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Hydroxycinnamic acid amide derivatives of polyamines reverse spermine-induced CNS excitation

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Abstract:

The aim of this study was to examine the acute effect of a range of novel hydroxycinnamic acid derivatives of spermine on the development of spermine-induced CNS excitation and convulsions in female Laca mice, and to assess the chronic adverse behavioural effect profile of these compounds over a 5 day period. Four of the six novel polyamine analogues dose-dependently inhibited body tremor and tonic convulsions caused by spermine, when administered centrally (icv) or peripherally (ip). BU43b was the most potent analogue tested, but BU31b, 33b, 36b were also effective ($p < 0.01$, Mann-Whitney U test). A similar profile of effectiveness was seen with peripheral and central administration, indicating that the analogues may cross the blood brain barrier. More chronic investigation of the adverse effects of the compounds administered alone over 5 days of observation indicated that the drugs were well tolerated, only causing reduced locomotor activity on the first day of the study and mild changes in behaviours linked to CNS and ANS function. It is likely that NMDA receptor NR2B subunit inhibition is involved in the anticonvulsant effect of these novel analogues, but other mechanisms may also be involved. These novel polyamine derivatives warrant further investigation of their therapeutic potential in treating epilepsy and CNS disorders where excitotoxicity is implicated.

Key words: Spermine; Novel hydroxycinnamic acid polyamine derivatives; BU43b; NMDA receptor; Behavioral observation; Tremor

1. Introduction

The polyamines are a family of di-, tri- and tetra-amines formed by the decarboxylation of ornithine. They are found in varied concentrations in different tissues in the body, and different regions of the brain (Shaw *et al.*, 1973). The polyamines are known to have several broad physiological functions, such as involvement in the growth, differentiation and death of cells. In the central nervous system, polyamines are involved in neurogenesis and in the maintenance of cell membrane excitability (Igarashi *et al.*, 2010; Malaterre *et al.*, 2004). Many of these functions are as a result of interactions with biomolecules, including ion channels that have been shown to have numerous physiological functions. The polyamines can interact with many receptor types, including ionotropic glutamate receptors (Williams, 1997). Their interaction with N-methyl-D-aspartate (NMDA) receptors has received particular attention. The NMDA receptor family is made up of three different subunit groups, NR1, NR2 and NR3 with the NR1 and NR2 subunits being the best characterised (Kew *et al.*, 2005). Through the arrangement of its subunits, the NMDA receptor has numerous binding/modulatory sites, including at least two polyamine binding sites (Williams, 1997).

Spermine has several effects on NMDA receptors, including glycine-dependent potentiation, a voltage-independent and glycine-independent potentiation and a voltage-dependent blockade (Benveniste *et al.*, 1993; Rock *et al.*, 1992). The effect of spermine which predominates depends to some extent on the pH (physiological or altered), the spermine concentration and the glycine concentration. However, at physiological concentrations, spermine will potentiate the activation of the NMDA receptor (Hackman *et al.*, 2010; Kirby *et al.*, 2005; Williams *et al.*, 1990).

It has been well established that the central delivery of spermine will result in CNS excitation. This excitation consists of two phases, the most significant of which appears to be the second phase (Doyle *et al.*, 1998; Doyle *et al.*, 1996; Kirby *et al.*, 2004a; Kirby *et al.*, 2005). This phase commences approximately 2 hours after injection, and is characterised by CNS excitation presenting as a tremor. The tremor increases in intensity with time, culminating in a fatal tonic convulsion, usually within 6-8 hours, unless effectively inhibited (Doyle *et al.*, 1998; Doyle *et al.*, 1996). Furthermore, over-expression of spermidine/spermine N1-acetyltransferase, (a polyamine degradation enzyme, thus resulting in reduced polyamine levels), has been shown to reduce susceptibility to seizures by elevating the pentylenetetrazol-induced seizure threshold in mice (Kaasinen *et al.*, 2003). This further implicates the polyamines in the development of CNS excitation and convulsions. While these excitatory effects of spermine are well recognized (Anderson *et al.*, 1975), their underlying mechanism still remains to be fully understood, though there is evidence of the involvement of NMDA receptors and L-type calcium channels (Doyle *et al.*, 2005).

Many compounds have been investigated for an ability to reduce convulsions through an action on the NMDA receptor, and some promise has been shown by novel polyamine analogues. Many of the spider and wasp toxins are polyamine conjugates, some of which are active at glutamate ion channels (Blagbrough *et al.*, 1994; Moya *et al.*, 1996). N¹-Dansyl-spermine is a lipophilic polyamine sulfonamide and has a simple structure related to the polyamine amide spider and wasp toxins (Seiler *et al.*, 1998). N¹-Dansyl-spermine was shown to be particularly effective at

blocking spermine-induced CNS excitation and also, in a different model, spermine enhancement of NMDA-induced CNS excitation (Kirby *et al.*, 2005). Other polyamine analogues have been synthesised and many have been tested *in vitro* for NMDA antagonistic activity. Toluenesulfonamide derivatives have been developed as water soluble NMDA antagonists and shown to be effective *in vitro* and to possess neuroprotective effects *in vivo*, similar to N¹-Dansyl-spermine (Kirby *et al.*, 2004b; Li *et al.*, 2004; Li *et al.*, 2005; Masuko *et al.*, 2012; Masuko *et al.*, 2010).

The hydroxycinnamic acid amides of polyamines, which are found in many plants are also similar in structure to the acylated polyamines found in spider and wasp toxins (Blagbrough *et al.*, 1992). As a result, these compounds have been shown to be effective NMDA antagonists *in vitro* and have also been shown to have neuroprotective activity following cerebral ischaemia, also thought to be through NMDA receptor antagonist activity (Fixon-Owoo *et al.*, 2003; Li *et al.*, 2006).

The aims of this study were to examine the effect of a range of putative polyamine/NMDA antagonists, all of which were hydroxycinnamic acid derivatives of spermine, on the development of spermine-induced CNS excitation in female Laca mice, and to assess the chronic adverse behavioural effect profile of these compounds over a 5 day period. The analogues were administered directly into the brain by the intracerebroventricular route, and were also given intraperitoneally to assess their ability to influence CNS excitation and behaviour having crossed the blood brain barrier.

2. Materials and Methods

2.1 Animals

Female *Laca* mice (20-25g) were housed in groups of 4-6 during the experiment with food and water available *ad libitum*. A 12-hour light:dark cycle was maintained (light hours 07.00 – 19.00) and all testing was carried out during the light phase. All experiments received ethical approval (Trinity College, Dublin) and were conducted under licence from the Department of Health and Children, in accordance with the requirements of the Cruelty to Animals Act, 1876 as modified by the European Community Directive, 86/609/EC.

2.2 Materials

All of the novel polyamine analogues (BU31b, BU 33b, BU36b, BU37b and BU43b) were provided by Dr. Jeffrey Atkinson, Brock University, Canada. (Figure 1). All compounds were dissolved in 0.9% sterile saline (with the addition of minimal Tween 80 to aid dissolution in two cases – BU31b & BU37b) for administration by either the intracerebroventricular (icv) or intraperitoneal (ip) routes. Spermine tetrahydrochloride was obtained from Sigma, UK and was dissolved in 0.9% sterile saline for icv administration.

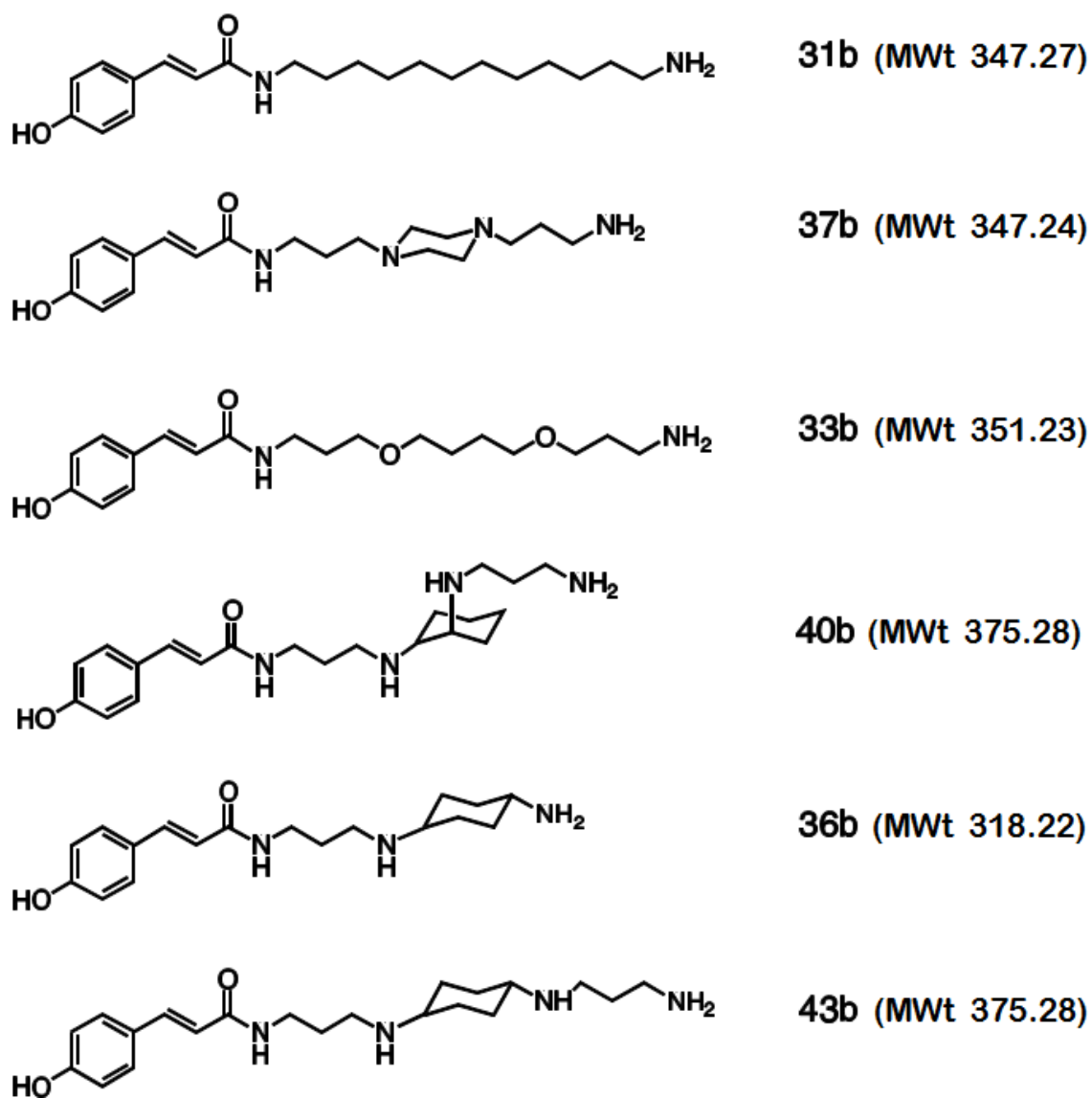


Figure 1: Illustration of the chemical structures and molecular weights of the hydroxycinnamic acid amide derivatives of polyamines utilised in this study.

2.3 Spermine-induced CNS excitation

Spermine (100µg in 20µl) dissolved in 0.9% sterile saline was administered, in a single injection, through the intracerebroventricular (i.c.v) route, directly into the left cerebral ventricle using the method described by Brittain (Brittain, 1966). This results in a characteristic behavioural profile that has been previously described in detail

(Doyle *et al.*, 1998; Doyle *et al.*, 1996; Kirby *et al.*, 2004a). Control animals administered saline behaved completely normally within 10 minutes of icv injection.

The novel polyamine analogues were either co-administered with the spermine (in the same single injection), by the icv route, or were given by ip injection 30 minutes prior to the icv spermine.

The assessment began 2 hours after the spermine administration and continued, at 30-minute intervals, for a further 5.5 hours at which point the experiment was terminated. During this period, CNS excitation is manifest as a body tremor, which increases in intensity with time, culminating in fatal tonic convulsions (Doyle *et al.*, 1996). A scoring system was established to monitor the degree of body tremor over this period. The scoring system used was 1: slight tremor, 2: moderate tremor, 3: severe tremor, 4: tonic convulsion - survived, 5: fatal tonic convulsion. Assessment of the degree of tremor was carried out by an experimenter blind to the administered treatment.

2.4 General behavioural observation

Locomotor activity and rotarod performance were assessed daily for 5 days, as were the animals weight and body temperature (rectal). In addition, mice were examined, by an observer blind to any treatment, for other behavioural abnormalities using a structured method similar to that described by Irwin and the SHIRPA protocol (Irwin, 1968; Rogers *et al.*, 1997).

Novel compounds were administered directly into the cerebral ventricles of the mice (icv) in a 20 µl volume or ip in a volume of 0.1ml/10g body weight. The highest doses from the spermine-induced CNS excitation study were used. Directly after administration of the novel compound, mice were placed in the locomotor apparatus and spontaneous locomotor activity was recorded for 1 hour. Mice were then immediately assessed for rotarod performance and for behavioural deficits using the behavioural observation assessment tool. The battery of tests was repeated on day 3 and 5. Body temperature and body weight were monitored daily.

2.4.1 Spontaneous locomotor activity (LMA) test

The mice were placed individually in an activity monitor, (AM1051 Activity Monitor, Benwick Electronics, Essex, UK), which consisted of 5 clear Perspex boxes, 42cm length x 21cm width x 20cm depth, with a caged top. Each of the boxes was positioned within a frame, equipped with infrared beams along the length and width of the frame. LMA was recorded automatically electronically on a computer by counting the number of breaks in the beam every 5 minutes over a 1 hour duration.

2.4.2 Motor Coordination – Rotarod assessment

The modified rotarod assessment consisted of examining balance and co-ordination when placed on a rotating rod (Palmer Recording Drum, UK) at two different speeds (2 or 4 revolutions per minute) (as described in (Li *et al.*, 2004)). Once the animal was balanced on the rod, the drum was switched on at the lower speed for one minute. If the animal maintained its balance at this speed, the speed was increased

for a second minute. At any stage during these two minutes if the animal fell off the rod the experiment was terminated and the time at which it fell was recorded. The time totals were added together as the final result for each mouse on that day (maximum of 120 seconds).

2.4.3 Behavioural observation schema:

The Irwin profile-based assessment tool quantified the following range of behaviours linked to CNS function: alertness, stereotypy, vocalisation, pelvic elevation, grooming, tail elevation, tremor and convulsion. In addition, the following ANS linked behaviours were also quantified: salivation, piloerection, palpebral closure, body posture, limb tone, abnormal gait and writhing.

Most notable changes were observed on the following behaviours: vocalisation, pelvic elevation, tremor, limb tone, body posture and abnormal gait.

The following scoring systems were used to quantify these behaviours:

Vocalisation: 0 none; 1 provoked during handling; 2 spontaneous vocalisation.

Pelvic elevation: 0 marked flattening; 1 pelvis touches observational plate floor; 2 normal (3mm pelvic elevation); 3 enhanced elevation.

Tremor: 0 none; 1 slight tremor; 2 moderate tremor; 3 severe tremor.

Limb tone: 0 no resistance; 1 slight resistance; 2 moderate resistance; 3 marked resistance (normal); 4 extreme resistance.

Body posture: 0 completely flattened; 1 lying on side; 2 lying prone; 3 sitting or standing (normal); 4 rearing on hind legs; 5 repeated vertical leaping.

Abnormal gait: 0 normal; 1 fluid movement but staggers; 2 limited movement only (staggers slowly); 3 incapacitated.

2.5 Data analysis

Spermine-induced CNS excitation: The median CNS excitation scores and interquartile ranges (IQR) were calculated. Results are shown as graphs of median CNS excitation scores versus time (hours). Statistical significance between test and control subjects was assessed by using the Mann-Whitney U-test with a Bonferroni correction for multiple comparisons.

Data for the LMA test and rotarod was expressed as mean and \pm Standard Error of Mean (SEM). Statistical analysis of differences between control and treated groups was carried out using One-Way ANOVA and subsequent *post-hoc* analyses were performed using Tukey HSD.

Scoring systems were used for the behavioural observation assessment. Data was expressed as median and interquartile ranges. Statistical significance between test and control subjects was assessed by using the Mann-Whitney U-test and a Bonferroni correction for multiple comparisons where appropriate.

3 Results

3.1 Spermine-induced CNS excitation, body tremor and tonic convulsions

3.1.1 Effect of novel analogues following icv administration

The initial dose for all of the novel polyamine analogues was 10 μ g/20 μ l which was co-administered with spermine (100 μ g). Depending on the result from using 10 μ g the succeeding doses were either lower or higher. This allowed the experimenter to observe dose-dependent effects.

Several of the hydroxycinnamic amide derivatives (Figure 2) showed dose-dependent effects on the spermine-induced CNS excitation, with BU43b having the most pronounced effects (Figure 2f). There was a significant reduction in the development of both body tremor and tonic convulsions for those mice treated with 31b, 33b, 36b or 43b (Figure 2). Further, there was a reduction in the total number of mice presenting with a tonic convulsion (data not shown). Finally, BU37b and BU40b did not show any significant reduction in CNS excitation, in fact, there was evidence of a small potentiation of the effects of spermine in both cases (Figure 2d & 2e).

Figure 2 (a – f): Effect of novel hydroxycinnamic acid amide derivatives of polyamines on spermine – induced CNS excitation when co-administered by intracerebroventricular injection with spermine (n=6 per group). Results are presented as median CNS excitation over time (hours). Statistical significance was assessed by Mann-Whitney U-test, with a Bonferroni correction for multiple comparisons.

3.1.2 Effect of novel analogues following ip administration

While the doses used in the ip administration were higher than those in the icv administration, the fact that an effect was seen was evidence of an ability of the novel derivatives to cross the blood brain barrier. Indeed, a similar profile of effectiveness was seen with peripheral administration as with central administration; BU43b was the most effective. BU31b, 33b, 36b also significantly reduced the development of CNS excitation caused by spermine, mirroring the effects of icv injection. BU37b and BU40b were ineffective. This provided further evidence of the range of ability of the hydroxycinnamic acid amide derivatives to reverse the spermine-induced CNS excitation (Figure 3 a - f).

Figure 3 (a – f): Effect of novel hydroxycinnamic acid amide derivatives of polyamines on spermine – induced CNS excitation when administered by intraperitoneal injection 30 minutes prior to spermine icv injection (n=6 per group). Results are presented as median CNS excitation over time (hours). Statistical significance was assessed by Mann-Whitney U-test, with a Bonferroni correction for multiple comparisons.

3.2 General behavioral observation over 5 day period

3.2.1 Effects of novel polyamine analogues on Locomotor activity (LMA), rotarod performance, body weight and temperature

In order to ensure the general safety of the novel derivatives a number of tests were performed for a 5 day observation period which would identify generalised behavioural impairment, if it were present.

3.2.1.1 Effect of novel analogues following icv administration

As can be seen in Table 1, there was a significant reduction in locomotor activity for all of the novel compounds on the first day of testing following icv administration. LMA had returned to control levels on day 3 and 5 following treatment (data not shown). None of the novel derivatives tested had any significant effect on rotarod performance on any day of testing, when administered icv (data for day 1 is represented in Table 1).

Table 1 Effect of novel polyamine analogues on locomotor activity and rotarod performance on day 1 of study following icv administration. Data presented is the mean +/- sem on day 1 (n= 6 per group); *p<0.05 vs control.

icv administration		
	LMA	Rotarod performance (seconds)
Control	1234 +/- 284	92 +/- 18
BU 31b	144 +/- 135*	39 +/- 18
BU 33b	291 +/- 83*	82 +/- 19
BU 36b	226 +/- 94*	120 +/- 0
BU 37b	228 +/- 106*	87 +/- 17
BU 40b	166 +/- 80*	120 +/- 0
BU 43b	469 +/- 147*	120 +/- 0

None of the drugs had any significant effect on body temperature. However, BU 31b caused a 15% reduction in body weight in the first 24h following treatment (average body weight at start of study: 23g). BU31b treated mice weighed 87 +/- 4% that of control animals on day 2. The weight loss remained evident on day 5, when animals treated with BU31b weighed 83 +/-4% that of control animals. None of the other drugs tested had a significant effect on body weight (data not shown).

3.2.1.2 Effect of novel analogues following ip administration

BU31b, BU33b and BU37b had no significant effects on any parameter assessed when administered by ip injection, so data is not shown.

Following ip administration, BU36b, BU40b and BU43b had a significant effect on LMA (Table 2). None of the novel derivatives tested had any significant effect on rotarod performance on any day of testing, when administered ip (data for day 1 is represented in Table 2).

Table 2 Effect of novel polyamine analogues on locomotor activity, rotarod performance and body temperature on day 1 of study following ip administration. Data presented is the mean +/- sem on day 1 (n= 6 per group); *p<0.05 vs control.

	ip administration		
	LMA	Rotarod performance (seconds)	Body temperature (°C)
Control	523 +/- 134	120 +/- 0	37.5 +/- 0.15
BU 36b	26 +/- 22*	120 +/- 0	36.6 +/- 0.2
BU 40b	139 +/- 22*	120 +/- 0	36.5 +/- 0.2
BU 43b	10 +/- 4*	120 +/- 0	30.9 +/- 0.4*

One of the novel compounds (BU 43b) caused a pronounced hypothermia on day 1 of the study following ip administration (Table 2). In this case, given the drop in temperature, the mice were placed under a heating lamp.

3.2 General effects of hydroxycinnamic amide derivatives: behavioural observational data

Table 3 contains a summary of the effects of the novel polyamine analogues on measures of behavioural functioning as assessed using the Irwin profile-based observational tool on day 5 of the study. The parameters most affected by the novel drugs were tremor, pelvic elevation, vocalisation, limb tone, body posture and abnormal gait. The profile of symptoms was most pronounced on day 5 (although similar behavioural effects were observed on day 1 and 3). Notably, all polyamine analogues tested caused the development of a low grade tremor that lasted the duration of the experiment (5 days) (Table 3). In addition, ip injection of the analogues caused more significant effects on pelvic elevation, body posture and abnormal gait than administration by the icv route (Table 3).

Table 3 Effects of the novel polyamine analogues on median (IQR) measures of CNS and ANS functioning as assessed using the Irwin profile- based observational tool on day 5 following treatment (n=6 per group); *p<0.05 vs control.

	Tremor	Pelvic elevation	Vocalisation	Limb tone	Body posture	Abnormal gait
Normal	0	2	0	4	3	0
BU31b lcv	1 (0.25)*	2 (1.25)	0 (0)	4 (0.75)	3 (0.5)	0 (0.75)
ip	1 (0.25)*	3 (1.25)	2 (0)*	2 (2)*	3 (0.25)	1 (0)*
BU33b lcv	1 (0.25)*	2 (0)	2 (2)*	4 (0)	3 (0)	0 (0)
ip	1 (0)*	3 (0)*	0 (0.5)	3 (2)	2.5 (1)	1 (0.25)*
BU36b lcv	1 (0)*	2 (0)	0 (0.5)	4 (2)	3 (2)*	1 (1.25)
lp	1 (1)*	3 (0.25)*	0 (0.5)	3 (1)*	2 (1)*	2 (1)*
BU37b lcv	1 (0.25)*	2 (0.25)	1 (2)	4 (0)	2 (0)*	0 (2)
lp	1.5 (1)*	1 (0)*	0 (0)	0.5 (1)*	1.5 (1)*	3 (0)*
BU40b lcv	1 (0.25)*	2 (0)	2 (0.5)*	4 (0)	2 (0)*	0 (0)
lp	2 (1)*	1 (0.75)	0 (2)	3.5 (1)	2.5 (1)	1 (0)*
BU43b lcv	1 (0.25)*	3 (0.25)*	0 (0.5)	2 (0.75)*	3 (0)	0 (0.25)
ip	0 (0.25)	3 (0.25)*	0 (0.5)	3 (1)*	3 (0)	1 (1)*

4 Discussion

Given that early studies showed raised hippocampal spermidine levels in patients with temporal lobe epilepsy (Laschet *et al.*, 1999) and elevation of polyamine metabolism has been seen in kindled rats, (Hayashi *et al.*, 1989) we investigated the inhibitory activity of hydroxycinnamic amide derivatives of the polyamines in the spermine-induced CNS excitation model. The majority of these compounds inhibited, in a dose-dependent manner, the CNS excitation (body tremor and tonic convulsions) caused by spermine.

Recent work with spermidine has highlighted a role for the NR2B NMDA receptor subunit in seizures. While spermidine produces a different profile of CNS excitation following icv administration compared to spermine, Napolini and co-workers demonstrated potentiation of seizures in rats given spermidine and sub-threshold concentrations of pentylentetrazol. This effect was reversed by the NR2B subunit selective antagonist traxoprodil (Napolini *et al.*, 2012).

There is evidence suggesting the involvement of the NMDA receptor in spermine induced tremor and tonic convulsions (Doyle *et al.*, 1996). However, an action solely through the NMDA receptor does not fully explain the behavioural effects caused by spermine and it has been demonstrated that spermine can bind to and interact with many different proteins (Johnson, 1996).

This work examined novel compounds, synthesised in Brock University, which have been shown to exhibit, among others, NMDA and AMPA receptor antagonist activity (Fixon-Owoo *et al.*, 2003). In the present study, BU 43b (both icv and ip) showed

very effective, dose-dependent inhibition of the development of spermine-induced convulsions. This highly effective activity is most likely due to its closer resemblance to the structure of spermine (3-4-3 arrangement of N-atoms) when compared to the other analogues. These results suggested that not only does BU 43b cross the blood brain barrier (as evidenced by its effectiveness ip as well as icv), but that it is also an antagonist of spermine-induced convulsions.

It has previously been proposed that the BU compounds may have biological activity as a result of the fact they are long chain polyamines. It has been posited that the polyamine tail could pass through the ion pore of the receptor and interact with the intracellular site, while the head group lodges in the channel (Fixon-Owoo *et al.*, 2003). This mechanism fits with that proposed from earlier work on polyamine analogues (Chao *et al.*, 1997; Igarashi *et al.*, 1995; Igarashi *et al.*, 1997). In a similar manner to BU43b, BU31b was effective in inhibiting the spermine induced CNS excitation, though it was less effective than BU43b. This is possibly due to the structure of BU31b, as it is of similar length to BU43b, but lacks the internal N-atoms and may therefore bind with less affinity or specificity.

In an *in vitro* study by Fixon-Owoo, these novel polyamine analogues were shown to have a high level of specificity for glutamate receptors and in particular the NMDA receptor. BU43b and BU31b showed similar high level of inhibition of NR1/NR2B NMDA receptor subunits (see Table 4, in part adapted from Fixon-Owoo, 2003). However, the effectiveness of the analogues cannot be completely explained by differences in inhibition of NMDA receptors. In our study, BU36b and BU33b were similarly effective to BU31b at inhibiting the development of spermine-induced CNS

excitation, but their ability to inhibit NMDA receptors is markedly less in vitro. In fact, BU33b was shown to have a negligible inhibitory effect on NMDA receptors in vitro (Table 4). It is also noticeable that BU40b, which was not effective our in model, exerts moderate inhibition of NMDA receptors in vitro (Table 4).

Table 4 (a) Summary of the relative effectiveness of the novel compounds at inhibiting spermine induced CNS excitation and tonic convulsions, and (b) relative inhibitory effect at NMDA and AMPA receptors in vitro (adapted from Fixon-Owoo, 2003)

Relative inhibitory effectiveness vs spermine excitation and tonic convulsions		In vitro NMDA receptor inhibition	In vitro AMPA receptor inhibition
BU31b	++	+++	-
BU33b	++	-	-
BU36b	++	+	-
BU37b	-	-	-
BU40b	-	+	-
BU43b	+++	+++	++

+++: most effective; ++ very effective; -: ineffective

+++: highly effective (>90% inhibition); +: moderate effect (25-60% inhibition); -: low effect (<20% inhibition) (adapted from Fixon-Owoo, 2003)

It is notable that BU43b showed a greater inhibition of AMPA subunits in vitro than any of the other analogues tested (Fixon-Owoo *et al.*, 2003), which may also be a possible explanation for the lesser effectiveness of the other analogues. Indeed, a number of groups have shown the effects of polyamine analogues on AMPA receptors and seizures. Takazawa *et al.* (Takazawa *et al.*, 1996) showed that a Joro spider analogue (1-naphthylacetyl spermine) had an anticonvulsant effect against amygdala-kindled seizures in rats, which was mediated through calcium-permeable AMPA receptors. Similarly, Masuko *et al.* (Masuko *et al.*, 2009) showed that tosyl-polyamine derivatives were able to inhibit currents in calcium permeable AMPA receptors. Therefore, this may, at least partially, explain the difference in effectiveness of the novel analogues.

NMDA antagonists have previously been shown to be effective in the spermine CNS excitation model used here, so have L-type calcium channel antagonists (Doyle *et al.*, 2005; Doyle *et al.*, 1996). Doyle *et al.* showed that verapamil, nisoldipine and nitrendipine inhibited spermine-induced CNS excitation in this model (Doyle *et al.*, 2005). This supports earlier work showing allosteric modulation of dihydropyridine binding to the L-type calcium channels by polyamines (Schoemaker, 1992). This, however, does not rule out a role for NMDA receptors, or indeed AMPA receptors, and it is most likely that a chain of events occurs resulting in CNS excitation and that a number of different ion channels and mediators are involved.

Furthermore, as the novel compounds were only examined versus glutamatergic receptors, one cannot rule out the possibility that they may act through other

receptor systems as well. Certainly given the varied roles of the polyamines, including effects on potassium channels and TRPV capsaicin receptors, this seems plausible (Ahern *et al.*, 2006; Lopatin *et al.*, 1994). The polyamines have been shown to be responsible for the block of inward rectifier potassium channels (Kir2.1) and, as such, play a role in membrane excitability (Lopatin *et al.*, 1994). Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification (Baronas *et al.*, 2014). Given that retigabine, a novel anti-epileptic, works through potassium channels (albeit different ones) to affect the M current, it is possible that the novel analogues examined here could exert an effect through potassium channels (Orhan *et al.*, 2012). Therefore, potential activity of the novel compounds at sites other than glutamatergic receptors or calcium channels cannot be conclusively ruled out.

These novel compounds, synthesised in Brock University, have to date only been subject to limited study of their biological effects. Fixon-Owoo *et al.* (Fixon-Owoo *et al.*, 2003) studied the compounds in insects, where it was found that they showed no notable toxicity. Our work has further demonstrated the relative safety and lack of adverse behavioural effects of the compounds. There was no evidence of hyperactivity (similar to that produced by MK-801), which may have been expected given that these compounds are putative polyamine/NMDA antagonists (Whishaw *et al.*, 1989). Other than reduced locomotor activity on the first day, the impact of the derivatives on behaviour was unremarkable. Particularly important was the lack of effect on motor coordination, demonstrated with the use of the rotarod.

It has been demonstrated that spermidine, following icv administration, produces a flaccid paralysis that develops over a number of days (Anderson *et al.*, 1975; Doyle *et al.*, 1994). The impact on pelvic elevation, limb tone, body posture and abnormal gait seen in this study shows the compounds may also possess some spermidine-like activity. However, the normal results on the rotarod, demonstrating normal motor coordination, is encouraging and highlights the low level of motor side effects of these drugs. In a previous study investigating the effects of the polyamine analogues and putative competitive antagonists, arcaine, 1,10-diaminodecane and DET in vivo, a similar finding of agonist-like activity was noted (Doyle *et al.*, 1998). Furthermore, the development of a lasting low grade tremor following the administration of the novel analogues is suggestive of a spermine-like proconvulsant effect. It is noteworthy that BU37b and BU40b did not have any significant inhibitory effect on spermine-induced CNS excitation following ip administration and in fact caused a marginal exacerbation of the development of convulsions following icv delivery, suggesting they possess agonist-like qualities.

In conclusion, four out of six novel derivatives tested significantly inhibited the development of spermine-induced tremor and tonic convulsions, with BU43b standing out as the most potent analogue. It is likely that NR2B subunit inhibition is involved in the anticonvulsant effect of these novel analogues, but other mechanisms may also be involved.

Furthermore, these results demonstrate that these novel polyamine analogues are well tolerated in mice and that, depending on chemical structure, it is possible for hydroxycinnamic amide derivatives of the polyamines to variably cross the blood

brain barrier. Further investigation of the mechanism of action of the novel compounds is needed, however, their anticonvulsant effects against the spermine-induced CNS excitation, and the earlier work on cerebral ischaemia (Li *et al.*, 2006), suggest that they certainly warrant further investigation in CNS disease models.

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5 References

- Ahern GP, Wang X, Miyares RL (2006). Polyamines are potent ligands for the capsaicin receptor TRPV1. *The Journal of biological chemistry* **281**(13): 8991-8995.
- Anderson DJ, Crossland J, Shaw GG (1975). The actions of spermidine and spermine on the central nervous system. *Neuropharmacology* **14**(8): 571-577.
- Baronas VA, Kurata HT (2014). Inward rectifiers and their regulation by endogenous polyamines. *Frontiers in physiology* **5**: 325.
- Benveniste M, Mayer ML (1993). Multiple effects of spermine on N-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones. *J Physiol* **464**: 131-163.
- Blagbrough IS, Brackley PT, Bruce M, Bycroft BW, Mather AJ, Millington S, *et al.* (1992). Arthropod toxins as leads for novel insecticides: an assessment of polyamine amides as glutamate antagonists. *Toxicon* **30**(3): 303-322.
- Blagbrough IS, Moya E, Taylor S (1994). Polyamines and polyamine amides from wasps and spiders. *Biochem Soc Trans* **22**(4): 888-893.
- Brittain RT (1966). The intracerebral effects of noradrenaline and its modification by drugs in the mouse. *J Pharm Pharmacol* **18**(9): 621-623.
- Chao J, Seiler N, Renault J, Kashiwagi K, Masuko T, Igarashi K, *et al.* (1997). N1-dansyl-spermine and N1-(n-octanesulfonyl)-spermine, novel glutamate receptor antagonists: block and permeation of N-methyl-D-aspartate receptors. *Mol Pharmacol* **51**(5): 861-871.
- Doyle KM, Kirby BP, Murphy D, Shaw GG (2005). Effect of L-type calcium channel antagonists on spermine-induced CNS excitation in vivo. *Neurosci Lett* **380**(3): 247-251.
- Doyle KM, Shaw GG (1998). Investigation of the actions and antagonist activity of some polyamine analogues in vivo. *Br J Pharmacol* **124**(2): 386-390.
- Doyle KM, Shaw GG (1996). Investigation of the involvement of the N-methyl-D-aspartate receptor macrocomplex in the development of spermine-induced CNS excitation in vivo. *Br J Pharmacol* **117**(8): 1803-1808.

Doyle KM, Shaw GG (1994). The mechanism of the neurotoxic effects of spermidine. *Biochem Soc Trans* **22**(4): 386S.

Fixon-Owoo S, Levasseur F, Williams K, Sabado TN, Lowe M, Klose M, *et al.* (2003). Preparation and biological assessment of hydroxycinnamic acid amides of polyamines. *Phytochemistry* **63**(3): 315-334.

Hackman JC, Holohean AM (2010). The effects of polyamine agonists and antagonists on N-methyl-D-aspartate-induced depolarizations of amphibian motoneurons in situ. *Brain Res* **1325**: 10-18.

Hayashi Y, Hattori Y, Moriwaki A, Saeki K, Hori Y (1989). Changes in polyamine concentrations in amygdaloid-kindled rats. *J Neurochem* **53**(3): 986-988.

Igarashi K, Kashiwagi K (2010). Modulation of cellular function by polyamines. *Int J Biochem Cell Biol* **42**(1): 39-51.

Igarashi K, Koga K, He Y, Shimogori T, Ekimoto H, Kashiwagi K, *et al.* (1995). Inhibition of the growth of various human and mouse tumor cells by 1,15-bis(ethylamino)-4,8,12-triazapentadecane. *Cancer Res* **55**(12): 2615-2619.

Igarashi K, Shirahata A, Pahk AJ, Kashiwagi K, Williams K (1997). Benzyl-polyamines: novel, potent N-methyl-D-aspartate receptor antagonists. *J Pharmacol Exp Ther* **283**(2): 533-540.

Irwin S (1968). Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacologia* **13**(3): 222-257.

Johnson TD (1996). Modulation of channel function by polyamines. *Trends Pharmacol Sci* **17**(1): 22-27.

Kaasinen SK, Grohn OH, Keinanen TA, Alhonen L, Janne J (2003). Overexpression of spermidine/spermine N1-acetyltransferase elevates the threshold to pentylenetetrazol-induced seizure activity in transgenic mice. *Exp Neurol* **183**(2): 645-652.

Kew JN, Kemp JA (2005). Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl)* **179**(1): 4-29.

Kirby BP, Ryder SA, Seiler N, Renault J, Shaw GG (2004a). N1-dansyl-spermine: a potent polyamine antagonist. *Brain Res* **1011**(1): 69-73.

Kirby BP, Shaw GG (2005). Effect of spermine and N1-dansyl-spermine on epileptiform activity in mouse cortical slices. *Eur J Pharmacol* **524**(1-3): 53-59.

Kirby BP, Shaw GG (2004b). The neuroprotective effects of N1-dansyl-spermine in the gerbil model of cerebral ischaemia. *Brain Res* **1011**(1): 74-83.

Laschet J, Trottier S, Leviel V, Guibert B, Bansard JY, Chauvel P, *et al.* (1999). Heterogeneous distribution of polyamines in temporal lobe epilepsy. *Epilepsy Res* **35**(2): 161-172.

Li J, Henman MC, Atkinson J, Fixon-Owoo S, Tatlisumak T, Shaw GG, *et al.* (2006). The pre-ischaemic neuroprotective effects of the polyamine analogues BU43b and BU36b in permanent and transient focal cerebral ischaemia models in mice. *Brain Res* **1076**(1): 209-215.

Li J, Henman MC, Doyle KM, Strbian D, Kirby BP, Tatlisumak T, *et al.* (2004). The pre-ischaemic neuroprotective effect of a novel polyamine antagonist, N1-dansyl-spermine in a permanent focal cerebral ischaemia model in mice. *Brain Res* **1029**(1): 84-92.

Li J, Henman MC, Tatlisumak T, Shaw GG, Doyle KM (2005). The pre-ischaemic neuroprotective effects of N1-dansyl-spermine in a transient focal cerebral ischaemia model in mice. *Brain Res* **1055**(1-2): 180-185.

Lopatin AN, Makhina EN, Nichols CG (1994). Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* **372**(6504): 366-369.

Malaterre J, Strambi C, Aouane A, Strambi A, Rougon G, Cayre M (2004). A novel role for polyamines in adult neurogenesis in rodent brain. *Eur J Neurosci* **20**(2): 317-330.

Masuko T, Namiki R, Nemoto Y, Miyake M, Kizawa Y, Suzuki T, *et al.* (2009). Neuroprotection by tosyl-polyamine derivatives through the inhibition of ionotropic glutamate receptors. *J Pharmacol Exp Ther* **331**(2): 522-530.

Masuko T, Suzuki T, Miyake M, Kusama-Eguchi K, Kizawa Y, Tomono K, *et al.* (2012). Antagonism of NMDA receptors by butanesulfonyl-homospermine guanidine and neuroprotective effects in in vitro and in vivo. *Neurosci Lett* **506**(2): 251-255.

Masuko T, Yoshida S, Metori K, Kizawa Y, Kusama T, Miyake M (2010). Synthesis of water-soluble polyamine derivatives effective as N-methyl-D-aspartate receptor antagonists. *Chem Pharm Bull (Tokyo)* **58**(6): 862-867.

Moya E, Blagbrough IS (1996). Efficient syntheses of polyamine and polyamine amide voltage-sensitive calcium channel blockers: FTX-3.3 and sFTX-3.3. *J Pharm Pharmacol* **48**(2): 179-182.

Naspolini AP, Cocco AR, Villa Martignoni F, Oliveira MS, Furian AF, Rambo LM, et al. (2012). Traxoprodil decreases pentylentetrazol-induced seizures. *Epilepsy Res* **100**(1-2): 12-19.

Orhan G, Wuttke TV, Nies AT, Schwab M, Lerche H (2012). Retigabine/Ezogabine, a KCNQ/K(V)7 channel opener: pharmacological and clinical data. *Expert opinion on pharmacotherapy* **13**(12): 1807-1816.

Rock DM, Macdonald RL (1992). The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons. *Mol Pharmacol* **41**(1): 83-88.

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE (1997). Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome* **8**(10): 711-713.

Schoemaker H (1992). Polyamines allosterically modulate [3H]nitrendipine binding to the voltage-sensitive calcium channel in rat brain. *Eur J Pharmacol* **225**(2): 167-169.

Seiler N, Douaud F, Renault J, Delcros JG, Havouis R, Uriac P, et al. (1998). Polyamine sulfonamides with NMDA antagonist properties are potent calmodulin antagonists and cytotoxic agents. *Int J Biochem Cell Biol* **30**(3): 393-406.

Shaw GG, Pateman AJ (1973). The regional distribution of the polyamines spermidine and spermine in brain. *J Neurochem* **20**(4): 1225-1230.

Takazawa A, Yamazaki O, Kanai H, Ishida N, Kato N, Yamauchi T (1996). Potent and long-lasting anticonvulsant effects of 1-naphthylacetyl spermine, an analogue of Joro spider toxin, against amygdaloid kindled seizures in rats. *Brain Res* **706**(1): 173-176.

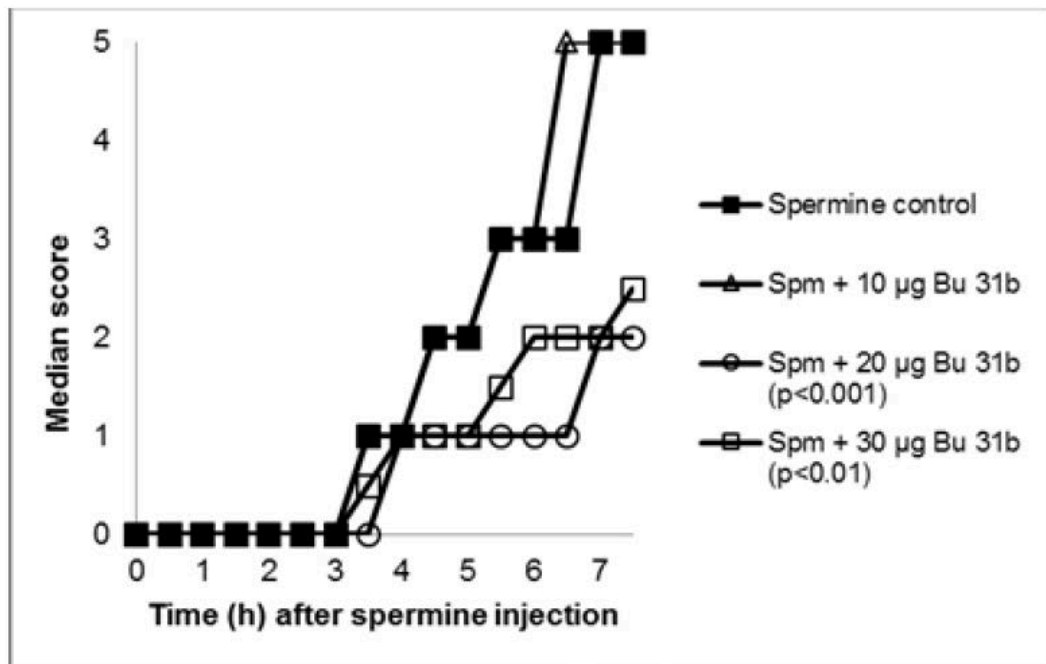
Whishaw IQ, Auer RN (1989). Immediate and long-lasting effects of MK-801 on motor activity, spatial navigation in a swimming pool and EEG in the rat. *Psychopharmacology (Berl)* **98**(4): 500-507.

Williams K (1997). Interactions of polyamines with ion channels. *Biochem J* **325 (Pt 2)**: 289-297.

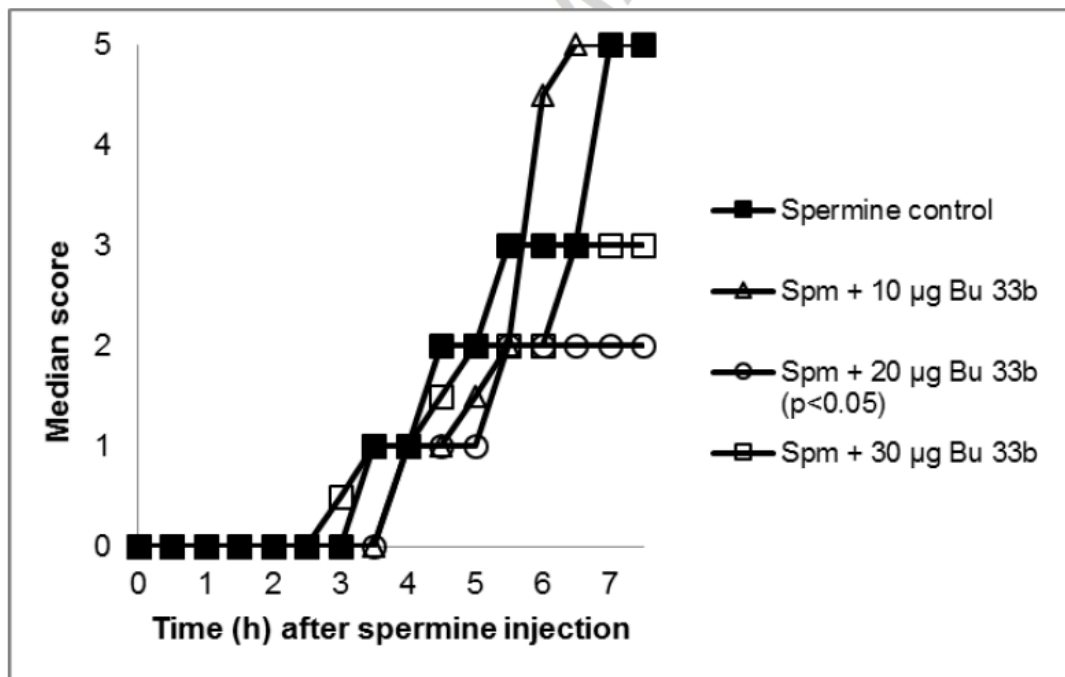
Williams K, Dawson VL, Romano C, Dichter MA, Molinoff PB (1990). Characterization of polyamines having agonist, antagonist, and inverse agonist effects at the polyamine recognition site of the NMDA receptor. *Neuron* **5(2)**: 199-208.

Figure 2

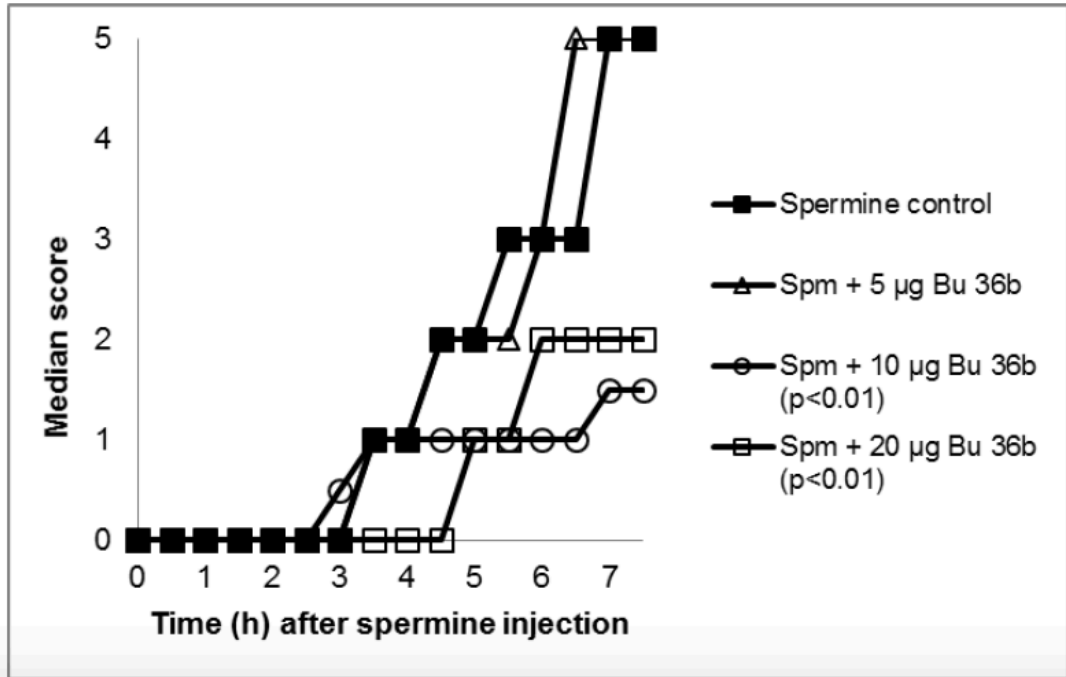
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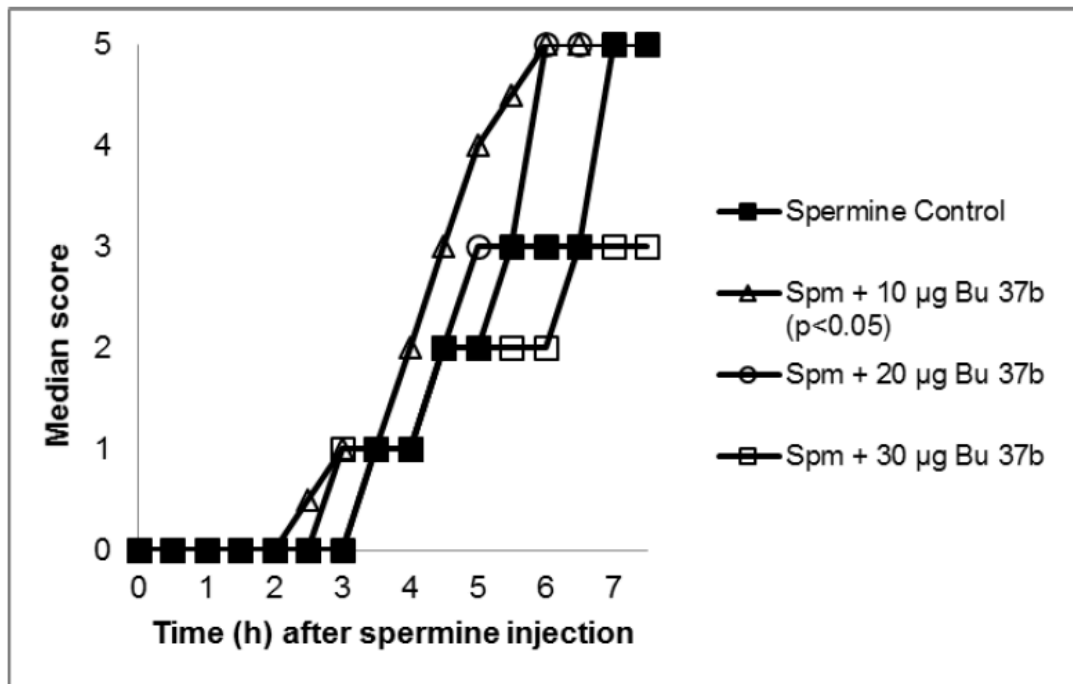
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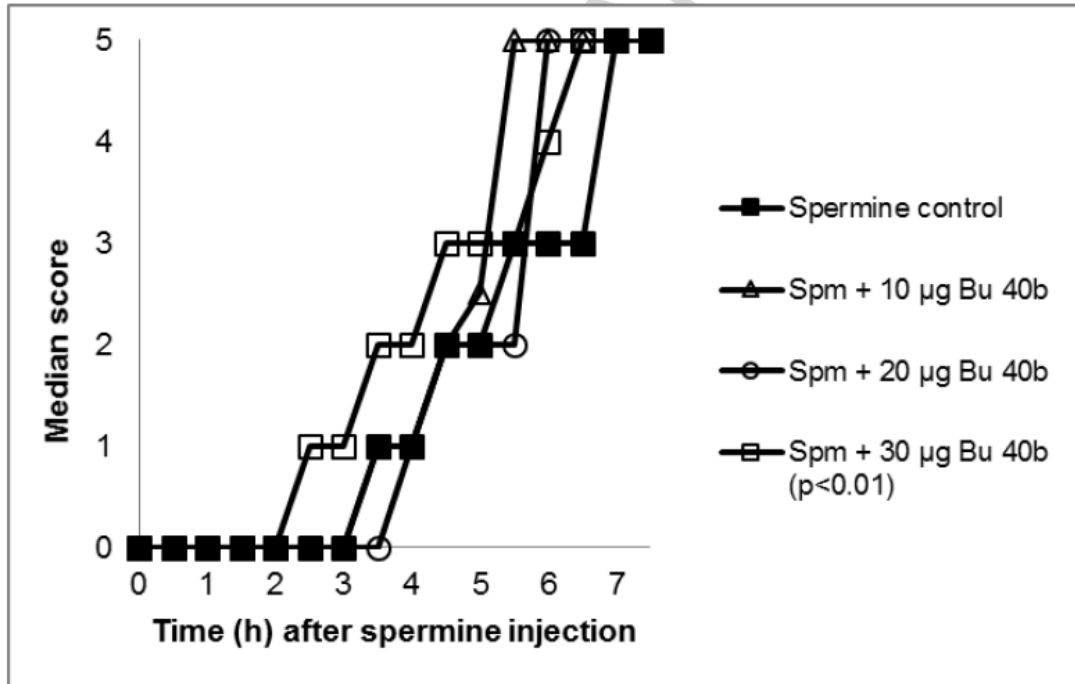
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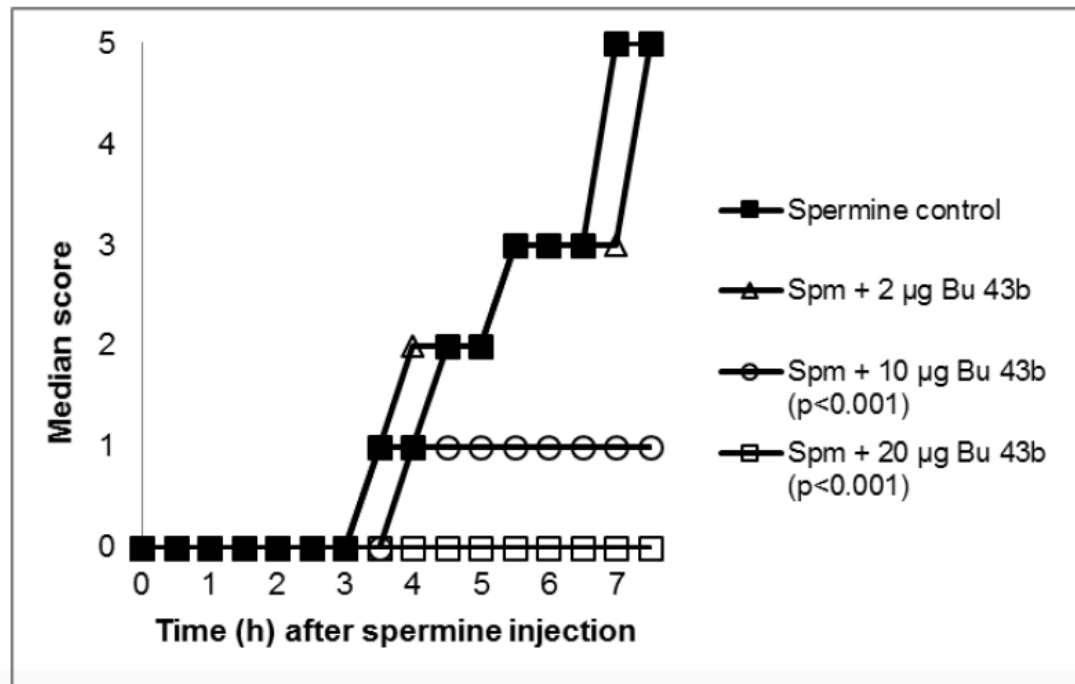
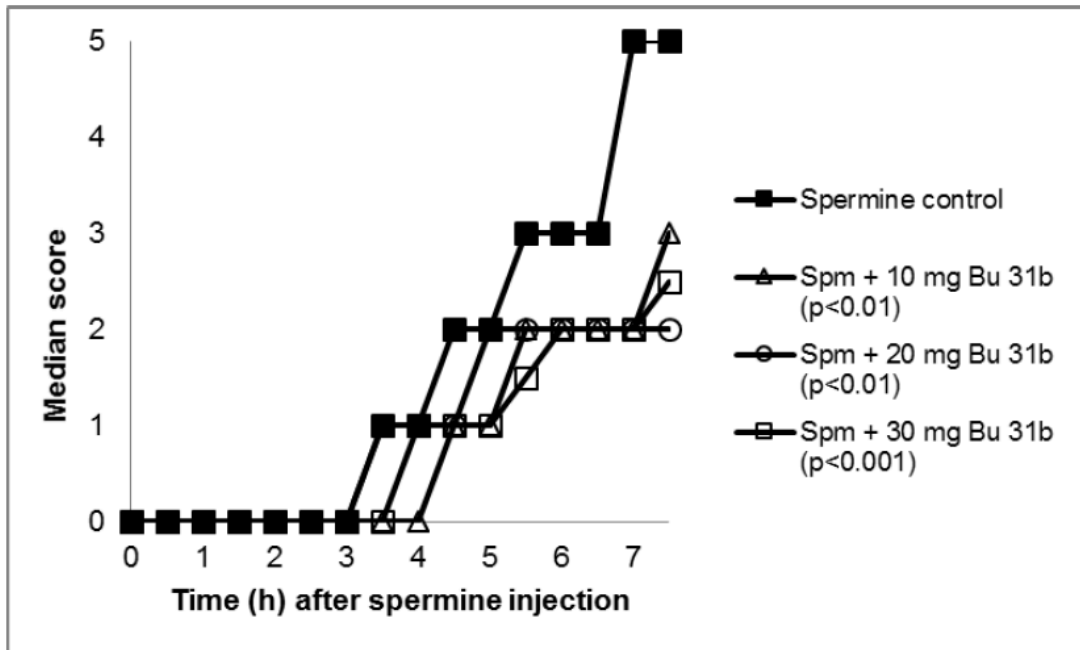
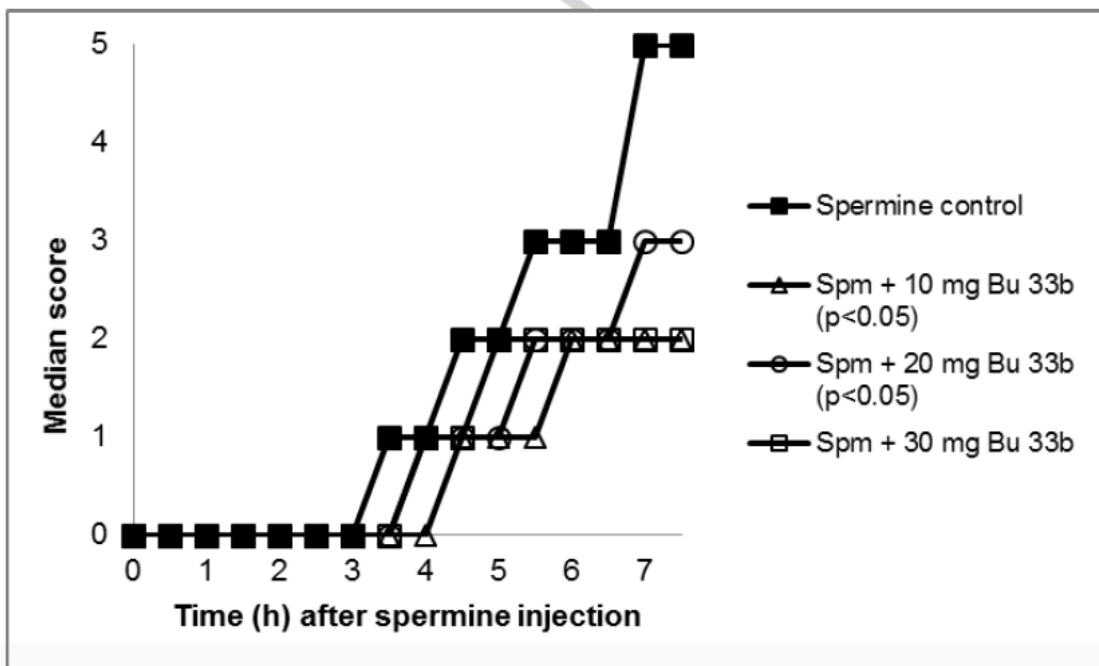


Figure 3

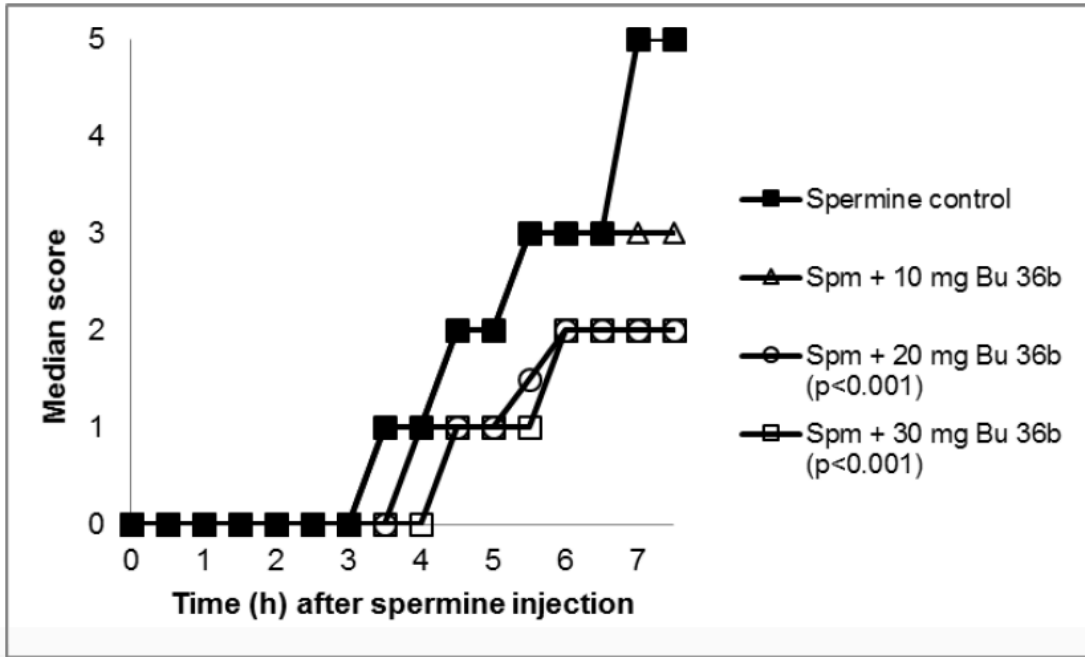
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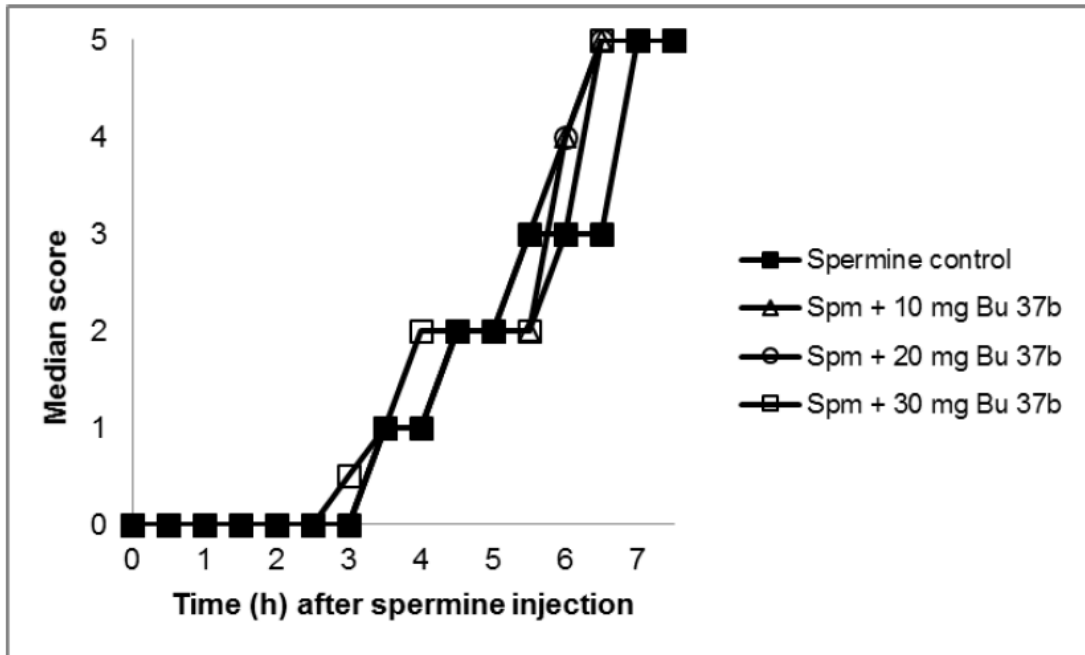
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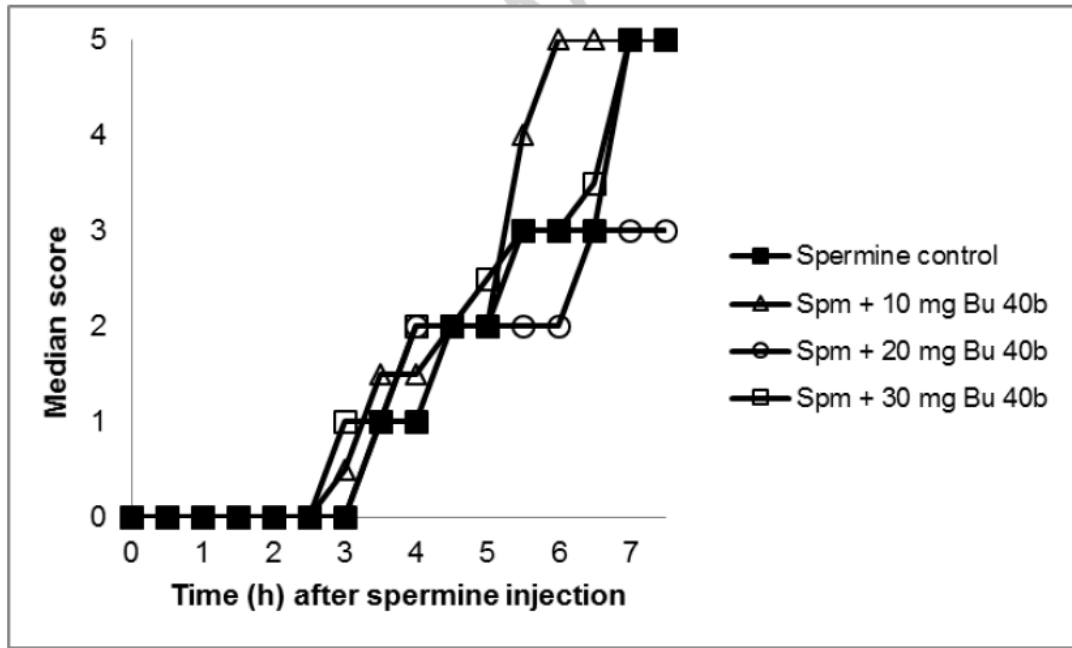
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