The use of zebrafish mutants to identify secondary target effects of Acetylcholine esterase inhibitors

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Abstract

We are confronted with a large and steadily growing number of bioactive compounds, including drugs, pesticides and industrial by-products. The assessment of target specificity and potential toxic effect on human health and the environment generates a strong demand for robust and cost-effective models with high predictive power. Here, we investigate the potential of the zebrafish embryo as a whole-organism, vertebrate model to assess the specificity of compounds that are known to inhibit Acetylcholinesterase (AChE). Inhibitors of AChE are widely used as drugs and pesticides. By application of simple assays and comparison with the phenotype of embryos with genetic lesions in the \textit{ache} gene, we demonstrate that only one of the AChE inhibitors (galanthamine) reproduces the phenotype of \textit{ache} mutant embryos. The other compounds produced additional effects indicating secondary targets. Our work demonstrates the power of a genetic system for toxicological evaluations. The combination of genetics and transgenesis with the other experimental virtues of the zebrafish embryo, such as small size and low cost, offers a whole organism platform for medium to high throughput compound testing.

Keywords: zebrafish, acetylcholinesterase, inhibitors, animal model, secondary drug targets
Introduction

A powerful methodology in development of bioactive chemicals has arisen from the synergy between structural information on the target molecules and combinatorial chemistry (Norin and Sundstrom, 2001). While the capability to synthesise novel compounds increased enormously in recent years, technologies to assess the biological activity and target specificities of these novel molecules did not match the pace of the modern synthesis capabilities (Balls, 2002). A good drug is potent and highly specific with a well-defined target. Ideally, the phenotypic consequences of an inhibitory compound should be the same as that of a genetic mutation causing loss-of-function of the drug target. Simple genetic models, such as yeast (Marton et al., 1998) or C. elegans (van Kessel et al., 1989) may help to identify primary and secondary targets but they are too distant in evolutionary time to provide a comprehensive toxicological profile for vertebrates.

Besides attracting ethical concern, the use of mammals is expensive and labour intensive, limiting an application in large-scale screening programs. Embryos and larvae of the lower vertebrate zebrafish (Danio rerio) may offer a cheap and effective alternative. Zebrafish embryos/larvae are small and can be obtained in large numbers throughout the year at a fraction of the cost of a mouse or a rat. Zebrafish embryos develop outside of the mother allowing systematic compound screens (Peterson et al., 2000) from earliest life stages onwards. Moreover, a large number of mutations affecting signalling pathways and physiological processes relevant to human pathology and pharmacology are available (Barut and Zon, 2000; Driever et al., 1996; Haffter et al., 1996; Langheinrich et al., 2002; Stern and Zon, 2003).

We have investigated here the application of wildtype and mutant zebrafish embryos in drug testing by phenotypic comparisons with a mutation in the acetylcholinesterase (ache)
AChE catalyses the hydrolysis of the neurotransmitter acetylcholine (ACh) and is crucial for cholinergic neurotransmission (Soreq and Seidman, 2001). AChE is a target of a plethora of toxins including snake venom, insecticides and chemical weapons (Soreq and Seidman, 2001). The pharmacological use of AChE inhibitors includes treatment of the autoimmune disease *myasthenia gravis*, glaucoma and Alzheimer’s disease (Soreq and Seidman, 2001).

We have previously identified a recessive mutation in the *ache* gene of zebrafish (Behra *et al.*, 2002). Zebrafish AChE is highly related to that of mammals (Bertrand *et al.*, 2001). A point mutation leading to replacement of a conserved serine at position 226 by an asparagine abolishes AChE enzymatic activity in homozygous mutant embryos. Mutants have impaired motility and develop a severe myopathy during the second day post-fertilisation (Behra *et al.*, 2002). The myopathy is due to an over-activation of the muscle by accumulating ACh; genetic impairment of the nicotinic acetylcholine receptor (nAChR) on the muscle cell prevents the development of the myopathy in *ache* mutants (Behra *et al.*, 2002).

The zebrafish presents a unique situation among vertebrates as the AChE is the only ACh hydrolysing enzyme in this organism. Homozygous *ache* mutant embryos and larvae lack detectable ACh hydrolysing activity and heterozygous adults show 50% of the ACh cleaving activity of wildtype siblings (Behra *et al.*, 2002). Moreover, the zebrafish genome does not encode a functional butyrylcholine esterase, a related enzyme that can also hydrolyse Ach (Bertrand *et al.*, 2001).

The phenotypic traits of the *ache* mutant provide criteria for a vertebrate that lacks ACh hydrolysing activity entirely and can thus serve as comparative standards for the specificity of AChE inhibitors. In this report, we used the zebrafish embryo as a whole organism model to test the specificity of chemicals. We chose four drugs known to be AChE
inhibitors (Cousin et al., 1998; Giacobini, 1998) and compared their effects with the consequences of genetic elimination of AChE activity. Only one test compound, (galanthamine, (GAL)) reproduced the ache mutant phenotype. The other three compounds (eserine (ESE), tacrine (TAC) and edrophonium (EDRO)) showed effects in addition to those observed in ache mutants demonstrating secondary target effects. In general terms, this work illustrates the power of the combination of zebrafish genetics with toxicological testing in the verification of drug target specificity and demonstrates the potential of the zebrafish embryo in the assessment of the toxicity of chemicals.
Experimental protocol

Fish stocks and embryo production

The wildtype zebrafish line wt ABO is a cross between fish purchased from a pet shop and the AB strain (Eugene, Oregon) and was maintained as inbred line for several years in the lab. Fish were bred and raised as described (Westerfield, 1993). The origin of the ache and the nicl mutant and the –3.1ngn1:gfp transgenic zebrafish were described (Behra et al., 2002; Blader et al., 2003; Sepich et al., 1998). To generate, transgenic ache mutant zebrafish, ache +/- fish were mated with –3,1ngn1:gfp transgenic fish and the off-spring with an ache +/- ; -3.1ngn1:gfp +/- genotype was crossed to ache +/- animals. Animal care and experimentation were done in compliance with French and European law (Authorization B67-218-5 from 23.02.1999).

Inhibitor treatment

Embryos were treated with the inhibitors (GAL (1,2,3,4,6,7,7a,11c-octahydro-9-methoxy-2methylbenzofuro[4,3,2-efg][2]benzazocin-6-ol), ESE (1’methylpyrrolidino (2’:3’:2:3) 1,3 dimethylindolin-5-yl N-methylcarbamate), TAC (1,2,3,4-tetrahydro9-aminoacridine) and EDRO ((3-hydroxyphenyl) dimethylethylammonium bromide) diluted in 10% Hanks’s solution (Westerfield, 1993) from the 5-somite stage onwards. Solutions were replaced each day. Unless stated otherwise 10^-4 M ESE, 10^-3 M GAL, 10^-2 M EDRO and 10^-5 M TAC were used. These concentrations were determined in motility assays and by morphological inspection of embryos to phenocopy the ache mutant phenotype with respect to motility most closely. Higher concentration of TAC and EDRO caused necrosis in the neural tube and were thus regarded to have a general toxic effect on the embryos.

Motility assays
For video analysis, embryos or young larvae were embedded in low melting point agarose as described (Behra et al., 2002; Westerfield, 1993). To allow recording of motility, the trunk and tail of embedded embryos was freed of agarose. Embryos were mounted under a dissecting microscope (Leica MZ FLIII) equipped with a CoolSnap video camera. Embryos were touched with a blunt metal rod at the body flank to trigger the startle response and swimming movements. Touch-evoked movements of embryos were monitored using the video camera recording 50 frames per second. Representative frames were overlaid to indicate the movement.

For measurement of the duration of swimming, individual embryos were placed in a 5 cm petridish. After touching the embryos, movement was recorded with the video camera. The duration of the swimming movements was measured with a stop watch from the recorded film. In these experiments only the swimming movements were scored. However, most inhibitor-treated and ache mutant embryos exhibited the startle response entailing a very fast bend of the body axis in the 10 millisecond range followed by a slow straightening of the body axis over the next 200 milliseconds. This movement was not scored as swimming movement since, with the exception of the initial bend, it does most likely not reflect an active movement. The data sets were subjected to statistical analysis with the Student’s t-test to assess the significance of the similarities and differences between wildtype, ache mutant and inhibitor treated embryos.

Measurement of AChE enzymatic activity

Detection of AChE activity in situ was adapted from Karnovsky (Karnovsky and Roots, 1964). For IC$_{50}$ measurements, AChE activity in supernatants of 4 day old larvae was carried out in triplicate as described (Behra et al., 2002; Bertrand et al., 2001; Ellman et al., 1961). Larvae were killed by incubation on ice for 5 min. Extract was prepared by passing
embryos ten times through a 26-gauge syringe in LST buffer (20 mM Tris/HCl pH 7.0, 5 mM EDTA, 1% Triton X-100). Afterwards extracts were centrifuged 1 min at 10000 rpm and supernatants were diluted in 100 mM phosphate buffer pH 7.0, 0.5 mM DTNB and pre-incubated with varying concentrations of inhibitors for 10 min (TAC, GAL, EDRO) or 60 min (ESE). Acetylthiocholine was added to a final concentration of 1 mM before real-time determination of acetylthiocholine hydrolysis at 412 nm (Ellman et al., 1961). IC$_{50}$ were deduced from the plots of activity versus inhibitor concentration from three independent measurements at every inhibitor concentration.

Measurement of AChE activity in 2 day old ESE-treated embryos was carried essentially as described above except that inhibitor was not added to the extract.

**Confocal microscopy and birefringence**

Transgenic embryos were mounted in Aqua Polymount and were analysed in lateral views with a confocal microscope. Optical sections were taken through the entire embryo. Stacks of images were used in 3D reconstruction, to generate lateral and transverse projections. Image processing was carried out with the programs tcstk and timt (Behra et al., 2002), further details and the programs are available from Jean-Luc Vonesch (jlv@igmbc.ustrasbg.fr).

Birefringence was analysed as previously described (Behra et al., 2002). Briefly embryos were embedded in methylcellulose (Westerfield, 1993), mounted under a dissecting microscope (Leica MZ FLIII) and illuminated with polarised light. The parallel alignment of normal muscle fibres reflects polarised light. The degree of reflection was translated into a colour code to reveal the differences in intensities between wildtype, mutant and inhibitor treated embryos.
Microinjection

Microinjection of $10^{-2}$ M GAL into the yolk of one cell stage embryos was carried out with a pressure driven microinjector (Eppendorf) as described (Westerfield, 1993). The injection volume was measured by injection into an oil droplet on an electron microscope grid and the volume of a zebrafish embryo was estimated to be approximately 1000 nl.

Application of penetration enhancers

In experiments, which were aimed to test the function of penetration enhancers, GAL was applied alone or together with the penetration enhancers from onset of gastrulation (50% epiboly; see (Westerfield, 1993) for staging). In pilot experiments, the range of concentration of each penetration enhancer, which did not show toxicity, was tested. Dimethylsulfoxide applied at 2% (v/v) did not reduce the effective concentration of GAL and caused toxic effects when administered alone at 5% (v/v). Caprate (0.005%), hexadecyltrimethylammonium bromide and cetyltrimethylammonium bromide gave a very weak enhancement of the GAL effect or were ineffective.
Results

Inhibitors of AChE phenocopy the motility defect of the *ache* mutation.

To validate a use of zebrafish embryos as model for testing the specificity of AChE inhibitors, we chose 4 compounds known to be inhibitors of AChE. These include the drugs ESE, GAL, TAC and EDRO (chemical names see Material and Methods, (Cousin *et al.*, 1998 and references therein).

We first assessed the effect of the compounds on motility of the embryo and young larva. Wildtype embryos show first signs of motility at 18 hours post-fertilisation (hpf). This initial beating of the tail is spontaneous, but becomes touch-evoked by about 36 hpf. Motility in response to touch has two phases: an immediate strong bend away from the source of stimulation, the so-called startle response, followed by a longer phase of swimming movements that varies in duration. Starting from 27 hpf, spasmodic contractions of the body axis can be observed in *ache* mutants while wildtype embryos perform smooth, wave-like movements of the trunk and tail (Behra *et al.*, 2002). By 48 to 72 hpf, motility of the mutants is strongly impaired (Fig. 1A, B). We determined the concentration of each of the four compounds, at which they affect motility in a similar manner as the *ache* mutation (Fig. 1, Tab. 1). Whole embryos were exposed to the compounds from the 5-somite stage onwards, the time, at which *ache* starts to be expressed (Bertrand *et al.*, 2001). Embryos bathed in $10^{-4}$ M ESE, $10^{-2}$ M EDRO, $10^{-3}$ M GAL or $10^{-5}$ M TAC showed reduced motility at 27 and 48 hpf in a manner similar to *ache* mutants (Fig. 1C, Fig. 2, Tab. 1). The embryos under all treatment conditions had beating hearts, indicating that impaired motility is not due to a lethal effect of the compounds. Increasing the concentration by tenfold did not elicit a stronger inhibition of motility and caused unspecific effects in the case of EDRO and TAC such as necrosis in the brain (data not shown).
To quantify the impairment of motility, we measured the duration of swimming movements after touching wildtype, *ache* mutant or inhibitor-treated embryos (Fig. 2, Tab. 1). Wildtype embryos carried out swimming movements for an average of 2.8 seconds after being stimulated. In contrast, *ache* mutants showed on average only a very short phase of swimming. Both GAL and ESE reduced the average duration of swimming by 95% (Fig. 2) while TAC and EDRO were slightly less effective in reducing the average swimming episode (86% and 70%, respectively (Fig. 2, Tab. 2)). The data sets were pairwise subjected to the Student’s t-test. In comparison to that of wildtype embryos, the duration of swimming of *ache* mutant and inhibitor treated embryos was significantly reduced (p<0.05, Tab. 2). The difference between *ache* mutant, ESE and GAL treated embryos is not significant (p>0.05, Tab 2). The lower efficiency of TAC and EDRO in reducing the duration of swimming is, however, significant (p<0.05, Tab. 2). Although *ache* mutant and inhibitor-treated embryos show strong reductions in swimming duration, they responded in many instances with one fast bend of the body axis. Moreover, the inhibitor treated embryos, like *ache* mutants, had beating hearts demonstrating that impaired motility is not due to a general toxic effect of the drugs (Tab. 2). Thus, zebrafish embryos treated with inhibitors of AChE have impaired motility similar to that of embryos that carry a loss-of-function mutation in the *ache* gene.

**Inhibitors differ in their ability to induce muscle degeneration**

Lack of AChE activity causes a progressive myopathy in homozygous *ache* mutant embryos. Mutant muscle fibers form vacuoles and myofibrils are not aligned in parallel as in wildtype embryos (Behra *et al.*, 2002). As a consequence, the reflection of polarised light (or birefringence) from the flank of mutant embryos is severely reduced at 48-72 hpf (compare Fig. 3A, B). We tested whether the AChE inhibitors would cause similar muscle defects. Embryos were exposed to the inhibitors from the 5-somite stage and were analysed under
polarised light at 72 hpf. GAL caused severe muscle defects in wildtype embryos (Fig. 3D) that were not increased by absence of the *ache* gene (Fig. 3E). Development of the myopathy in *ache* mutants requires the nicotinic acetylcholine receptor (nAChR), since mutations in the α1 subunit of nAChR (*nic1*) suppress the myopathy of *ache* mutants (Fig. 3J to L and (Behra et al., 2002) for details). In contrast to wildtype embryos, GAL-treated, *nic1* mutant embryos that lack functional nAChR (Sepich et al., 1998) do not develop a myopathy (Fig. 3F compared with C, D). Thus, the GAL-induced muscle degeneration depends on a functional nAChR similar to the myopathy caused by lack of *ache* gene activity. In comparison to GAL (Fig. 3D), EDRO and TAC induced a weaker reduction in birefringence (data not shown) in agreement with their slightly weaker effect on motility (Fig. 2, Table 2).

Curiously, ESE, even though it impaired motility strongly (Fig. 2), did not cause a significant reduction in birefringence (Fig. 3G). ESE may not inhibit AChE at all in the zebrafish or may have additional activities. To exclude the first possibility, we performed histochemical and spectrophotometric measurements of AChE activity in extracts or intact embryos treated with ESE. Embryos exposed to 10⁻⁵ ESE showed a reduction of AChE histochemical activity and exposure to 10⁻⁴ M ESE almost completely abolished the histochemical reaction (Fig. 4A to D) comparable to that seen in the *ache* mutant (Fig. 4D). Similarly, when measured by the colorimetric Elman assay, the AChE activity was reduced by 72% and 91% in extracts from embryos treated with 10⁻⁵ and 10⁻⁴ M ESE, respectively. Hence, ESE is able to inhibit zebrafish AChE efficiently. The lack of a myopathy is thus likely due to a secondary target effect of ESE. The lack of the myopathy in embryos that lack a functional nAChR renders the nAChR a possible secondary target of ESE. If this notion is correct, the myopathy of *ache* mutant embryos should be suppressed by treatment with ESE. Indeed, treatment of *ache* mutants with ESE almost completely abolished the myopathy (Fig. 3H) observed in untreated *ache* mutants (Fig. 3B). These results show that ESE has a
secondary effect. This is most likely caused by antagonistic interaction with the nAChR. In vitro studies suggest that ESE can act as an antagonist of the Torpedo nAChR (Kawai et al., 1999).

**Secondary effects of inhibitors in the nervous system**

To assess a possible effect of the AChE inhibitors on the developing nervous system, we utilised a transgenic line that expresses the green fluorescent protein (gfp) gene under control of the regulatory sequences of the zebrafish neurogenin1 gene (-3.1ngn1:gfp) (Blader et al., 2003). The neurogenin1 gene is a neural determination gene, which controls differentiation of Rohon Beard sensory neurons involved in the perception of touch (Korzh and Strahle, 2002). The transgene is strongly expressed in Rohon Beard neurons (Fig. 5A). In addition, a few scattered interneurons located immediately below the dorsal longitudinal fascicle (dlf) are marked by weak GFP expression (Fig. 5A). The overall pattern of GFP expression is unchanged in ache-/- embryos (n=20) at 27 hpf (Fig. 5B) suggesting that lack of ache activity does not affect the expression of the transgene and the pattern of neurogenesis at this stage. When embryos were exposed to either EDRO (Fig. 5C) or TAC (Fig. 5D) from the 5-somite stage to 27 hpf, the pattern of GFP-expressing neurons was abnormal (69% treated embryos, n=19; 70% treated embryos, n=37, respectively). Ectopic, GFP-expressing neurons were noted at the ventral aspects of the neural tube in the drug-treated embryos suggesting that the two drugs caused aberrant neurogenesis or cell migration (Fig. 5C, D). An abnormal pattern of neurogenesis was not observed in the ache mutants (Fig. 5B; n=20). This effect is not the result of inhibition of AChE activity and is likely to reflect a secondary target effect.

These drastic changes in neuronal pattern were also not observed in embryos treated with ESE or GAL (Fig. 5F, E, n=35 and n=33, respectively). Occasionally, ESE or GAL-treated
embryos were noted that had slightly displaced cells (Fig. 5F, E, arrow, 23% and 21%, respectively). However, these cells were also observed in untreated embryos at a similar frequency.

The inhibitors block zebrafish AChE effectively at micro- to nanomolar ranges.

When assessed by their dose-dependent effect on motility (Fig. 1, Tab. 1) or by in situ analysis of AChE activity in the case of ESE (Fig. 4B,C), the effective blocking concentration of each of the 4 inhibitors is high in comparison to reported IC₅₀ values of AChEs from other species (Ariel et al., 1998; Harvey, 1995; Shafferman et al., 1992; Stein and Lewis, 1969). Three reasons could account for this: first, the inhibitors have lower affinities to the AChE of zebrafish than to that of other vertebrates. Second, the zebrafish embryo may take up these chemicals from the environment rather inefficiently hence requiring higher doses of inhibitor. Finally, one could also envisage that the compounds are metabolised in the embryo.

To assess the first possibility, the IC₅₀ of the four inhibitors was determined in extracts of zebrafish larvae. The extracts were pre-incubated with varying concentrations of the inhibitors before AChE activity was measured spectrophotometrically (Ellman et al., 1961). ESE and TAC inhibited zebrafish AChE half-maximally at 125 +/- 15 nM and 114 +/- 23 nM, respectively (Fig. 6). The respective IC₅₀ values of GAL and EDRO were 2800 +/- 21 M and 1300 +/- 100 nM. These IC₅₀ values are in the range of those reported for AChEs of other species (Ariel et al., 1998; Harvey, 1995; Shafferman et al., 1992; Stein and Lewis, 1969). Thus, the zebrafish AChE is as sensitive to the inhibitors as those of other vertebrates.

To exclude the possibility that the drugs are metabolised in the embryo, GAL was injected into the yolk cell of one-cell stage embryos. We injected approximately 1 nl of 10⁻² M GAL per embryo resulting in an intraembryonic concentration of about 10⁻⁵ M assuming...
free diffusion within the embryo and no reduction due to growth of the embryo and diffusion out of the embryo until the 72 h-stage. All injected embryos (n=50) showed impaired motility and reduced reflection of polarised light at 72 hpf in comparison to mock injected controls (Fig. 7A, B). Assuming no losses due to dilution or diffusion between the injection and examination of the effect 72 h later, the estimated intra-embryonic concentration is at least 10-fold lower than that required in bath-application to “chemocopy” the ache mutant phenotype. Thus, intra-embryonic degradation of the drugs is unlikely to account for the relative insensitivity of whole embryos.

Taken together, these results suggest that GAL penetrates the zebrafish embryo ineffectively. As microinjection is not the most convenient way to deliver compounds to a large number of embryos, we next assessed whether the drug effect could be improved by co-applying penetration enhancers. Dimethylsulfoxide, caprate, cetyltrimethylammonium bromide applied at various concentrations (see Materials and Methods) did not increase the sensitivity to GAL efficiently (data not shown). However, when GAL was applied together with 0.05 or 0.01% (v/v) Tween 80, a significant effect could be noted. While 10^{-5} M or 10^{-6} M GAL administered alone was ineffective (100% embryos; n=30, Fig. 7E, G), the same concentrations of GAL co-applied with 0.05% Tween 80 impaired motility and reduced the birefringence (Fig. 7F, H; 60% embryos; n=30). Administration of Tween 80 (0.01% or 0.05%) alone did not affect development or viability in any detectable way (data not shown). Thus, impaired penetration is a likely explanation for the relative insensitivity of whole embryos and the barrier can be overcome by applying the penetration enhancer Tween 80.
Discussion

We assessed here the potential of wildtype and mutant zebrafish embryos as model for the specificity of inhibitors of AChE. Using this class of inhibitors, this work was also aimed at evaluating in general the use of wildtype and mutant zebrafish embryos in testing the toxic or teratological effect of chemicals. Among the four compounds tested, only GAL “chemocopied” the ache mutant phenotype. Like genetic loss of ache function, GAL treatment impaired motility, caused a myopathy, but did not affect the differentiation of neurons in the spinal cord. Moreover, the myopathy in ache mutants (Behra et al., 2002) and GAL treated embryos (this study) require both an intact nAChR. The other inhibitors had additional effects not seen in ache mutants. Both TAC and EDRO caused impaired neuronal development that was not detected in ache mutants. ESE even cured the muscle defects of the ache mutants. As a similar suppression of the muscle phenotype of ache mutants is observed, when the AChR is genetically removed (Behra et al., 2002), this suggests that ESE acts as an antagonist of the AChR. This notion is in agreement with previous in vitro studies (Kawai et al., 1999), in which it was shown that ESE acts as an antagonist of the nAChR. In summary, our work demonstrates the power of wildtype and mutant zebrafish embryos to assess the target specificity of AChE inhibitors.

We noticed also a drawback in that the concentrations required to “chemocopy” the ache mutant phenotype in intact embryos are higher in comparison to the reported in vitro inhibitory concentration for AChEs from other species (Ariel et al., 1998; Harvey, 1995; Shafferman et al., 1992; Stein and Lewis, 1969). This does not appear to be due to a low affinity of the inhibitors to zebrafish AChE or degradation of the compounds in the embryos. A likely explanation is the inefficient uptake of the compounds. While this relative insensitivity of zebrafish embryos may be a hindrance in primary screening programmes of compound libraries where the available amounts of chemical are rather limited, it may not
present an obstacle for second phase toxicological assessment of candidates or the analysis of the toxicological mechanism. Interestingly, not all penetration enhancers were equally effective, suggesting that the type of penetration enhancer may have to be taken into consideration for each compound class to be tested.

The effective in vivo dose to impair motility appears to be linked to the chemical structure of the AChE inhibitors. EDRO is the least efficient (10^{-2} M) to inhibit motility in bath applications even though its in vitro IC_{50} is in the m range. ESE “chemocopies” the ache mutant phenotype less effectively than TAC, but both compounds inhibit the AChE activity with an IC_{50} around 100nM. Other compounds were previously applied to zebrafish embryos at micro- and nanomolar concentrations and caused significant effects (Peterson et al. 2000; Peterson et al., 2001). We show that, at least in the case of GAL, direct injection of the compound or the co-administration of the penetration enhancer Tween 80 can reduce the effective dose in the embryos suggesting that the up-take of the AChE inhibitors may represent a barrier for the AChE inhibitors.

Our work illustrates the potential of zebrafish in assessment of toxicological and teratological effect of compounds in that zebrafish mutants and mutant phenotypes act as blueprints of drug effect to assess target specificity and to gain insight into the toxic mechanism in a whole organism set-up. Ideally, if an inhibitor is specific it should reproduce the phenotype of a loss-of-function mutation faithfully with no additional effects. However, reality can be more complex than this theoretical concept. Complications may be imposed by the complexity of the responses of intact organisms. First, the phenotypes of a loss-of-function mutation may not reflect the effect of an inhibitor that blocks its target only partially as sub-maximal inhibition may have different phenotypic consequences. Second, detoxifying pathways may break down a chemical to metabolites that act unspecifically, even though the administered inhibitor is perfectly specific for its target. Another complication may arise by
the fact that genetically altered organisms can have varying phenotypes depending on the specific lesion and the genetic background. We have not observed variation of the *ache* mutant phenotypes utilised here, even when crossed into distantly related strains such as the WIK mapping strain (MB and US, unpubl.). However, this does not mean that this may not present a problem in other genetic backgrounds and for other genetic lesions in the zebrafish.

Despite these caveats, we believe that, with the increasing number of cloned mutant loci, the zebrafish will provide a simple cost-effective platform to assess the effects of chemicals on many developmental and physiological processes. The potential of the zebrafish is even further increased by the steadily growing number of transgenic lines that permit monitoring of many physiological processes in the living animal. The small size, transparency and extra-uterine development of the zebrafish embryo allow the application of simple assays, which can easily be modified to satisfy medium- and possibly also high-throughput applications. In summary, even though the concentrations required to achieve an effect may be relatively high for certain chemicals, the zebrafish embryo can provide a valuable whole-organism-assay to satisfy the demand for efficient toxicological test systems by regulators and industry.

**Legends**

Figure 1

Comparison of touch-evoked motility

A to C: Videomicrographs showing the touch-evoked movements of wildtype (A), *ache* mutant (B) and $10^{-3}$ M GAL-treated (C) embryos at 48 hpf. The heads of the animals were embedded in agarose and embryos were touched with a blunt metal rod. Wildtype embryos reacted with a strong bend away from the source of stimulation (A) and maintained swimming movements for several seconds. In contrast, *ache* mutant and GAL-treated
embryos responded with a single bend of the tail away from the source of stimulation and returned slowly back to the starting position without swimming movements over the next 200 ms (middle panels in B and C). Wildtype embryos (A, bottom) continue swimming for several seconds, while mutants (B, bottom) or GAL-treated embryos (C, bottom) are motionless. The individual panels represent overlays of selected video frames. The time of each sequence is indicated in the right bottom corner in milliseconds. The middle and bottom panels of B and C represent 200 ms periods to document the slow straightening of the tail (middle panels) or the motionless state (bottom panels). In contrast, the middle and bottom panel of A represent 40 ms periods.

Figure 2
Duration of swimming movements is reduced by application of AChE inhibitors

Wildtype embryos were treated with 10^-4 M ESE, 10^-3 M GAL, 10^-2 M EDRO, and 10^-5 M TAC and the duration of the swimming response (seconds) was measured after a tactile stimulus to the body flank. Embryos were exposed to the compounds from the 5-somite stage onwards. Movement was recorded by video microscopy at 48 hpf and the duration of the swimming response was determined. The results were averaged from 48 to 70 embryos for each condition and standard deviations are indicated.

Figure 3
GAL but not ESE causes a myopathy similar to that observed in ache mutants

Wildtype (A, D, G), ache mutant (B, E, H) and nAChR (nic1) mutant (C, F, I) embryos (72 hpf) were either untreated (A to C) or treated with 10^{-3} M GAL (D to F) or 10^{-4} M ESE (G to I). Lack of ache causes defects in the axial musculature, which are visualised by illuminating embryos with polarised light. Strong reflection of polarised light translated to
red/yellow colours by the spectral representation indicates a normal parallel alignment of myofibrils (A, C, F, G to I). In contrast, myofibrilar arrangement is disrupted in *ache* mutants (B, represented by a blue green colour code). This defect is phenocopied by treatment of wildtype embryos with GAL (D) but not with ESE (G). The GAL-induced myopathy is suppressed by lack of a functional nAChR (nic1) (F). Application of ESE to *ache* mutant embryos suppresses the myopathy of *ache* mutants (H). View onto the axial musculature at the hindgut extension. Dorsal is up and anterior to the left. The scale bar represents 50 µm. The rainbow scale indicates the quantitative differences in reflected polarised light represented by the colour code. J to L: Schematic summary of the epistatic relationships of AChE and AChR in wildtype (J), in the development of a myopathy in *ache* mutants (K) and its suppression in *ache;nic1* double mutants (L). ACh released from the nerve terminal accumulates in the neuromuscular cleft of *ache* mutants and leads to over-activation of the muscle by interaction with the nAChR on the muscle membrane. As a consequence, the myofibers are damaged. Over-activation of the muscle is prevented in *ache;nic1* double mutants in which the nAChR is missing to transmit the effect to the muscle cells. As a consequence the myopathy does not develop. The phenotype of the double mutant is chemocopied by treatment of *ache* mutants with ESE (H).

**Figure 4**

*In situ* determination of AChE activity in embryos treated with ESE

Determination of AChE activity in untreated (A), $10^{-5}$ M ESE (B), $10^{-4}$ M ESE-treated WT wildtype embryos (C) and *ache* mutant embryos (D). View onto the axial musculature at the hindgut extension. Dorsal is up and anterior to the left. The scale bar represents 30 µm.

**Figure 5**
Edrophonium and tacrine alter the pattern of neurogenesis in the spinal cord

Wildtype (A), 

*ache* mutant (B), 10^{-2} M EDRO (C) 10^{-5} M TAC (D), 10^{-3} M GAL (E) and 10^{-4} M ESE (F) treated embryos carrying the *3.1ngn1:gfp* transgene. This transgene expresses GFP in Rohon-Beard sensory neurons (rb) and weakly in some interneurons (i) of the dorsal spinal cord. It also marks the dorsal longitudinal fascicles (dlf) at the dorsolateral aspect of the neural tube. The pattern of GFP expression is indistinguishable in wildtype and *ache* mutant embryos. In contrast, both EDRO- and TAC-treated embryos show ectopic GFP expression in ventral neural tube regions (arrows in C, D). Arrows in E and F show examples of cells that are only slightly ventrally displaced in GAL and ESE treated embryos. These deviations are variation of the normal body plan as they occur with a similar frequency also in wildtype embryos. Embryos are 27 h old and are shown in lateral views. Dorsal is up and anterior to the left. The scale bar represents 15 μm.

Figure 6

Efficient inhibition of zebrafish AChE by GAL, ESE, TAC and EDRO *in vitro*

Extracts from 4 day old embryos were incubated with increasing concentrations of ESE, GAL, EDRO and TAC and AChE activity was measured using the Ellman assay. IC_{50} are in the nano- to micromolar range.

Figure 7

Microinjection of GAL or co-application of a penetration enhancer reduces the concentration required to “chemocopy” the myopathy of *ache* mutants

A, B: Birefringence of uninjected (A) or embryos (B) injected with GAL (10^{-2} M) giving an approximate intra-embryonic concentration of 10^{-5} M GAL.
C to H: Birefringence of embryos treated with $10^{-4}$ (C, D), $10^{-5}$ (E, F) and $10^{-6}$ M GAL (G, H) in the absence (C, E, G) or presence (D, F, H) of 0.05% (v/v) Tween 80 (Tw80). The penetration enhancer Tween 80 reduces the GAL concentration, which is required to induce a myopathy, by 10- to 100-fold. Embryos are oriented anterior left and dorsal up. Views onto the trunk at the level of the yolk extension are shown. Colour coding of the reflection of polarised light as described in legend to Fig. 3.
Acknowledgement

We are grateful to N. Fischer, D. Biellmann, C. Vialle, J.L. Vonesch, D. Hentsch, M. Boeglin for technical assistance, artwork and animal care. We thank S. Rastegar and C. S. Lam for comments and discussion and D. Dembele for help with the statistical analysis. US was supported by CNRS/INSERM/ULP/HUS, AFM, ARC, Volkswagen Stiftung and AICR.

References


residues affecting signal transduction from the surface to the catalytic center. *Embo J* 11, 3561-8.


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Table 1 Dose effect of AChE inhibitors on motility of 27h old embryos.
Table 2. Reduction of duration of swimming relative to control/wildtype (CO/WT) in *ache* mutant, 10^{-4} M ESE, 10^{-3} M GAL, 10^{-5} M TAC and 10^{-2} M EDRO treated embryos. Heart beat served as survival control. ND: Not determined

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BEHRA & ETARD Fig. 2