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ORIGINAL PAPER

Isopathic *versus* enantiomeric inhibition of U-50488 HCl toxicity – experimental studies

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Background: **Previous studies have investigated toxicity inhibition of optically active compounds by potentized preparations of their enantiomers. It was hypothesised that inhibition of toxicity may be stereospecific. This paper presents 2 studies investigating stereoisomer potencies in terms of their ability to counteract toxicity of the (−) stereoisomer. The stereoisomers used were (−)-*trans*-(1S,2S)-U-50488 HCl and (+)-*trans*-(1R,2R)-U-50488 HCl.**

Materials & methods: **Designs were prospective, blind, randomised, intention-to-treat and compared the efficacy of 2 indistinguishable treatments. The outcome was the difference in survival. Potency 'chords' consisting of 4th, 12th and 30th approximately centesimal dilutions were prepared, representing concentrations of 1.08×10^{-10} M. One study compared inhibition of (−)-U-50488 toxicity injected ip at the estimated LD50 into male ICR mice, treated with a potency chord of the same stereoisomer, with control ('isopathic' study). The other study compared inhibition of toxicity by potency chords made from the stereoisomers (+)-U-50488 and (−)-U-50488 ('enantiomer' study), Treatments were administered orally on 11 occasions: twice before and nine times after ip injections.**

Results: **The isopathic study did not yield a significant result. In the enantiomer study, comparison of isopathy with enantiomer potency treatment showed a highly significant difference odds ratio 1.97 (95% CI: 1.23–3.14).**

Conclusion: **We conclude that enantiomeric potencies are superior to identically produced isopathic potencies, in inhibiting toxicity of (−)-U-50488 HCl. Homeopathic inhibition of toxicity may be stereospecific.** *Homeopathy* (2009) **98**, 83–87.

Keywords: homeopathy; isopathy; stereoisomer; optical isomer; patent; mice; enantiomer

Introduction

U-50488 is a potent inhibitor of peripheral and visceral pain. This is mediated by kappa opiod receptors, but non-opioid mechanisms are also involved, such as blocking of sodium channels in neurons.[1,2]

This paper reports the results of 2 experiments. The first looks at the ability of a homeopathic dilution of (−)-U-50488, to inhibit the toxicity of intraperitoneal injections of the same stereoisomer, and uses the same protocol as an earlier paper.[3] These results were used to calculate sample size for the study comparing isopathic with enantiomeric treatment, also reported here. It was calculated that between 150 and 450 mice would be required in each arm to achieve statistical significance; i.e., a total of between 300 and 900 male mice were needed, for power of 0.975 at the 5% level.[4] Since the vivarium we used can only house about 300 mice, it was decided prospectively to stop the experiment if it achieved significance after the first batch of 300 mice. If significance had not been achieved, the experiment would have been stopped after 600 mice or, if necessary, after 900 mice. In fact significance was achieved with the 300 mice.

An experimental model using bioassay with other stereoisomers such as α-methylbenzyl isocyanate, verapamil,

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BAY K8644 and a nicotine salt has been reported,[5] using the method of Kozlova.[6] More recently other bioassays have been used.[7,8]

Previous papers have described the background of stereoisomers in homeopathy[9,10] and we will not reiterate this here. (Patents applied for.*) RMK speculated that use of stereoisomer potencies of a toxic chemical compound, may more accurately reflect the Law of Similars than isopathy. This study was not intended as a test of homotoxicology, although the use of potency chords is common in homotoxicology.[11,12] A potency chord was used purely on empirical grounds in this study, since previous work demonstrated efficacy in mice and Aspergillus awamori.[5] More recent bioassay work, currently unpublished, in other microorganisms, suggests that simplexes were more efficacious than a potency chord in these organisms.

The purpose of the present studies was to confirm the null hypothesis of equal survival proportions, comparing the difference between the efficacy of identically prepared potencies of enantiomers of U-50488, in terms of their ability to counteract the toxicity of (−)-U-50488. Enantiomers are stereoisomer molecules which are non-superimposable mirror images of each other. The study was prospective, blind, randomised and intention-to-treat, using indistinguishable experimental arms.

Materials and methods

Experiment 1: isopathic experiment

In the isopathic experiment survival of ICR mice treated with a potency chord of (−)-U-50488 HCl was compared with mice treated with indistinguishable control. Intraperitoneal injections of (−)-U-50488 HCl at the estimated LD50 dose of 25 mg/kg were given. This estimated LD50 was in accordance with a previous study.[3] In the subsequent isopathy *versus* enantiomer study, described below as experiment 2, an estimated LD50 of 30 mg/kg was used, since it was deemed to more accurately reflect LD50. Other than using different treatments and different doses of (−)-U-50488 HCl, the protocols were the same.

Preparation of control

Fresh control was prepared on each day of the experiment immediately before preparation of the potency, H2, by adding 3 drops of 40% ethanol from a hematocrit capillary tube to a 10 ml test tube containing 7 ml 40% ethanol. This was given 20 succussions. The same ethanol BP stock bottle was used for control and potency preparation in both experiments 1 and 2. The same person made both potencies and controls.

Experiment 2: enantiomer experiment

The protocol is as reported elsewhere.[3,9,10] Male ICR mice were bred in Slivnitza animal house, and transferred to the laboratory animal house (temperature 20–22°C, photoperiod 7am–7pm), 1 week before the experiment to allow acclimatization. Experiments were conducted in accordance with laws prevailing in Bulgaria. People who worked on a given arm of the experiment for a given batch of mice, did not touch, handle or contaminate the other arms. The different arms were in this sense physically separated.

Mice were allowed food and water *ad libitum* at all stages of the experiment up until just before the injection of (−)-U-50488 HCl, with the exception of the brief period of abstinence (10 min) after administration of oral liquids.

All glassware used more than once during the experiment was washed with tap water, copiously rinsed with distilled water, hot-air dried and heated for 2 h at 200°C before being considered clean and reusable. The same glassware was used for preparation and storage of (−)- and (+)-U-50488 potencies. In addition, as in previous experiments[3,9,10] cages and water bottles were washed after each experiment with "Pur + Aloe Vera" (pH neutral, Henkel, Austria, 245605), mixed 50:50 with Belina ACE (Greece). Bottles were copiously rinsed with distilled water and treated according to the protocol mentioned above for glassware in general.

Preparation of (+)-U-50488 HCL and (−)-U-50488 HCL potencies

Stereoisomers of U-50488 were supplied by Sigma, solid, ≥98% (HPLC). The (−) and (+)-U-50488 potencies were manufactured on different days, to avoid the theoretical risk of contaminating the (−)-potencies with the (+)-potencies and *vice versa*. One person was responsible for manufacture of potencies.

To make the (+)-potencies, 10 mg (+)-*trans*-(1R,2R)-U-50488 HCl ((+)-*trans*-(1R,2R)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide hydrochloride), or (+)-U-50488, was added to one ml distilled water in a 10 ml Hamilton Laboratory Glass test tube with a glass stopper. The test tube was given 20 forceful downward succussions in a vertical line at a rate of between 0.75 and 1 Hz. A non-heparinized disposable hematocrit capillary tube (75microlitre, Hirschmann Laborgeräte, 1 drop = 0.025 ml) was used to add 3 drops of this solution to another test tube containing 7 ml 40% ethanol, the dilution ratio was thus approximately centesimal 93.33:1. Capillary tubes were discarded after use. The test tubes were not more than two-thirds full, allowing sufficient room for the fluid to collide violently with the test tube walls on succussion. The process was repeated until the 30th dilution was reached.

To make the 'potency chord', 1.5 ml of liquid was removed from the tubes containing the 4th, 12th, and 30th dilutions and added sequentially to a 10 ml Hamilton test tube. The mixing was done with minimal delay, the contents were quickly mixed and then succussed. The contents were given 20 forceful downward succussions at 0.75–1 Hz. This complex was called "H1", and was used for the experiment. It was stored carefully in the dark wrapped in white paper and was minimally handled. Potencies were stored

*Australian patent no. AU2003208170, Indian patent application no. 2704/DELNP/2004, European patent application no. EP1487448, US patent application no. 20050119350, Canadian patent application no. CA 2505984, Hong Kong patent application no. 05105229.7, New Zealand patent application no. 535654, International application no.: PCT/AU2003/000219.

in the dark away from direct sun, and avoiding sources of electromagnetic energy and strong odours.

On each day of the experiment, 3 drops of the verum chord (H1) were added to a test tube containing 7 ml 40% ethanol. This was immediately given 20 succussions at 0.75–1 Hz to produce 'H2'. The test tube containing H2 was then wrapped with white paper, and tubes were handled only by the wrapped section. H2 was administered to mice. The final molarity was 1.08×10^{-10} M. The (−)-U-50488 HCL potency corresponding to H1 described above was produced in the same way but on a different day.

Randomisation and blinding

The initial step in randomisation and blinding of the H2 potencies involved affixing the letters A or B to the paper wrapped test tubes according to coin toss. After the initial randomisation, the tubes were given to a second person (person 2). Person 2 performed the final randomisation and blinding of solutions of (+)- and (−)- potencies just before the experiment, and did not handle the mice during the experiment and was absent from the vivarium during the experiment. Person 2 covered the previous A and B stickers affixed to the paper wrapper covering the 2 test tubes, by wrapping another layer of paper secured with tape around the tubes, thereby completely obscuring the A and B codes underneath. Person 2 then repeated the coin toss and allocated new A and B stickers to the test tubes. The blind randomisation codes thus produced were kept in the personal possession of the respective randomisers, and were not revealed until data had been collected. In this way nobody involved in the experiment knew the order of randomisation and the experiment was blinded.

Mice were given the oral potency chords on a total of 11 occasions: approximately 18 and 1 h before and at 5, 10, 15, 20, 30, 40, 50, 80 and 110 min after the ip injection: the first time shortly after their selection at 4.30 pm on the day prior to intraperitoneal U-50488 injection, and again at the following day immediately after weighing. The manner of administration of potencies after ip injection was different from that prior to injection, due to the unconscious state of the mice after ip injection. After injection each mouse was given only 1 drop (0.025 ml) of fluid, by hematocrit capillary tube, instead of almost the full contents of the capillary tube (0.075 ml) used for doses prior to ip injection. This was done to avoid aspiration.

To give oral fluids to a conscious mouse, the mouse was taken from its cage by an experienced handler and held in the supine position. Another person took 0.075 ml of the respective solution from the storage tube with a hematocrit tube, which was introduced just behind the incisors of the mouse such that it was impelled to drink quickly. The tube was removed as soon as possible and the mouse held for another 20 seconds in the supine position, then returned to its cage. The drinking water bottle and food were not placed in the cage until 10 min later. All cages used in the experiment contained a constant amount of softwood (pine) bedding just covering the cage floor. One mouse at a time was removed from this cage, and a coin was tossed to decide if it was to be allocated to group "A" or group "B". Group A and B mice were then administered their respective A or B fluids, as per randomisation.

Mice were housed in 3 different sizes of cages depending on the stage of the experiment, with stainless steel mesh roof, cradle for inserting a water bottle, and grill onto which could be placed dry food. The cage sizes are (width × length × height):

I. 30 × 33 × 15 cm for adaptation of the mice prior to the onset of the experiment
II. 18 × 33 × 15 cm – for selection of the mice prior to injection, and
III. 15 × 24 × 15 cm into which mice were individually placed after injection of ip (−)-U-50488

Two investigators and four laboratory assistants were involved in the study.

One of the investigators administered ip injections for every experiment. One laboratory assistant performed randomisation and blinding of solutions only and was referred to as 'person 2', and the remaining 3 laboratory assistants were mouse handlers. One of the investigators also acted as a mouse handler, a fifth person administered ip injections. The person giving ip injections did not otherwise handle the mice.

The four mouse handlers took animals from cages, and held them while they received ip injections or oral solutions were divided into two groups of two on each day, by blind drawing of cards. Thus, the arms of the experiments for a given batch of mice were kept separate. This was done to avoid cross-contamination of the (+)- and (−)-isomer potencies.

At 9.30 am mice were placed in a clean strong plastic bag one at a time and suspended from a Pesola 30 g or 60 g scale (Pesola, Switzerland). The weight was used to calculate dosage of intraperitoneal (−)-U-50488.

The weighed mice were sequentially removed from their cages between 10 and 11 am and held gently but firmly in the supine position. Each was injected ip with a solution (−)-U-50488 HCl, diluted in Normal Saline for injection BP at the estimated LD 50 of 30 mg/kg. The volume administered was 0.1 ml/10 gm body weight, such that each mouse received an estimated LD50 of (−)-U-50488 HCl ip. Injections were given using disposable 0.5 ml insulin syringes with 27-gauge needles, intraperitoneally into the left lower quadrant of the abdomen. After injection the mice were held in the supine position for 2–5 min until heavily sedated. (−) and (+) Isomer potencies were subsequently administered at 5, 10, 15, 20, 30, 40, 50, 80 and 110 min after U-50488 injection. After receiving ip injections mice were sequentially placed into individual cages. The number of mice alive at 09.00 the next morning was the end-point of the experiment.

Results

In the isopathy study, in which the mice were treated with a 'potency chord' of the (−) isomer, compared to control, 47/79 (59%) were alive approximately 23 h after receiving the ip injection, compared with 41/79 (52%) for

Table 1 Cross tabulation of 'arm' *versus* 'alive/dead' for isopathy study

Table of outcome by treatment for isopathy study

Outcome alive/dead	*Treatment*		*Total*
Frequency Row Pct	*(−)-isomer potency (Medicine)*	*Control*	
Alive – number	47	41	88
Percentage	59.49	51.90	
Dead – number	32	38	70
Percentage	40.51	48.10	
Total number	79	79	158

$\chi^2 = 0.9215$ with 1 degree of freedom, $p = 0.3371$.

control. ($\chi^2 = 0.9215$, 1 degree of freedom, $p = 0.3771$) (Table 1).

In the enantiomer experiment comparing treatment with potency chords of the two stereoisomers, 99/150 (66%) of the mice treated with the (+) stereoisomer were alive approximately 23 h after receiving the ip injection, compared with 74/150 (49%) of those receiving the (−) stereoisomer. $\chi^2 = 8.534$, 1 degree of freedom, $p = 0.0035$, odds ratio (+ *versus* −) 1.9936 (95% confidence Interval 1.2518–3.1751) (Table 2).

Although animals were administered ip U-50488 according to body weight, drug elimination is not linearly related to weight, since smaller animals have a higher metabolic rate.[13] Therefore we adjusted the results for weight by logistic regression. As in previous studies, this was part of the design. Logistic regression is a statistical method for analysing dichotomous response data (in this case 'outcome' = alive/dead) while adjusting for covariates (treatment and weight). A model with a treatment, weight and treatment by weight interaction was fitted. The interaction was not significant so a model with weight and treatment was fitted, this yielded $p = 0.0047$, odds ratio 1.97, 95%. Confidence Interval: 1.23–3.14 (Table 3).

We conclude that enantiomeric potencies were superior to identically produced isopathic potencies, in terms of their ability to inhibit toxicity of (−)-U-50488 HCl.

Discussion

Many natural biomolecules are stereoisomers. Stereoisomers are molecules, which, in terms of structure, are identical to each other in all regards except for their spatial configuration. Enantiomers are molecules that are non-superimposable mirror images in 3-dimensional space. Living organisms are very specific in terms of their sensitivity to stereoisomers due to stereospecificity and stereoselectivity. Enantiomers, mirror image molecules, are referred to with (+) and (−) prefixes. The application of chemical nomenclature to stereoisomers and some of the difficulties associated therewith are discussed elsewhere.[14] It could be noted here that the R and S notation of the Cahn-Ingold-Prelog convention, used in stereochemical nomenclature, was incorrectly described in an earlier paper[9] and is correctly presented by Smith.[14]

Table 2 Cross tabulation of 'arm' *versus* alive/dead for isopathy/enantiomer study

Table of Outcome by Treatment

Outcome alive/dead	*Treatment*		*Total*
Frequency Row Pct	*(+)-isomer*	*(−)-isomer*	
Alive – number	99	74	173
Percentage	66.00	49.33	
Dead – number	51	76	127
Percentage	34.00	50.66	
Total number	150	150	300

$\chi^2 = 8.534$ with 1 degree of freedom, $p = 0.0035$. Odds ratio (+ *versus* −) 1.9936 (95% Confidence interval 1.2518–3.1751).

Experiments using isopathy have been previously reported,[15–23] and at least 2 have reported use of stereoisomers.[17,24] Jonas *et al* reported effects of potencies of (−)-glutamate isopathically inhibiting (−)-glutamate toxicity in the nervous system of rats.[17] Bonamin *et al* reported very significant inhibition of dexamethasone activity by administration of isopathic potencies of dexamethasone in a small sample of BALB/c mice.[24] Usually, effects in isopathy are relatively small. To treat the toxicity of intraperitoneal injections of (−)-U-50488 in an isopathic way, one would use potencies of (−)-U-50488. When the enantiomer is used, i.e., (+)-U-50488, we refer to it as enantiomeric or stereoisomeric treatment. Stereospecificity and stereoselectivity dictate that the pharmacokinetics, pharmacodynamics, physiological actions and therefore symptoms produced by stereoisomers tend to be very different from one another. Accordingly, it is misleading to think of enantiomeric treatment as being semi-isopathic. Also, as demonstrated in this study, the effects of enantiomeric treatment are different from isopathic treatment. The reason for using U-50488 in this study, rather than another stereoisomer, is that it was commercially available and provided the possibility of a humane method for testing the hypothesis.

In the present experiments, as in previous experiments using enantiomeric potencies, results were obtained with minimal treatment, and multiple tests were not performed to determine optimal potencies. That it was possible to obtain evidence of effects under such conditions supports the robust nature of the hypothesis. Molecules were selected purely on the basis of stereochemistry and commercial availability.

A higher proportion of (−)-isomer treated mice survived in the isopathic experiment than in the enantiomer/isopathy study – see Tables 1 and 2. One would expect more (−)-isomer mice to survive in the first, isopathic, experiment than in the second experiment comparing enantiomer and

Table 3 Parameter estimates from Logistic regression (+)-isomer treatment *versus* (−)-isomer treatment

Parameter	*Estimate*	*Standard error*	*Pr > ChiSq*
Intercept	−1.7654	0.8552	0.0390
Treatment + *versus* −	**0.6755**	**0.2392**	**0.0047***
Weight (gm)	0.0896	0.0433	0.0383*

'treatment +' factor p-value = 0.0047. Odds ratio 1.97 (95% Confidence interval: 1.23–3.14).

isopathy, because the second experiment used a higher dose of intraperitoneal (−)-U-40588, so higher mortality is expected.

The experimental model has a clearly defined unequivocal end-point. Mice were not moribund at the end of the experiment and thus did not complicate interpretation of the outcome. There are no inherent problems with poor remedy selection, which can plague clinical studies and effectively render them under powered, since all subjects receive the same preparation. It is possible to quickly achieve data on relatively large sample sizes. Our experiment on 300 mice took 17 days.

It should be noted that mice were administered potencies and control in 40% ethanol. Mice accepted this well. We doubt that potencies in water are stable, and wonder if they lose biological activity within hours if not minutes. This question could possibly be answered using stereoisomer potencies in bioassay.[5–8]

If one were to generalize the application of stereoisomers as used in the present study, then one could design experiments using any stereoisomer where it was sought to modulate that isomer's toxicity. Any substance can be toxic depending on the dose and context. Therefore, possible experiments investigating the present effect could involve many types of compounds including many pharmaceuticals, drugs of addiction, cytokines, neurotransmitters, enzymes, hormones, proteins, amino acids, etc. However, some molecules are so complex, e.g., proteins, that the necessary technology is currently not available to synthesize their stereoisomers.

Conflict of interest

All authors have a financial interest in some patents quoted in this study.

Funding statement

RM Kuzeff provided all funding.

Animal ethics

Experiments were conducted at the Institute of Zoology, Bulgarian Academy of Sciences, in accordance with laws prevailing in Bulgaria.

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