paraplegia in dogs (0.5–1.0 mg/kg body weight) [24]. In the case of an approximately 500 g guinea pig, this would result in a total dosage of about 0.25 mg. A stock solution for gavage was prepared where 0.2 cm^3 of an aqueous solution contained 0.2 mg of drug. This allowed the relative standardization of total concentration given to animals, and was facilitated by the lack of significant variation in their weights (for the 10 animals that were tested with 4-AP the mean weight was 421.5 ± 20.9 g; for methyl carbamate **9** the mean weight was 411.6 ± 23 g; $P \geq 0.5$). The effective dosage for 4-AP was sufficient to produce an improvement in the electrophysiological record equal to 0.47 ± 0.04 mg/kg. By comparison, the effective dosage for ethyl carbamate **10** appeared to be slightly lower (0.37 ± 0.2 mg/kg) but this difference was not statistically different given the small sample size ($P \geq 0.05$).

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