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Original article

Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs

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Abstract

Introduction: Seizure liability is an adverse property of new candidate drugs typically detected only in later stage pre-clinical safety studies. Consequently, pharmaceutical discovery needs small scale (microplate-based), rapid throughput screens to 'front-load' such adverse endpoints in order to reduce associated attrition rates. Of the potential methods available, previously published studies have shown that the quantification of seizure-associated locomotion in the larval zebrafish (*Danio rerio*) offers high potential for development into such a screen. Here, we present methodology and validation data (on 25 compounds) from a larval zebrafish (Zf) convulsant assay, based on the quantification of high speed locomotion after exposure to a range of test compounds. **Methods:** All assays were undertaken in 7 days post fertilization (dpf), WIK-strain Zf larvae, at 27±1 °C. The blinded validation test set consisted of 17 positive and 8 negative controls, based on literature evidence for seizure liability. Initially, a Maximum Tolerated Concentration (MTC) assay was undertaken on each compound to identify the maximum concentration not causing general toxicity, sedation or overt neuromuscular effects. Next, the convulsant assay was undertaken on 5 concentrations from the MTC down, plus a dilution water control. Exposed larvae were videotracked for 1 h, using the Viewpoint Videotrack for Zebrafish™ system, and high speed movements, typically associated with seizure-like locomotor activity, were quantified. **Results:** According to classification criteria proposed by the European Centre for the Validation of Alternative Methods (ECVAM), the data generated appeared to offer "sufficient" predictivity (72% overall), particularly considering the potential for throughput and likely positioning within a safety pharmacology front-loading screening cascade. **Discussion:** Possible reasons for the misclassifications are discussed, and potential improvements to increase sensitivity and specificity outlined. In all, these initial validation data suggest that this assay offers potential as a medium throughput screen aimed at the early drug discovery detection of this complex safety pharmacological endpoint.

Keywords: Convulsant; *Danio rerio*; Drug discovery; Epilepsy; Front-loading; Methods; Safety assessment; Screen; Seizure; Throughput; Videotracking; Zebrafish

1. Introduction

Over recent years, zebrafish (*Danio rerio*) have been identified as a potential 'new' vertebrate model for assessing drugs *in vivo*, with respect to a wide range of toxicological and safety pharmacological endpoints (see Goldsmith, 2004; Langheinrich, 2003; Parng, Seng, Semino & McGrath, 2002; Rubinstein, 2006 for reviews). Of the potential applications for a relatively high throughput *in vivo* zebrafish (Zf) screen, one of the most promising areas from the literature is regarding seizure liability. In this respect, recent papers have identified the potential of the embryo-larval Zf model for quantifying seizure-like locomotor

Abbreviations: COV, Coefficient Of Variation; dpf, days post fertilization; DWC, Dilution Water Control; ECVAM, European Centre for the Validation of Alternative Methods; HPTC, Human Plasma Therapeutic Concentration; LOEC, Lowest Observed Effect Concentration; MTC, Maximum Tolerated Concentration; PTZ, Pentylenetetrazole; SC, Solvent Control; TL, Tübingen long fin; WIK, Wild-type India Calcutta; Zf, Zebrafish.

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activity (Baraban, Taylor, Castro & Baier, 2005), in addition to outlining screening methodologies for anticonvulsive therapies (Berghmans, Hunt, Roach & Goldsmith, 2007). To date, however, there are to our knowledge no published methods with validation data that use Zf larvae as a tool to identify seizure liability in development drugs.

The need for such a screen is clear. Examples of many marketed compounds have been associated with an increased risk of seizure in the clinic, including various antidepressants, antipsychotics, anaesthetics, antiarrhymics, analgesics, antibiotics and even, rather paradoxically, antiepileptics (see Ruffmann, Bogliun & Beghi, 2006; Zaccara, Muscas & Messori, 1990 for reviews). Obviously, when developing new therapies, seizure liability is an undesirable property, and is typically detected only in later stage pre-clinical safety studies conducted on higher vertebrates (Redfern & Wakefield, 2006). Consequently, safety pharmacologists would benefit from a small scale (thus reducing compound requirements) rapid throughput (thus allowing front-loading) *in vivo* screen to address seizure liability, and reduce associated compound attrition. Here we present methodology and initial validation data from a potentially useful medium throughput *in vivo* Zf-based screen for the identification of seizure liability in development drugs, at an early stage in pre-clinical safety assessment.

The method currently presented is based on the original work of Baraban et al. (2005), who reported that pentylenetetrazole (PTZ; a commonly applied experimental convulsant) will induce seizures in a concentration-dependent manner in 7 day old (post fertilization) TL-strain (Tübingen long fin) Zf larvae, and that these could be quantified with the aid of videotracking and image analysis. More specifically, larvae in normal exposure media showed infrequent darting swimming, whereas those exposed to PTZ exhibited predictable locomotory patterns: dramatically increased swimming speed/activity (named stage I); rapid "whirlpool" motion circular swimming (stage II); loss of posture and loss of motion for 1–3 s (stage III). The recorded behavioural observations were reinforced with electroencephalogram recordings showing epileptiform activity, along with upregulated *c-fos* gene expression.

This basic method for locomotor activity measurement was adapted in the current study to allow the videotracking and image analysis assessment of the convulsive (single compound concentration–response) activity of a coded set of compounds. These compounds were from a range of pharmacological classes that exhibited either positive (17 compounds), or negative (8 compounds), seizure profiles in mammals. Here we present the methodology (Maximum Tolerated Concentration and convulsant propensity) and data from this assay validation exercise.

2. Methods

2.1. Experimental animals

Adult Zf (Wild-type India Calcutta or WIK-strain, broodstock obtained from the Zfin strain repository, Oregon, USA) were held under optimal spawning conditions (16 hour light: 8 hour dark cycle, with 20 minute dusk–dawn transition periods, 28±1 °C), in groups of males and females in flow through aquaria at AstraZeneca, Brixham, UK. WIK larvae were chosen, as previous work suggested good reproduction in the laboratory with few spontaneous developmental abnormalities. In addition, this strain appeared to offer superior performance in safety pharmacology method development work, when compared with other strains (Richards et al., submitted for publication).

The culture water used was dechlorinated tap water, which had been passed through activated carbon, coarsely filtered to remove particulate material and dechlorinated with sodium thiosulphate. Salts were added to maintain minimum hardness levels, and the treated water then passed through an UV-steriliser. The supply was then delivered to a temperature controlled header tank in the husbandry unit and finally re-filtered to 5 μm before use. All dechlorinated tap water was routinely monitored for general water quality parameters (e.g. pH, hardness, conductivity) and also for specific trace metal and organic contaminants (including Polychlorinated Biphenyls (PCBs) and organochlorine pesticides). This water was used for all adult Zf and embryo-larval culturing, and is referred to as 'culture water' throughout this paper. The routinely monitored physicochemical properties of this culture water are summarised in Table 1.

To obtain embryos for use in the assays, fish were mated in discrete male–female pairs to allow individual clutch use. When sufficient embryos were deposited, they were subjected to an antifungal 'bleaching' procedure: 1% $^{w}/_{v}$ chloramine T (Sigma-Aldrich, Poole, UK) in culture water for 1 min, then two rinses in culture water followed by transfer (of fertilized, viable embryos) to a Petri dish containing 0.8 μm sterile-filtered culture water (25 embryos in 45 ml per Petri dish).

Petri dishes were held in an alarmed, temperature controlled room at 27.1±0.3 °C (mean±SD, n=7003, recorded from a beaker), in which additional temperature logging was undertaken in a Petri dish (26.7±0.4 °C, mean±SD, n=3597). Embryo-larvae were held on a black background, under a 16-hour light: 8 hour dark cycle (0.42±0.07 Klux cosine, mean±SD, n=9), with 20 minute dusk–dawn transition periods.

Throughout the culture period, a Petri dish water change was undertaken every 24 h to maintain water quality. From 4 days post fertilisation (dpf) to 6 dpf, larvae were fed euryhaline rotifers (*Brachionus plicitalis*. After Hutchinson & Williams, 1994) at a density of 25 rotifers per ml of culture water/Petri dish/day. Prior to addition, the rotifers were well rinsed in culture water to remove any residual salts.

Table 1
Physicochemical properties of the Zf embryo-larval culture water

Parameter	Mean	SD	N
pH	7.62	0.18	21
Dissolved O_2 (mg/l)	8.19	0.22	21
Conductivity (μSeimens/cm)	243.57	33.32	21
Alkalinity (mg $CaCO_3$/l)	30.08	5.17	21
Hardness (mg $CaCO_3$/l)	43.41	4.16	21
Chlorine (total μg/l)	<0.2	–	21
Salinity (‰)	<0.5	–	21
Light intensity (Klux cosine)	0.42	0.07	9

2.2. Test compounds and reagents

All test compounds were supplied as coded, pre-weighed vials, containing enough to make 100 ml of 1 mM test solution. Test compound identities were only revealed after the completion of all experiments and draft reporting of the study results. All test compounds and reagents were obtained from Sigma-Aldrich (Poole, UK) except bemegride (Fisher Scientific, Loughborough, UK) and bicuculline methoiodide (Tocris Cookson Ltd, Bristol, UK).

Test compounds were either classified as positive or negative controls based on the evidence of seizure liability (in mammals and/or the clinic) from the published literature. No emphasis was given to the frequency or intensity of seizures in the clinic, with respect to the positive controls, and as such the positive control materials represented a wide range of seizure liabilities. Negative controls were selected from a table of drugs that are well established in clinical use (Brunton, Lazo, Parker, Goodman & Gilman, 1990), using a random number generator. The selected drugs were then checked on the Medline public literature database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed) to confirm the absence of seizure liability. Some negative control materials were found, at a later date, to have isolated incidences of convulsions associated with them, although in such cases the weight of evidence for positive seizure liability was still considered low (see Discussion section).

2.3. Experimental procedures

2.3.1. Maximum Tolerated Concentration (MTC) determination

To determine an appropriate concentration regimen for subsequent videotracking, a Maximum Tolerated Concentration (MTC) was determined for each test compound. MTC assessment was undertaken in 7 dpf larvae, for 1 h, to mirror the videotracking experiments. The MTC served to establish the toxic concentration of the test compound, and also those concentrations that would adversely affect the locomotor activity of the larva (e.g. sedation or neuromuscular effects).

Approximately 24 h (PM) prior to test compound exposure, 6 dpf larvae were individually loaded into each well of a pre-leached polystyrene, opaque-walled, transparent-bottomed 24 well plate (Arctic White, Bethlehem, USA) in a total volume of 2 ml of culture water. These plates served to eliminate inter-well cross-talk and thus potential behavioural cues. Following loading, larvae were cultured as before, except without feeding to avoid water fouling. For the MTC, 3 plates were prepared, initially providing 12 animals per test concentration (in the majority of cases). On each plate, 5 treatments (test compound concentrations) were assigned randomly to columns (to avoid

Table 2
Summary of the physicochemical properties of all test solutions used in the current study

Test compound	CAS number	pH			Dissolved oxygen (mg/L)			Conductivity (μSeimens/ cm)			Temperature (°C)		
		Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Penicillin G	69-57-8	7.76	0.24	18	7.94	0.27	18	285.33	75.44	18	27.43	0.77	18
Maprotiline hydrochloride	10347-81-6	7.65	0.19	18	8.09	0.10	18	284.94	120.21	18	27.07	0.37	18
Bemegride	64-65-3	7.76	0.05	12	8.09	0.18	12	222.00	18.23	12	27.16	0.36	12
Bicuculine methoiodide	55950-07-7	7.82	0.06	12	7.95	0.34	12	277.08	27.60	12	27.12	0.42	12
Strychnine hemisulphate	60-41-3	7.73	0.13	18	7.70	0.26	18	269.28	6.42	18	26.83	0.80	18
Cefazolin sodium	27164-46-1	7.68	0.11	12	7.93	0.35	12	295.50	19.38	12	27.37	0.26	12
Isoniazid	54-85-3	7.70	0.04	12	7.92	0.10	12	276.83	16.21	12	27.93	0.34	12
Physostigmine salicylate	57-64-7	7.63	0.27	18	7.92	0.10	18	260.56	33.89	18	27.33	0.64	18
Amoxapine	14028-44-5	7.81	0.10	18	8.03	0.16	18	349.50	183.38	18	26.93	0.23	18
Enoxacin	74011-58-8	7.75	0.14	12	7.38	0.38	12	260.17	12.50	12	27.63	0.33	12
Semicarbazide hydrochloride	563-41-7	7.72	0.04	12	8.00	0	12	270.33	41.39	12	26.95	0.56	12
Bupropion hydrochloride	31677-93-7	7.81	0.18	18	8.08	0.10	18	289.39	17.64	18	26.76	0.27	18
Picrotoxin	124-87-8	7.64	0.13	12	7.91	0.09	12	218.42	13.78	12	27.48	0.41	12
Pentylenetetrazole	54-95-5	7.88	0.14	12	7.73	0.30	12	246.33	70.35	12	27.67	0.27	12
Aminophylline hydrate	317-34-0	7.94	0.10	12	7.68	0.46	12	322.75	27.95	12	27.58	0.20	12
4-Aminopyridine	504-24-5	7.70	0.24	12	7.65	0.27	12	313.75	28.89	12	27.68	0.38	12
Acetylsalicylic acid crystalline	50-78-2	7.65	0.12	12	8.07	0.10	12	245.11	74.94	12	27.37	0.24	12
Verapamil hydrochloride	152-11-4	7.78	0.12	12	7.93	0.18	12	265.42	24.15	12	26.97	0.14	12
Naproxen	225204-53-1	7.88	0.06	12	8.10	0.34	12	334.67	100.59	12	27.04	0.30	12
Dexamethasone	50-02-2	7.88	0.05	12	7.93	0.10	12	280.92	23.52	12	26.97	0.54	12
(−)-Scopolamine hydrochloride	55-16-3	7.68	0.18	12	7.88	0.26	12	268.00	33.86	12	26.91	0.58	12
Tobramycin	32986-56-4	7.88	0.06	12	8.30	0.31	12	307.67	55.53	12	27.18	0.28	12
Acetaminophen	103-90-2	7.83	0.07	12	7.93	0.40	12	242.67	6.37	12	26.62	0.13	12
4-Aminophenyl sulfone	80-08-0	7.72	0.11	12	7.73	0.29	12	394.83	195.19	12	26.64	0.32	12
Protriptyline hydrochloride	1225-55-4	7.89	0.04	12	8.12	0.25	12	267.15	41.44	12	27.10	0.11	12

For simplicity, data are shown as the mean, standard deviation and *n* of all test solutions (all dilutions) used in all MTC and convulsant screen studies. All of these data are after all dilutions except the final small dilution of 10%, in the microplate well. Those compounds that exhibited stock solution pHs outside of the range of 6–8 were adjusted using NaOH or HCl. In cases of poor water solubility, this was aided by gentle warming, sonication, and/or vigorous stirring.

positional bias), along with a dilution water control (DWC) or solvent control (SC) as appropriate.

Treatments were assigned equally to individual clutches of animals. For example, where two clutches were used, 1.5 plates were prepared with each clutch, the third plate consisting of rows A and B from clutch one, and rows C and D from clutch two (thus covering all treatments, and plate positions with each clutch). This study design ensured that any inter-clutch variability in the results obtained would be accounted for within the overall variability of the dataset generated. Wherever possible, and in most cases (80%), 2 or more clutches were used in each experiment (MTC and convulsant assay) to reduce the chances of erroneous results being obtained due to clutch-related locomotor defects. Further to this, and considering one would expect any positive clutch effect on locomotion to be exhibited also in the DWC animals, in those cases where only 1 clutch was used, only 1 compound was classified as a negative (penicillin G), and this effect was repeated using a different clutch of animals (see Results section).

On 7 dpf, the stock test compound was freshly prepared in culture water (or an appropriate solvent as required), and the pH checked and adjusted as necessary (to approximately 7.5) using 1 M NaOH/1 M HCl. Once subsequent treatment dilutions were prepared, each was checked for basic physicochemical properties (Table 2 — note that the data shown are, for simplicity, a summary of all dilutions used in all studies, and as such the SD reflects the variation in the parameters described across the concentration ranges employed). Normally, the dilution series consisted of 1, 0.5, 0.25, 0.125 and 0.0625 mM plus a DWC, although this was modified if high toxicity was suspected from the limited information available in the censored Material Safety Data Sheet (MSDS).

Test compound exposure commenced on the afternoon of 7 dpf, to minimise any temporal variability in activity. To achieve this, 1.8 ml of culture water was carefully removed from each well (leaving 200 µl and the larva), and 1.8 ml of the appropriate treatment was added. This exposure procedure introduced an additional small dilution to the treatment concentration such that the top concentration tested in most cases, 1 mM (nominal), corresponded with a nominal 0.9 mM well concentration after plate loading.

The MTC was then assessed using the following criteria. After 10 min and then periodically throughout the exposure period, each well was observed for PTZ-like seizure activity (very rapid swimming and circling) or generally erratic locomotion; loss of posture (e.g. loss of dorso-ventral balance); and abnormal morphology (e.g. contortion). The results were recorded as presence or absence of such an effect suggesting a positive effect (seizure), sedation, overt toxicity or neuromuscular effects at that concentration. In addition, at 30 min of exposure, larvae were tested for responsiveness by gently approaching each larva with the tip of a seeker, and scoring the response: 1 for an uncompromised escape response; 2 for an escape response following a touch; and 3 for an absence of an escape response despite a touch. After 1 h of exposure, mortality was assessed microscopically by the absence of a heartbeat.

Collectively, these data defined the MTC according to the following criteria: seizure incidence was a 'desired' effect to inform the choice of the top concentration; loss of posture should be seen in no more than 20% of animals (some loss of posture was often evident with epileptiform activity) at the MTC; no unusual morphology should be observed in any animals at the MTC as this was normally associated with moribund animals; an average responsiveness score of <2 was desirable at the MTC, as a score above this was generally indicative of sedation; and finally, no mortalities should be observed at the MTC. At the end of the MTC assessment period, all remaining larvae were humanely killed using an overdose of anaesthetic (benzocaine).

In addition to undertaking an MTC on all test compounds, a set of limited n-number MTC assessments were undertaken on range of commonly used (in aquatic toxicology) solvents, using the same classification criteria. This was undertaken to provide information on solvent toxicity in the event of low test compound solubility.

Following the determination of the MTC, the concentration regimen for the subsequent convulsant assay experiments extended from the MTC across a further 4 concentrations, each 50% of the preceding concentration, alongside a DWC. This was considered appropriate to provide a high-resolution concentration–response curve, whilst allowing accurate determination of the lower threshold for seizure induction.

2.3.2. Convulsive effect determination

The quantification of seizure-type locomotor activity was achieved using the VideoTrack for Zebrafish™ (Version 2.5 with background subtraction, Viewpoint, France) videotracking and movement quantification system. This consisted of a video camera (maximum 25 frames per second) to capture movements within predefined tracking areas (one area = one well of the microplate, internal diameter approximately 15 mm), and software to analyse data to give numbers of movements, distances travelled and total durations of movements, within 3 predefined 'speed' categories. These 3 speed categories were: low-medium speed movements of <5 mm/s; medium speed movements of between 5 and 20 mm/s; and high speed movements of >20 mm/s. For tracking the (black) animal, the detection threshold was set at 16 (on a scale of 0–255), although on occasions, this needed modification according to the contrast between the animal and the background.

For the classification of seizure-like locomotor activity, only the distance travelled in the high speed category (≥ 20 mm/s) was analysed. This approach was taken as in preliminary experiments, these very high speed movements were associated (in appreciable numbers) only with animals that had been exposed to convulsant compounds, and the distance data appeared to be representative of both counts and durations of such movements.

To assess convulsive potential, larval culture, plate loading, test compound preparation and exposure were as previously described for the MTC assessment, except that on 7 dpf (test day), only one microplate was exposed at a time to allow videotracking assessment. As with the MTC, test compound

physicochemical parameters were recorded and are also summarised in Table 2.

For technical reasons (high background detection), clear polystyrene 24 well microplates from Corning (UK) had to be utilized. These plates were leached overnight and externally frosted using acetone to reduce cross-talk and improve image contrast.

Prior to the exposure period, each plate was subjected to a 10 min baseline assessment period to ensure that each animal was tracking correctly, and also to highlight any unusual activity prior to the exposure period to aid the interpretation of the resultant data. Following this, the test compound was added to the wells of the first plate, and videotracking commenced after 5 min, for 1 h. All data collected during this 1 h period was divided into 10 min recording epochs to maintain data resolution, and highlight any temporal variations in activity.

During this exposure tracking period, any wells showing incorrect tracking (e.g. detection of the cell wall instead of the animal) were noted, and discarded from the final dataset prior to analysis. The same tracking procedure was undertaken for each plate in turn, and following the completion of each plate, larvae were humanely killed using an overdose of anaesthetic (benzocaine).

Fig. 1 shows an example screenshot from a 24 well plate in which larva have been exposed to a concentration range of PTZ.

2.4. Test compound classification and data analyses

No statistical analyses were undertaken on any of the MTC data, as these were considered qualitative in nature. Prior to statistical analyses on the videotracking data, two quality-control measures were applied. Firstly, any wells in which erroneous tracking was observed (as previously described), were discarded. Secondly, data were discarded for any wells for which an animal was undetected for ≥30% of any 10 min tracking period.

Following this, datasets were analysed using Mintab™ statistical analysis software. Initially each dataset was assessed for deviations from a normal distribution using the Anderson–Darling normality test, and for equality of variances using the Bartlett's statistic. If either of these parameters indicated that a dataset deviated from normality/equality of variances (the vast

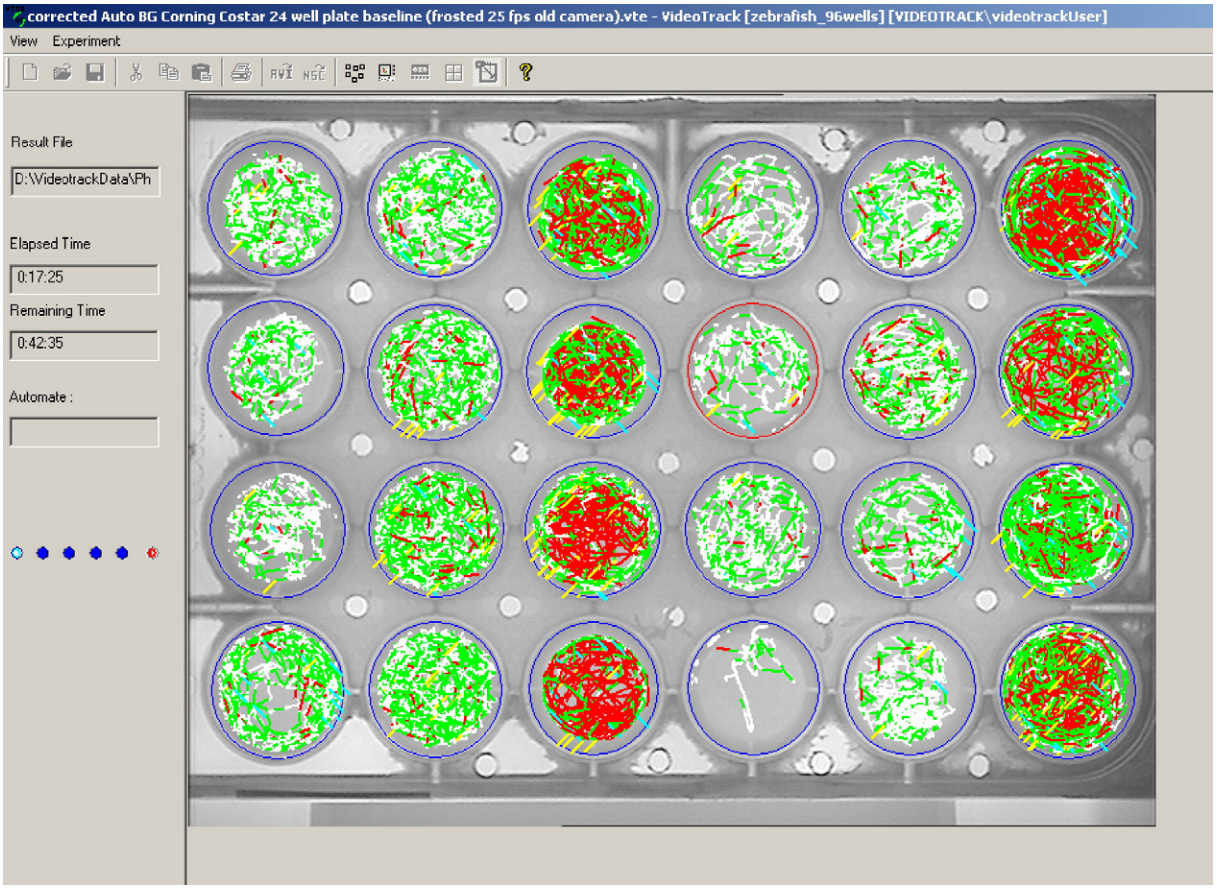

Fig. 1. Screen shot from the Viewpoint Videotrack for Zebrafish™ system. A 24 well plate is shown, with the tracking from one 7 dpf larva per well, after 17 min of exposure to PTZ (from left to right, each column shows 4 replicates of PTZ exposed animals at concentrations of 0.625, 1.25, 2.5, 0, 0.3125 and 5 mM). White tracking lines signify low speed movements (<5 mm/s), light grey (green) lines are medium speed movements (5–20 mm/s) and dark grey (red) lines are high speed movements (>20 mm/s), according to the thresholds set in the user interface. Images were captured using a 25 frames per second infrared detecting camera. Note the much greater levels of activity at higher concentrations of PTZ, especially in the high speed movement category (>20 mm/s). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

majority of cases), then non-parametric methods were applied. For all other datasets, parametric methods were appropriate. For parametric analyses, a one-way analysis of variance (ANOVA) was employed across the concentration range followed by a Dunnett's post-hoc test to assess which treatments differed from the DWC (both within each 10 minute epoch, and across the 1 h experimental period as a whole). For non-parametric datasets, a Kruskal–Wallis test followed by a Dunn's comparison of each treatment with the DWC was conducted on the same data.

The Lowest Observed Effect Concentration (LOEC) was defined as the activity at the lowest concentration of that test compound, from the videotracking data, that was statistically significantly different from that of the DWC group, when all time point data were combined. To distinguish between strong and weak positive control compounds, an overall convulsive assay 'rating' was given to each test compound, which was based on a 4-point scoring system. For this, each test compound obtained a '+' for each of the following: a statistically significant increase in activity compared with the DWC during 2 or more of the 10 minute recording epochs; a clear positive concentration-related response curve across the whole 1 h experimental period (e.g. see Fig. 2); a high magnitude of effect (greater than 4×DWC level for at least 2 of the 10 minute recording epochs; and a positive LOEC of ≤0.45 mM during 2 or more of the 10 minute recording epochs. From this, a score of '++++' signified a strong positive result, and a score of + a weak positive effect. If a test compound failed to attain any points, then it was assigned a '−'. In a small number of cases, a negative concentration-related response was observed (i.e. there was a statistically supported reduction in activity associated with increased test compound concentration), and here, a score of '−/−' was assigned. From this scoring system, the overall predictivity was judged against the success criteria proposed by ECVAM (European Centre for the Validation of Alternative Methods) for the validation of new toxicological methods (described by Genschow et al., 2002). These authors proposed that an overall predictivity of 65–74% was sufficient, 75–84% was good, and >85% was excellent.

The MTC:LOEC ratio was defined as the ratio between the MTC attained for that test compound, and the LOEC for all time points grouped. This figure was designed to give an indication of the difference between the concentration required to elicit a seizure, and the concentration required to elicit generalised systemic toxicity (a so-called 'seizure liability margin'). For a number of compounds, no MTC and/or LOEC were obtained (i.e. was >0.9 mM nominal). In such cases, a value of 1.8 mM (nominal) was assigned in subsequent calculations, to allow clear distinction from compounds at which an MTC or LOEC was attained at 0.9 mM. Acetylsalicylic acid gave a LOEC of 1.8 mM after a repeat run, and for continuity, this was also assigned a score of 1.8 mM to allow comparison with all other compounds at which the maximum concentration tested was 0.9 mM.

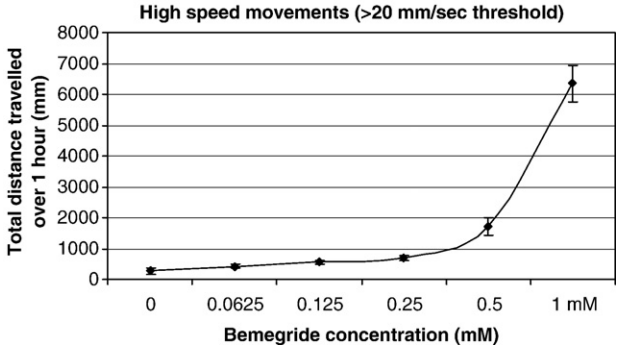

Fig. 2. An example positive concentration–response curve generated following exposure to bemegride, and tracking for 1 h. Data shown are the total distance travelled over 1 h, in the high speed category (>20 mm/s), by larvae following exposure to a range of concentrations of bemegride. Data are mean ± standard error, $n=12$ animals per concentration.

The estimated pharmacological potency of the compound was calculated as the maximum distance travelled in the high speed category (as an average across the whole 1 h), divided by the concentration at which that activity was observed. This index was only calculated for those compounds that exhibited a positive concentration-related response curve.

In all cases, a significance level of $p<0.05$ was considered indicative of a statistically significant difference between a treated group and the relevant control group (both within each 10 minute recording epoch and when all time points were grouped together).

3. Results

3.1. Maximum Tolerated Concentration (MTC) determination

The data used to set the MTC for all test compounds and solvents is summarised in Table 3. From these data, it can be seen that with many of the test compounds under investigation, there were few signs of toxicity even at the top nominal concentration tested. This meant that no MTC was determined, and as such seizure liability was measurable only at this concentration or at the limit of water solubility for a given compound.

3.2. Convulsive effect determination

Data generated from the convulsant assay are summarised in Table 4. The tabulated data include the test compound identity, pharmacological class, (suggested) mechanism of seizure induction, and overall convulsant assay score. In addition, the LOEC (as the mean of all time points) and MTC:LOEC ratios are shown, along with the calculated relative pharmacological potency of each test compound. A wide range of seizure liabilities was observed ranging from strong concentration-dependent convulsive effects (e.g. Table 3 — physostigmine salicylate), to a complete absence of any discernable effect up the maximum concentration tested (e.g. protriptyline hydrochloride). In addition, two compounds showed some evidence of a negative concentration-related response: bupropion hydrochloride and 4-aminophenyl sulfone. Fig. 2 shows an example positive concentration–response curve following exposure to bemegride.

The LOEC:MTC ratios ('seizure liability margin') obtained for each of the test compounds revealed a wide range of values:

Table 3
Summary of data used to define the MTC for each test compound along with that used to define the MTC of some commonly used solvents

Test compound	MTC well nominal concentration (mM)	% Mortality	Mean response score	% Showing abnormal posture	% Showing unusual morphology	% Appearing to exhibit PTZ-like locomotion
Penicillin G (sodium salt)	**>0.9**	0	1.00	0	0	10
Maprotiline hydrochloride	**0.014**	0	1.17	0	0	0
Bemegride	**>0.9**	0	1.56	0	0	77.77
Bicuculine methoiodide	**>0.9**	0	1.50	0	0	58.33
Strychnine hemisulphate	**0.014**	0	1.17	0	0	8.33
Cefazolin sodium	**>0.9**	0	1.00	0	0	41.67
Isoniazid	**>0.97**	0	1.17	0	0	8.33
Physostigmine salicylate	**0.49**	0	1.92	8.33	0	25
Amoxapine	**0.028**	0	1.63	37.50	0	100
Enoxacin	**>0.9**	0	1.75	0	0	16.67
Semicarbazide hydrochloride	**>0.9**	0	1.18	0	0	60
Bupropion hydrochloride	**0.056**	0	1.25	16.67	0	0
Picrotoxin	**0.24**	0	2.25	33.33	0	83.33
Pentylenetetrazole	**>0.9**	0	1.50	0	0	75
Aminophylline hydrate	**0.9**	0	1.38	25	25	100
4-Aminopyridine	**>0.9**	0	2.00	0	0	75
Acetylsalicylic acid crystalline	**>0.9**	0	1.00	0	0	0
Verapamil hydrochloride	**0.09**	0	1.25	8.33	0	0
Naproxen	**>0.45**[a]	0	1.50	10	0	30
Dexamethasone	**>0.9**[a]	0	1.38	0	0	83.33
(−)-Scopolamine hydrochloride	**>0.9**	0	1.58	100	0	8.33
Tobramycin	**>0.9**	0	1.83	0	0	0
Acetaminophen	**>0.9**	8.33[b]	1.58	8.33[b]	0	0
4-Aminophenyl sulfone	**>0.45**[a]	0	1.83	0	0	0
Protriptyline hydrochloride	**0.009**	0	1.08	0	0	0
DMSO	**4% v/v**	0	1.5	0	0	0
Ethanol	**1% v/v**	0	1.5	0	0	0
Acetone	**1% v/v**	0	1.25	0	0	0
DMF	**1% v/v**	0	1.5	0	0	0
Trigol	**4% v/v**	0	1.5	0	0	0
Methanol	**4% v/v**	0	1.75	0	0	0

DMSO (dimethylsulphoxide), DMF (dimethyl formamide), Trigol (triethylene glycol). > Signifies that the MTC was not reached at that concentration according to the outlined classification criteria.

[a] Limit of solubility.

[b] Larva likely to have been damaged upon transfer to test solution.

for example the highest was for the positive control physostigmine salicylate (32) suggesting a wide margin between general toxicity and convulsant effects; and the lowest was for the negative control protriptyline hydrochloride (0) suggesting a narrow margin. This pattern was not true, however, for all positive and negative controls. For example, a number of low scores were obtained for designated positive controls, and tobramycin, a designated negative control, gave a value of 16. This highlights that caution should be applied when using this proposed 'scoring system' for assessing seizure liability at a glance.

In terms of the estimated pharmacological potency of positively acting test compounds, there was also a wide range of values obtained, with high potency recorded for compounds such as amoxipine and strychnine hemisulphate, through intermediate potency exhibited by compounds such as bemegride and 4-aminopyridine, to low potency shown by compounds such as semicarbazide hydrochloride and pentylenetetrazole.

From the data obtained in the convulsant assay, there were a number of compounds that were 'misclassified' based on literature-reported seizure liability, and our method of classification. The compounds that were considered 'positive' controls according to the literature evidence, but failed to evoke a positive effect in the convulsant assay at these concentrations were penicillin G (sodium salt), cefazolin sodium, isoniazid and bupropion hydrochloride. Similarly, a number of compounds that were considered 'negative' controls according to the (relative) absence of reports of seizure induction in the literature returned a positive response in the convulsant assay. These were naproxen, verapamil hydrochloride and tobramycin. Possible reasons for these misclassifications are detailed in the Discussion section.

The overall predictivity of the convulsant assay was 72%, consisting of a positive control predictivity rate of 77% (4 'false' negatives out of 17), and a negative control prediction rate of 63% (3 'false' positives out of 8).

3.3. Convulsant assay reproducibility

The inter-assay coefficient of variation (COV) for the high speed distance data measured in dilution water control animals, during all convulsant assays undertaken here (including 1 solvent control group), was 59%, based on 34 replicate experiments (mean of all time points). Furthermore, the actual values

Table 4
Summary of data obtained from the Zf convulsant assay along with test compound identity and pharmacological details

Test compound	Pharmacological class	(Pro)convulsive mechanism	Convulsive assay rating	LOEC (mM) all time points	MTC:LOEC score	Estimated pharmacological potency
Penicillin G (sodium salt)	β-lactam antibiotic	GABA-A antagonism	–	>0.9	1	–
Maprotiline hydrochloride	Tetracyclic antidepressant	Proposed antihistamic antimuscarinic	++	0.014	1	4441
Bemegride	Analeptic	Proposed GABA-A antagonism	++++	0.22	8	1175
Bicuculine methoiodide	Analeptic	GABA-A antagonism	++	0.9	2	313
Strychnine hemisulphate	Analeptic	Glycine antagonism	+++	0.0035	4	9264
Cefazolin sodium	Cephalosporin antibiotic	Proposed GABA-A antagonism	–	>0.9	1	–
Isoniazid	Anti-tuberculosis antibiotic	GABA depletion (GAD inhibition)	–	>0.97	1	–
Physostigmine salicylate	Cholinesterase inhibitor	Cholinesterase inhibition	++++	0.0151	32	5292
Amoxapine	Tricyclic antidepressant	GABA interaction and effects on Ca	++++	0.014	2	13286
Enoxacin	Quinolone antibiotic	GABA-antagonism	++++	0.45	4	450
Semicarbazide hydrochloride	Amine oxidase inhibitor	Amine oxidase inhibition	++	0.9	2	174
Bupropion hydrochloride	Tricyclic antidepressant	Blocks serotonin, dopamine and noradrenalin reuptake	−/−	–0.028	–2	–
Picrotoxin	Analeptic	GABA-A antagonism	++++	0.03	8	5883
Pentylenetetrazole	Analeptic	GABA-A antagonism	++	0.9	2	96
Aminophylline hydrate	Methylxanthine bronchodilator	Adenosine antagonism	++++	0.45	2	702
4-Aminopyridine	Avicide	K+ channel blocker, enhances glutamate level	++++	0.45	4	964
Acetylsalicylic acid crystalline	NSAID	Hypoglycaemia and metabolic acidosis	+	1.8	1	122
Verapamil hydrochloride	L-type Ca^{2+} channel blocker	–	++++	0.028	3	3739
Naproxen	NSAID	–	+++	0.45	4	186
Dexamethasone	Corticosteroid	–	–	>0.9	1	–
(−)-Scopolamine hydrochloride	Antimuscarinic	–	–	>0.9	1	–
Tobramycin	Aminoglycoside antibiotic	–	++++	0.113	16	1193
Acetaminophen	Anti-inflammatory	–	–	>0.9	1	–
4-Aminophenyl sulfone	Antibiotic	–	−/−	>0.9	1	–
Protriptyline hydrochloride	Tricyclic antidepressant	–	–	>0.9	0	–

> Signifies LOEC not attained. For the MTC:LOEC score, any compounds in which an MTC or LOEC was not attained was given a concentration value of 1.80/1.94 mM (dependent on supplied compound weight), to distinguish from those compounds for which a value at 0.9 mM was attained. Acetylsalicylic acid attained a LOEC at 1.8 mM in a repeated study. Grey shaded boxes represent designated positive control test compounds based on literature evidence. Convulsive assay rating based on the scoring system: + for a statistically derived positive Lowest Observed Effect Concentration (LOEC) at ≥2 time points (mandatory); + for a clear positive concentration-related effect; + For a high magnitude of effect (≥4 × DWC at least 2 time points); + for a positive LOEC of ≤0.45 at least 2 time points; - No evidence of a positive effect (i.e. none of the above); −/− Evidence of a negative concentration–response. NSAID ~ non-steroidal anti-inflammatory drug, GABA ~ γ-aminobutyric acid.

obtained for the DWC animals were very low compared to most test compounds that showed a clear positive effect in these assays, and as such this background variability did not appear to be a hindrance to detecting a positive effect.

From the experiments undertaken using PTZ, further assay reproducibility data can be calculated. At the subthreshold PTZ concentration of 0.3125 mM (see Section 4.4), the COV for the same parameter was 52%, based on 26 replicate experiments (mean of all time points data normalised to the corresponding DWC). The COV for a 0.9 mM concentration of PTZ was 43% based on 4 replicate assays undertaken on separate occasions (calculated for 0.9 mM assuming a linear concentration–

response between 0.625 and 1.25 mM where appropriate, with the mean of all time point data normalised to the corresponding DWC).

Example intra-assay variability figures, from an experiment employing PTZ as the test compound, were 28% at high (5 mM), 35% at medium (1.25 mM) and 47% at low (0.3125 mM) test compound concentrations (mean of all time points, $n=12$ animals).

4. Discussion

4.1. Overview

Here we present validation data from a novel Zf embryo-larval assay intended for the seizure liability screening of development drugs. The studies undertaken were an MTC, and a single compound concentration–response (convulsant) assay. Overall, the MTC assay used appeared to be suitable for identifying a suitable concentration range to use in the subsequent convulsant assay. Secondly, the convulsant assay appeared to offer "sufficient" predictivity of eventual (pre)clinical outcome, considering the potential for throughput, with an overall prediction rate of 72%. The validity of this experimental approach, its applicability to the front-loading of drug safety pharmacology assessment, and possible reasons for the misclassifications are discussed.

4.2. MTC assessments

The MTC endpoints used here appeared to offer an appropriate qualitative method for determining the toxicity and sedative properties of each of the test compounds. Such a 'pre-screen' is imperative with this type of work, as any general systemic toxicity, neuromuscular effects or sedation is likely to have an seizure-independent effect on the locomotory performance of the larvae. There are few published studies directly proposing the application of any Zf-based toxicological/pharmacological endpoint screens for drug safety assessment (e.g. Parng et al., 2002; Ton & Parng, 2005), and of these even fewer in which details of MTC-type range finding assays are outlined. Although the primary aim was to screen for anti-convulsant efficacy, rather than drug safety, Berghmans et al. (2007) did employ an MTC in their PTZ-induced Zf seizure model. Interestingly, these authors used a startle response amongst the endpoints assessed, although details of the other parameters used to classify the MTC were not given.

The MTC endpoints/classification criteria proposed here appear to be appropriate in most cases, although additional endpoints and/or classification criteria could be added/modified according to the ultimate assay being applied, or in light of details regarding the mode of action of the compound under investigation. For example, in the case of bupropion hydrochloride, a statistically significant negative concentration–response was observed in the convulsant assay, which was not detected by the stimulus response test during the MTC. This could mean that exposure to bupropion hydrochloride, at these concentrations, resulted in the inhibition of spontaneous motor activity without compromising any stimulus response mechanisms (see also below). This is perhaps an area in which an additional endpoint could be incorporated, for example the assessment of spontaneous locomotion during the MTC, although it should be ensured that such changes do not affect the simplicity and relatively high throughput of the MTC method, both of which are key advantages of the assay.

4.3. Overall performance of the convulsant assay

After compound unblinding, it was revealed that of the 25 compounds supplied, 17 were considered positive regarding epileptogenic potential, and 8 were considered negative. The classifications were based on seizure incidences in the literature, but with no consideration of potency, specificity, seizure frequency, exposure length or seizure mechanism. Similarly, in the case of negative controls, the lack of seizure liability was assumed through the relative absence of accounts only within the literature that was consulted. With these considerations in mind, the overall predictive power of the convulsive assay was 72%, comprising a positive control prediction rate of 77% (4 'false' negatives out of 17 positives), and a negative control prediction rate of 63% (3 'false' positives out of 8 negatives). Therefore, based on the original success criteria we used (Genschow et al., 2002), the overall predictivity was considered "sufficient" (65–74% sufficient, >75% good).

For general comparison with another frontloaded safety pharmacology endpoint, a screen for human ether-á-go-go related gene (hERG) as a predictor of torsadogenic risk exhibited 84% predictivity following the imposition of a 30-fold margin cut-off between hERG IC_{50} and unbound maximum therapeutic plasma concentration (Redfern et al., 2003).

As stated, a number of compounds were 'misclassified' according to their control classification: false negatives were penicillin G (sodium salt), cefazolin sodium, isoniazid and bupropion hydrochloride; and false positives were verapamil hydrochloride, naproxen and tobramycin.

4.4. False negatives

Penicillin G (a β-lactam antibiotic, used as sodium salt), despite being a widely reported convulsant in both animal models and the clinic (Grondahl & Langmoen, 1993; Ruffmann et al., 2006), failed to elicit a positive response in the Zf convulsant assay. There are a number of possible reasons for this insensitivity. Firstly, the incidence of seizures in the clinic is reportedly low (e.g. <1%), and often associated with predisposed patients and/or the administration of high doses (Zaccara et al., 1990). Secondly, the oral bioavailability of penicillin G in mammals is known to be low (e.g. around 30%; Parfitt, 1999), resulting in low systemic exposure at a given dose. This may also be the case in Zf, and would be supported by the absence of effects in the MTC (up to 0.9 mM). Certainly, pharmacokinetic measurements of test compound absorption, distribution, metabolism and elimination in embryo-larval Zf would be extremely valuable for interpreting the data generated by such assays, particularly where negative results are obtained (Berghmans et al., 2007; Parng et al., 2002).

Another potentially confounding factor influencing the response to penicillin G, and indeed the performance of the Zf assay as a whole, is the issue of test solution stability. For example, Vella-Brincat et al. (2004) reported that penicillin G exhibited a 40% reduction in activity after 24 h at 26 °C. Although the stability of penicillin G is unlikely to be a major issue in the current study as all solutions were freshly prepared, low test compound stability combined with poor bioavailability may be sufficient to reduce plasma exposure to sub-seizure threshold levels.

In the case of the cephalosporin antibiotic cefazolin sodium, it is not known why the Zf convulsive assay was not sensitive to the effect of this known convulsant. The absence of an MTC and any obvious symptoms of toxicity at up to 0.9 mM, however, again suggest that systemic exposure may have been low in these animals. Indeed, gastrointestinal tract absorption is reported to be low (Parfitt, 1999), as is the (often high dose associated) clinical incidence of seizures (Zaccara et al., 1990). Indeed, the concentrations employed here were relatively low when compared with Human Plasma Therapeutic Concentrations (HPTC of 315 μM; Regenthal, Krueger, Koeppel & Preiss, 1999). In this respect, it is worth noting that the top tested concentration would be user-definable during pre-clinical use, and as such could be related to the predicted HPTC or any relevant *in vitro* EC_{50s} (e.g. 100 times the predicted HPTC in the MTC, dependent on inherent toxicity).

In common with cefazolin sodium, the absence of an MTC for isoniazid (an anti-tuberculosis antibiotic) and a lack of any obvious symptoms of toxicity up to 0.9 mM perhaps suggest that systemic exposure was also low, or that the reportedly low clinical incidence of seizure resulted in a negative classification. Certainly, the relatively close amino acid sequence homology between Zf and human glutamic acid decarboxylase (GAD) enzymes (between 71.9 and 95.7%), and expression of GAD isozymes in Zf CNS tissue as early as 1 dpf (Martin, Heinrich & Sandell, 1998) would suggest the capacity for some pharmacological activity, although differences in protein expression levels may infer differences in terms of species sensitivity.

As with the other false negatives, the reported clinical incidence of seizures associated with the adrenergic/dopaminergic reuptake inhibiting antidepressant bupropion hydrochloride (Jefferson, Pradko & Muir, 2005) is low (e.g. <1%. Ruffmann et al., 2006), and often concomitant with predisposition, high doses, and chronic administration (Ross & Williams, 2005). In addition, oral bioavailability is also reportedly low (Parfitt, 1999). Consequently, any of these factors could have served to mask the Zf seizure liability of this compound. Interestingly, there were overt neuromuscular effects (manifested as abnormal posture) apparent at high concentrations during the MTC study, and so comparatively low concentrations were used in the convulsive screen run. Consequently, the low occurrence of seizures may have been exacerbated by the need to use lower concentrations, thus further decreasing the chances of attaining a positive response in these animals. Furthermore, bupropion hydrochloride has been associated with anti-seizure activity in mice at low concentrations (Foley, DeSanty & Kast, 2006), which may explain the apparent negative concentration-related relationship shown here.

The observed neuromuscular effects of bupropion hydrochloride could also have affected locomotor activity in a seizure-independent fashion. In support, the modulation of fish swimming activity has been relatively well documented in response to the application of serotonergic and dopaminergic compounds (e.g. Kemnitz, Strauss, Hosford & Buchanan, 1995; Svensson, Woolley, Wikstrom & Grillner, 2003), and preliminary experiments using the dopamine antagonist (and known convulsant) chlorpromazine failed to induce seizures even at the MTC (Winter et al., unpublished data). Consequently, it is conceivable that dopamine modulators may have seizure-confounding effects on Zf locomotion, and as such this could preclude their applicability in this assay.

Another potential explanation for the absence of seizures associated with exposure to bupropion hydrochloride is the issue of metabolism. Bupropion hydrochloride is known to be metabolised extensively in several mammalian species, with the formation of a range of metabolites of differing pharmacological activity (Suckow, Smith, Perumal & Cooper, 1986). Consequently, it is conceivable that the metabolism of this compound in the Zf contributes to its effects in this species, as with other species, and as such has served to reduce the level of pharmacological activity exhibited.

Regarding the issue of false negatives, additional work was undertaken in our laboratory on a PTZ-induced subthreshold proconvulsive assay to investigate the effect of the test compounds on seizure induction in predisposed individuals. However, the variability in responses shown between animals, to the subthreshold concentrations of PTZ and the test compounds, limited the usefulness of this approach (Winter et al., unpublished data).

With respect to all of these suggested reasons for misclassifications, if this assay were to be deployed as a medium throughput *in vivo* screen in early drug discovery, it is likely that some data on water solubility, partition coefficients, stability, and potentially on absorption/bioavailability, primary pharmacodynamics and inherent toxicity may be available. Consequently, the assay could be tailored for the requirements of the test compound: for example, alternative routes of administration could be applied (e.g. microinjection), solvents could be used, longer exposure times employed or the temperature of the assay adjusted to increase compound stability. Furthermore, some classes of compound may be considered unsuitable for use in the assay, for example dopamine modulators due to potential confounding effects on locomotor activity. Therefore, it is conceivable that the overall predictivity of the seizure liability assay could increase when positioned in such a way.

4.5. False positives

Of the false positives, verapamil hydrochloride is a potent L-type calcium channel blocker, a class of compounds known to inhibit seizures in rodent models (Campbell & Hess, 1999). Despite this, there have been a small number of positive associations with seizure-like activity (e.g. Parfitt, 1999; Passal & Crespin, 1984), and also reports of Parkinsonism-type tremor in elderly patients following long-term administration (Parfitt, 1999). Even more interestingly, Popoli, Pezzola, Sagratella,

Zeng and Scotti de Carolis (1991) revealed epileptiform bursting in the rat hippocampal slice preparation at concentrations of 1.5–2 mM. In this respect, it is possible that the zebrafish is more sensitive than existing mammalian models, to the effects of this compound.

The non-steroidal anti-inflammatory drug (NSAID), naproxen, gave a relatively strong positive effect in the convulsive assay despite being considered a negative control compound. In support, although regarded as having a safe seizure profile, a small number of seizures associated with naproxen (and other NSAIDs) have been recorded in the literature in association with metabolic acidosis (Martinez, Smith & Frankel, 1989). It is conceivable that the same mechanism could have played a role here, especially if the zebrafish was more sensitive to the effects of weak acids and associated acidosis.

The aminoglycoside antibiotic tobramycin was also classified as a strong positive in the convulsive assay, although the majority of previously published literature suggests a safe seizure profile. Despite this, other members of this class are well known convulsants, and as such it may be that the Zf model is particularly sensitive to the CNS effects of aminoglycosides. Indeed, there are a limited number of previously reported positive associations with seizure attributed to high doses of tobramycin in the literature (Wold, Turnipseed, Broddle & Owen, 1977), and the concentrations used here were certainly high compared with human plasma concentrations (toxic at 25 μM in humans; Regenthal et al., 1999).

Finally, it is unlikely that the positive response exhibited by these proposed negative control compounds was due to irritancy, as none have been associated with such in the clinic. For example, tobramycin does not cause ocular irritancy and is used as an inhalational antibiotic in cystic fibrosis patients (Salva, Costa, Andreu, Notivol & Martinez, 1999); and verapamil has been shown to reduce contact hypersensitivity in the mouse (Wille, Kydonieus & Kalish, 1999). Naproxen has been publicised as a potential gastrointestinal irritant (as with other NSAIDs, Devane, Butler & Mulligan, 1996), although this is negated somewhat by other studies in which naproxen has been reported as not inducing contact dermatitis (Matthieu et al., 2004). Having said this, it is clear that assay performance needs to be assessed further with a wider range of negative control test compounds, than has been employed in this initial validation exercise.

4.6. Assay performance compared with other seizure liability screens

The sensitivity of the current WIK-strain Zf assay can be compared qualitatively with those of Baraban et al. (2005), and Berghmans et al. (2007), as all three studies employed pentylenetetrazole (PTZ). The current convulsant screen showed a statistically defined LOEC of 0.9 mM (nominal, mean across all time points), which compares favourably with the data from the previous studies:Baraban et al. (2005) tested an effective concentration range of 2.5 to 15 mM in TL-strain Zf larvae, although the LOEC was not clear; and Berghmans et al. (2007) reported an approximate 2-fold increase in total distance moved at 5 mM compared with control, in WIK-strain animals. Interestingly, both previous studies employed total distance moved as the defining parameter, and our data suggests that other parameters (e.g. consideration of higher speed movements only) can result in an increase in sensitivity, and potentially specificity. Moreover, the use of different strains in at least two of the three published Zf (anti)seizure studies raises the issue of inter strain variability, a factor shown to be important in the response of Zf to the developmental effects of ethanol (Loucks & Carvan, 2004).

Comparisons between published Zf assay data regarding other convulsant test compounds is not possible, however, as although Baraban et al. (2007) mentioned use of 4-aminopyridine and picrotoxin, the responses obtained and concentrations used were not discussed.

The sensitivity of the Zf convulsant assay can also be compared with other potential screens for seizure liability, for example the rat hippocampal slice preparation. This *in vitro* assay offers some characteristics that are amenable for use as a prescreen, such as medium throughput capability (at least compared with rat *in vivo* proconvulsive assays) and low compound requirements. In terms of comparative sensitivity, the current Zf convulsant screen exhibited a LOEC of 0.9 mM for PTZ (nominal, mean across all time points), as opposed to the spontaneous bursting activity in the rat hippocampal slice at 2–10 mM (Baraban et al., 2005); a LOEC of 0.03 mM picrotoxin compared with epileptiform activity in the slice at 0.05 mM (Benini & Avoli, 2005); a LOEC of 0.45 mM for 4-aminopyridine compared with epileptiform activity at 0.05 mM in the slice (Benini & Avoli, 2005); and a LOEC of 0.45 mM for aminophylline compared with 0.1 mM antagonizing the depression of epileptiform activity caused by propofol in the slice (Ohmori, Sato & Namiki, 2004). Collectively, these data would suggest that the Zf assay has broadly similar sensitivity when compared with the rat hippocampal slice assay, especially considering that the Zf assay represents a true *in vivo* test system. In this respect, further work in comparing in-house data from the two assays is currently under way, in addition to methodological refinement in an attempt to improve predictivity further.

4.7. Concluding remarks

Assuming the predictivity of the Zf convulsant assay was suitable for inclusion as an early drug discovery screen for seizure liability (and with any model, some misclassifications are inevitable), it is worth discussing the positioning of such an assay in a screening cascade. It is likely that this type of screen would be the first tier in the cascade, possibly preceded by *in silico* structure activity relationship models. It is also conceivable that any 'positives' from the Zf convulsant assay would then pass into a slightly lower-throughput, second tier assay, for confirmation prior any mammalian *in vivo* studies. A suitable assay in this respect could be the rat hippocampal slice assay. Related to this, there is clearly a need to minimise the false negative classification rate, something that is likely to be heavily impacted with increased knowledge of the pharmacokinetics of compound exposure in larval fish. This type of cascade has been suggested previously by Berghmans et al. (2007) in respect of their anticonvulsant screen, and appears to be the most

appropriate strategy to adopt. Indeed, the key attributes of the Zf convulsant screen are its *in vivo* nature and throughput, and as a tool for reducing late stage attrition rates, this model could prove highly useful in the drug development process.

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