Zebrafish Models for Neuroscience

Drug Discovery

THESIS

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by

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CERTIFICATE

This is to certify that the thesis entitled **Zebrafish Models for Neuroscience Drug Discovery** and submitted by **PUSHKAR KULKARNI** ID No **2013PHXF0118H** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

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To

My Family

Especially my wife Swati

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ABSTRACT

Zebrafish models are rapidly becoming popular in pharmaceutical and disease biology research. Several neuroscience models have been developed and recent regulatory decisions have created a strong case for their use for neuropharmacology and drug discovery research. Furthermore, zebrafish genome has been fully sequenced with high conservation to humans. This has furthered the scope to use this model for disease biology, target identification & validation, genetically modified models and similar research areas.

The major shortcoming in zebrafish research has been the ability to correlate the zebrafish data to humans or other species for drug discovery decision making. Especially for “phenotypic drug discovery”, where efficacy/potency and therapeutic index are critical parameter to decide the fate of drug there has been a major gap in zebrafish research. Therefore, our focus in this thesis work and manuscript was to generate the efficacy data in terms of milligrams per kilograms using oral or intra-peritoneal drug administration and then correlate this data to higher species including human beings. We used the adult zebrafish model, over the more popular embryo-larval model, for this work due to several advantages; especially; the fact that the blood brain barrier is fully developed in adult fish and behavioral phenotypes are clearer in an adult.

The models studied are described below:

1. Multiple Sclerosis Model: A novel experimental autoimmune encephalomyelitis (EAE) model in adult zebrafish was developed and validated and is the first such model reported and published by our laboratory. We proposed a simple protocol and validated it using known drugs for efficacy. Further the data between zebrafish has been correlated with the published data in rodents and humans for dose and to some extent for mechanism.

2. Pentylenetetrazole (PTZ) induced Epilepsy Model: This model is a well characterized and validated model of epilepsy and is gaining acceptance by regulators as well. We have evaluated known antiepileptic drugs and correlated the data on dose with humans for dose. We further tried to demonstrate that drugs that work though certain mechanisms and show selective efficacy in rodent PTZ model also show efficacy in zebrafish model. Similarly drugs that do not show efficacy in rodent PTZ model (attributed to different mechanisms) did not show efficacy in zebrafish model with one drug as an exception that showed partial efficacy.

3. Light/Dark Model of Anxiety: The study was conducted to evaluate known anxiolytic drugs in adult zebrafish light/dark or scototaxis model. Similar to other models the data from zebrafish was correlated with rodents and humans.

4. Pharmacokinetics & Brain Penetration: Understanding pharmacokinetics and brain penetration is critical component of neuropharmacology

program. We conducted adult zebrafish PK and brain penetration studies on two known compounds (irinotecan and lorcaserin) with distinct PK and brain penetration properties and correlated key parameters with the data from rodents, primates and humans.

We have also attempted to extrapolate the dose from zebrafish to humans have proposed a formula for dose conversion between zebrafish and humans. The formula that has emerged out of real time data is very similar to the theoretical extrapolation using the formulae from body surface area based extrapolations. In summary, we have attempted to address a major gap in zebrafish research; that of; data correlation with humans. This work and publications from it, we believe, will contribute to further improve the utilization of zebrafish for neuropharmacology research and drug discovery.

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LIST OF ABBREVIATIONS/SYMBOLS

µl	:	microliter
µl	:	microliter per kilogram
ADME	:	Absorption, Distribution, Metabolism and Excretion of drug
AED	:	Anti Epileptic Drug
ANOVA	:	Analysis Of Variance
AUC	:	Area Under the Curve
AUMC	:	First Moment Curve in Non-Compartmental Analysis
BCS	:	Biopharmaceutical Classification System
BLAST	:	Basic Local Alignment Search Tool
Ca	:	Calcium
CBT	:	Cognitive Behaviour Therapy
CBZ	:	Carbamazepine
CFA	:	Complete Freund's adjuvant
Cl	:	Clearance
Clast	:	Last Measurable Concentration
Cmax	:	The peak plasma concentration of a drug after administration
DBS	:	Dried Blood Spot
DPBS	:	Dulbecco's Phosphate Buffer Saline
dpf	:	days post fertilization
EAE	:	Experimental Autoimmune Encephalomyelitis
ED_{50}	:	Median Effective Dose
EEG	:	Electroencephalogram
et al.	:	and others
ETS	:	Ethosuximide
F	:	Bioavailability
FBM	:	Felbamate

GABA	:	Gamma Amino Butyric Acid
h	:	hour/s
HPLC	:	High Performance Liquid Chromatography
i.p.	:	intra-peritoneal
i.v.	:	intravenous
ICH	:	International Conference on Harmonization
kg	:	kilogram
Kp,brain	:	Partition Coefficient to predict drug brain penetration
Kp,uu,brain	:	Partition Coefficient to predict unbound drug brain penetration
Lambda_z	:	Terminal Elimination Rate Constant
LCMS/MS	:	Liquid chromatography tandem-mass spectrometry
LLOQ	:	Lower Limit of Quantification
LTG	:	Lamotrigine
LVT	:	Levetiracetam
MAG	:	Myelin Associated Glycoprotein
MBP	:	Myelin Basic Protein
MED	:	Minimum Effective Dose
MES	:	Maximal Electroshock
mg/kg	:	milligram per kilogram
mg/ml	:	milligram per milliliter
mg	:	milligram
ml/kg	:	milliliter per kilogram
ml	:	milliliter
mm	:	millimeter
MOG	:	Myelin Oligodendrocyte Glycoprotein
MRT	:	Mean Residence Time
MS	:	Multiple Sclerosis

n	:	sample size
Na	:	Sodium
NCBI	:	National Center for Biotechnology Information, USA
NCTR	:	National Center for Toxicological Research, USA
ng/ml	:	nanograms per milliliter
ng	:	nanograms
NIH	:	National Institutes of Health, United States of America
p value	:	calculated probability when null hypothesis is true
p.o.	:	per os (oral)
PD	:	Pharmacodynamic
PHT	:	Phenytoin
PK	:	Pharmacokinetics
PLP	:	Proteolipid Protein
PTZ	:	Pentylenetetrazole
s.c.	:	sub coetaneous
S.D.	:	Standard Deviation
S.E.M.	:	Standard Error of Mean
t1/2	:	Elimination half-life
TGB	:	Tiagabine
Tlag	:	Lag Time
Tmax	:	Time to reach Cmax
USFDA	:	United States Food and Drug Administration
Vz	:	Volume of Distribution
WHO	:	World Health Organization
WMH	:	World Mental Health

CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1 Zebrafish

The zebrafish (*Danio rerio*), is a small fish, belonging to the genus Danio, which is a part of the Cyprinidae family. It is a tropical fish that is found mainly in the Indian sub-continent in various rivers, lakes and freshwater bodies. The important characteristics that have made zebrafish popular in research are as follows:

(1) Fully sequenced genome with high conservation to humans (Howe et al., 2014)

(2) High spawning frequency (Kimmel et al., 1995; Briggs, 2002)

(3) Ex-vivo fertilization (Kimmel et al., 1995; Briggs, 2002)

(4) High fecundity (Kimmel et al., 1995; Briggs, 2002)

(5) Transparent embryos (Kimmel et al., 1995; Briggs, 2002)

(6) Short generation time (Kimmel et al., 1995; Briggs, 2002)

(7) Ease of genetic/chemical manipulation (Scholz et al., 2008)

(8) Ease of housing and low husbandry time and costs (Grone & Baraban, 2015)

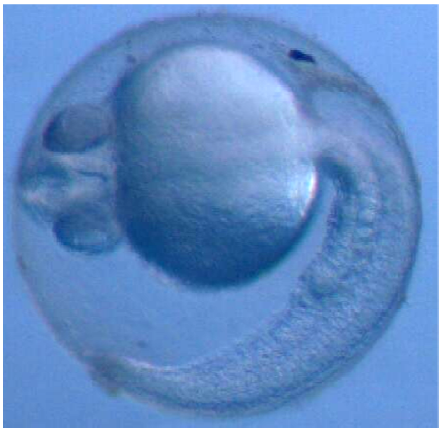
(9) Well documented anatomical characteristics (ZFIN Atlas)

(10) Increasing use in drug discovery (MacRae & Peterson, 2015)

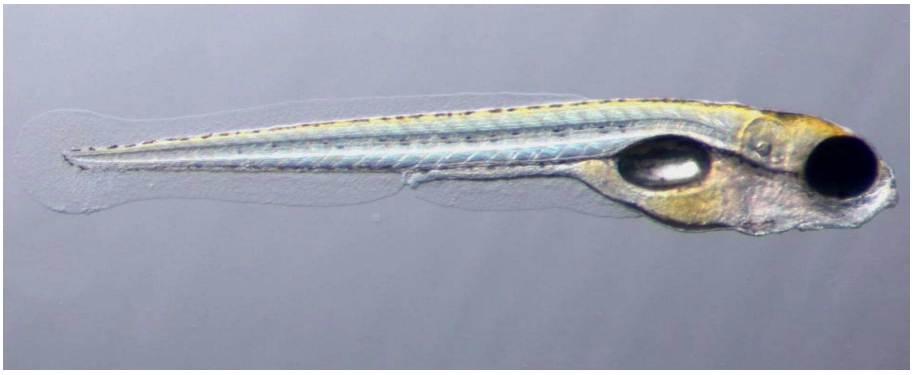
All the above characteristics make zebrafish very interesting model for drug discovery and disease biology. In terms of disease areas, zebrafish models have been developed and are being used by various researchers in academia and industry for neurology, cardiology, gastroenterology, nephrology, infectious diseases, inflammation, immunology and many other areas (MacRae & Peterson, 2015).

Figure 1.1 shows the pictures of various stages of zebrafish individually and in groups.

(A)



(B)



(C)

Figure 1.1.: Zebrafish pictures from Dr. Reddy's Institute of Life Sciences: (A) 24 hour old embryo; (B) 7 day old larvae; and; (C) adult (> 3 months old).

1.1.2 Zebrafish in Pharmaceutical Research

Zebrafish models have been increasingly being used in pharmaceutical industry worldwide and Table 1.1 provides a snapshot of research areas studied using the zebrafish models by major pharmaceutical companies. The table represents excerpts from Fleming & Alderton, 2013. The authors point out the following major advantages seen by the pharmaceutical companies for using zebrafish:

(a) Time advantage to screen drugs in zebrafish as compared to other conventional mammalian models.

(b) Zebrafish offer an ethical advantage as the larval model is considered as humane alternatives for in-vivo research.

(c) Zebrafish are being increasingly used for drug repurposing to identify new uses for existing or clinical candidate molecules.

(d) As zebrafish are amenable and easy for genomic/proteomic manipulations, they are being used for target identification and target validation

Recently, the United States Food and Drug Administration (USFDA) allowed Orphan Drug Designation for a drug discovered using zebrafish. In this case, lorcaserin, a marketed drug is being repurposed for patients suffering from Dravet Syndrome, a rare epileptic disease and has shown efficacy in a small pediatric trial. This was the first instance of “aquarium to bedside approach”, wherein a drug, though repurposed, was tested in humans directly after establishing efficacy in zebrafish model (NIH, 2017). This research was sponsored by the National Institute of Neurological Disorders and Stroke (NINDS) which is a part of the National Institutes of Health (NIH), USA. Furthermore, the NIH has ranked zebrafish as the third most important experimental organism after rats and mice, and the US FDA's National Center for Toxicological Research (NCTR) has set up a zebrafish laboratory to study "predictive toxicology" (USFDA, 2013).

These developments suggest the growing acceptance of zebrafish in scientific community, pharmaceutical industry as well as by regulators.

Table 1.1. Snapshot of research areas studied using the zebrafish models by major pharmaceutical companies. The table represents excerpts from compilation of publications (2006–2012) by pharmaceutical companies using zebrafish assays by Fleming & Alderton, 2013.

Assay	Pharmaceutical company
Cardiac function	Abbott
Visual function	AstraZeneca
Seizure liability	AstraZeneca
Tauopathy	AstraZeneca
ADME	AstraZeneca
Ototoxicity	AstraZeneca
Embryotoxicity/ teratogenicity	Bristol-Myers Squibb
Bone formation	Eli Lilly
Primordial germ cell	Eli Lilly
Embryotoxicity/teratogenicity	GlaxoSmithKline
Hepatotoxicity	J & J; Pfizer
Embryotoxicity/ teratogenicity	J & J
Embryotoxicity/ teratogenicity	Merck KGaA
Developmental biology	Novartis
Gastrointestinal motility	Novatis
Toxicology; MoA	Novartis
Safety pharmacology	Pfizer
ADME	Pfizer

1.1.3 Zebrafish Models of Neurosciences

Researchers, for a long time, considered fish behaviors as stereotyped or simple (Rose, 2002, 2007), however, voluminous research in last decade has demonstrated that context-dependent complex behavioral responses can be elucidated and modeled in zebrafish (Kalueff et al., 2014; Stewart et al., 2014). Furthermore, it has been discovered that zebrafish possess all major neurotransmitters, hormones and receptors and many pathways of neurological disorders are conserved when compared to humans (Alsop & Vijayan, 2009; Mueller et al., 2004; Panula et al., 2006). Wide range of the neurodegenerative and neurobehavioral diseases that are currently being studied in zebrafish include, Alzheimer's, Parkinson's, Huntington's, Dravet's, multiple sclerosis, epilepsy, anxiety, sleep, sociality, cognition, etc.. Though most of the well established zebrafish models are in larvae, recent evidence using adult zebrafish strongly supports the use of adult zebrafish for phenotypic drug discovery and disease biology research (Maximino et al., 2010; Stewart et al., 2014).

Neuropharmacological correlation of various biomarkers and neurochemicals with the behavioral phenotypes suggests a strong correlation of both phenotypes as well as molecular mechanisms between zebrafish models and their analogous of other mammals (especially rodents) and humans. (Egan et al., 2009; Lau et al., 2011; Teles et al., 2013).

The risk- benefit evaluation of various zebrafish neuropharmacology models has been carried out in a recent review by Kauleef et al. (2014), where the authors have compiled the data on large number of studies and stated that "the advantages of using zebrafish in neuroscience outweigh the risks and limitations". In this review the authors have also compiled and compared the number of zebrafish publications with publications using other common species such as dogs, mouse, rats, fruit flies and

worms (C. elegans) over a 10 year period between 2004 and 2013. They report that zebrafish based publications have doubled over the decade which is the highest incremental increase in all in-vivo based publications. This suggests a wide acceptance of zebrafish models amongst academicians.

In summary, zebrafish models have emerged over years as useful models for translational neurosciences. As the research community overcomes the gaps in existing research and further reduced the limitations of this model, its use in pharmaceutical industry will become more visible.

1.2 GAPS IN EXISTING RESEARCH

Although zebrafish have become very popular in recent years, a major gap in research has been the fact that predictive value of these models has not been established (Peterson & McRae, 2012). Apart from factors associated with genetics and physiological systems, most important aspect in establishing predictivity is validation of robust zebrafish protocols and correlation of data to conventional animal models (Kulkarni et al. (2014)). The inability to correlate data from zebrafish to humans makes it difficult use data from zebrafish studies for taking decisions in drug discovery research, especially with respect to selecting hits or leads for further investigation. This has hampered the optimal utilization of zebrafish in drug discovery research.

Therefore, this thesis work has been focused on developing and validating robust protocols for select neurological diseases and correlating the data with other mammalian models including humans. The aim of this work is to contribute towards understanding the predictivity and optimum utilization of zebrafish for neuropharmacology research.

1.3 OBJECTIVES

The objectives of this thesis work were as follows:

1. Standardizing adult zebrafish based neuropharmacology models for Multiple Sclerosis, Epilepsy, Anxiety and Blood Brain Barrier Penetration.
2. Evaluating known and approved drugs in these models to validate these models as relevant models for these diseases and to determine efficacy dose for the drugs in these models.
3. To correlate the data between zebrafish and higher mammals including humans based on published literature.

1.4 GENERAL METHODOLOGY

1.4.1 Statement of Animal Ethics

All zebrafish experiments were performed following institutional guidelines of Dr. Reddy's Institute of Life Sciences, Hyderabad, India; as per the animal ethics laws of India; and; under the supervision of a licensed veterinarian.

1.4.2 General Care and Maintenance of Zebrafish

Zebrafish maintenance was performed based on guidelines published by the National Institutes of Health for care and use of zebrafish and procedures mentioned in the Zebrafish book (Westerfield, 2000). Wild type zebrafish were procured from Vikrant Aquaculture, Mumbai, India and maintained in re-circulatory system with controlled environment conditions with a temperature of 28ºC, and a light/dark cycle of ~ 14/10 hours. They were fed thrice with live hatched brine shrimp and dry food. The age, sex and other experimental specifications for each model have been described in the relevant chapters.

1.4.3 Rationale for use of Adult Zebrafish

Adult zebrafish have been used for the experiments conducted for this thesis work, despite of the fact that larval model has been more popular in drug screening research, based on the following rationale:

a) The larval brain at five days post fertilization (dpf) is less than 500 µm thick and 1.5 mm long (Friedrich et al. 2010; Banote et al. 2013), while the adult zebrafish brain is 4.5 mm long and between 0.4 and 2 mm thick (Rup et al., 1996; Wullimann, 1996) making almost all neurons accessible.

b) Adult zebrafish has fully developed brain regions (Ullmann et al., 2010) including neurotransmitter systems (Kily et al., 2008; Norton & Bally-Cuif, 2010; Panula et al., 2006; Rico et al., 2009; Rico et al., 2011) and blood-brain-barrier (BBB) (Eliceiri et al., 2011; Jeong et al., 2008), whereas larval zebrafish has an incompletely developed brain and the BBB starts functioning after 10 dpf (Fleming et al., 2013).

c) Moreover, poorly soluble drugs cannot be tested in larval zebrafish owing to precipitation and non-absorption, whereas, in adult zebrafish such experiments can be conducted by oral (Kulkarni et al., 2014) or intraperitoneal administration (Chaudhari et al., 2013).

1.4.4 Models, Studies and Analysis

1. Multiple Sclerosis Model: Development, standardization and validation of novel Multiple Sclerosis model in adult zebrafish and correlating data with humans.
2. Epilepsy Model: Standardizing and evaluating known antiepileptic in the Pentylenetetrazol induced Epilepsy model and correlating data with humans.
3. Anxiety Model: Standardizing and evaluating known anxiolytic compounds in the Dark/Light Box model and correlating data with humans.

4. Blood-Brain-Barrier Penetration Model: Standardizing and validating Blood-brain-barrier penetration model and correlating data with conventional models including humans.
5. Conclusions comprising of robust protocols and data correlation and proposing formula for data extrapolation from zebrafish to humans.

1.4.5 Minimum Efficacy Dose

The efficacy dose that was established and used for correlation for each pharmacological model was based on the principle of Minimum Effective Dose (MED). “The MED has been defined by the Encyclopedia of Biopharmaceutical Statistics as the lowest dose level of a pharmaceutical product that provides a clinically significant response in average efficacy, which is also statistically significantly superior to the response provided by the placebo” (Liu, 2010). This was chosen over the older concept of ED_{50} as ED_{50} is a "median effective dose" and represents an all or nothing effect (quantal effect) in 50% of population, and, has limited use to understand efficacy for the entire sample size. Guidelines from International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use regarding Dose-Response Information to Support Drug Registration (ICH-E4, 1994) state the use of MED, along-with other safety and kinetic parameters for determining the therapeutic regimen. The ICH-E4 guidelines are accepted by all major regulatory agencies across the globe including the USFDA (USFDA, 2003).

Therefore, for each drug evaluated in various zebrafish models, we have tried to identify parameters to establish the MED and correlate it with human MED.

1.4.6 Data Correlation and Extrapolation

We have tried to correlate the data obtained from zebrafish studies to higher mammals and humans based on the parameters of potency ranking, MED and mechanism of action. In the last chapter on conclusions, we have also attempted to and proposed a formula to extrapolate zebrafish dose data to humans.

1.5 CONCLUSION

This thesis work is focused on validating robust protocols for selected neuropharmacological models and correlating the data with higher species, especially humans. The anticipated impact of this work is to plug some of the gaps in the existing research and propose a framework for future research. Overall, the work will contribute towards better understanding of zebrafish neuropharmacology models and help their optimal utilization in drug discovery, pharmaceutical research as well as disease biology.

CHAPTER 2:

ZEBRAFISH MULTIPLE SCLEROSIS MODEL

CHAPTER 2: ZEBRAFISH MULTIPLE SCLEROSIS MODEL

2.1 INTRODUCTION

2.1.1 Background:

Multiple Sclerosis (MS) is neurodegenerative disease and is the leading cause of autoimmune neurological disability with a prevalence of 2.3 million patients worldwide as per the World Health Organization (WHO) Atlas of MS, 2013. The data shows that the prevalence of this disease in growing not just amongst developed countries but also amongst developing ones (Browne et al., 2014).

MS is disease wherein there is neurodegeneration which is mediated by autoimmune response. The disease affects the central nervous system and is mainly characterized by inflammation and demyelination in the brain and spinal cord leading to dysfunction of motor and sensory and disability (Compston & Coles, 2008). The clinical presentation of the disease is seen by affecting the following (Compston & Coles, 2008): (a) sensation, examples: loss of sensitivity, numbness; (b) muscle strength and mobility, examples: muscle weakness, spasms, speech & swallowing problems, bladder and bowel difficulties; (c) balance, examples: ataxia, feeling tired, acute or chronic pain; and; (d) vision, examples: blurred vision, nystagmus, optic neuritis or double vision

The pathophysiology of this disease is not fully understood; however, T lymphocyte (T-cell) mediated autoimmune mechanisms has be considered as the major trigger for disease initiation (Nylander & Hafler, 2012; Hartley et al., 2014).

2.1.2 Animal Models of Multiple Sclerosis:

Experimental autoimmune encephalomyelitis (EAE) is the condition wherein immune and neuropathological pathways cause disease features similar to MS (Prineas et al.,

1984; Raine & Wu, 1993; Constantinescu et al., 2011). Many self antigens such as myelin-associated glycoprotein (MAG), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or proteolipid protein (PLP) have been used as immunizing agents to create EAE in rodent models (Tuohy et al., 1988; Amor et al., 1994; Johns et al., 1995). EAE models are routinely used in drug discovery for MS and many approved drugs were tested in this model (Constantinescu et al., 2011).

EAE is known to be a T-cell dependent disease and it has been shown in-vitro that adoptive transfer of activated myelin-reactive CD4+ T cells induces the disease (Olsson, 1995; Weir et al., 2002). Myelin oligodendrocyte glycoprotein peptide residues 35-55 (MOG) is a commonly used protein to induce EAE. MOG is a myelin component that activates T-cells in mice and humans which results in CNS tissue destruction due to T-cell trafficking to the brain and spinal cord (Koehler et al., 2002; Weir et al., 2002).

2.1.3 Zebrafish Models of Multiple Sclerosis:

Although there have been several studies on myelination/de-myelination, including the ones for drug screening as well as pathobiological understanding (Buckley et al., 2008; Raphael & Talbot, 2011; Karttunen et al., 2017); there wasn't a published zebrafish model for MS. We were the first group to develop, standardize, validate and report the zebrafish EAE model with a robust protocol and detailed methodology. This chapter is recreated based on our publication i.e. Kulkarni et al., 2017.

2.1.4 Zebrafish EAE Model of Multiple Sclerosis:

Zebrafish, have emerged as a promising model to study neuro-degeneration related to autoimmune demyelination (Buckley et al., 2008; Fang et al., 2015). This work reported here, demonstrates the standardization and validation of a zebrafish model

of EAE. We report a robust protocol comprising of the regimen of MOG for disease induction; designation of the clinical scores and, other assessments like body weight and histopathology changes in the fish. The model has been standardized by assessing the rescue of the clinical symptoms by using approved drug fingolimod hydrochloride. Furthermore, the model has been validated using approved drugs or drugs under development for the treatment of MS which have shown efficacy in the rodent EAE model.

Mouse models of EAE are laborious, expensive and take long time they take 4-8 weeks for induction of symptoms and monitoring of drug responses, as well as require high amounts of drug for long duration studies (Merrill, 2009; Getts et al., 2012). Thus, the experiments conducted here were to develop a model that will be useful as a quick in vivo screen for MS.

2.2 GAP IN EXISTING RESEARCH

Zebrafish have been known to be a good model to study the myelin sheet, however, there has been no published protocol for use of zebrafish as a model for MS, and therefore, the gap in existing research was as follows:

1. Lack of a robust zebrafish EAE model for MS.
2. No report on efficacy of known MS drugs and their dosage.
3. No data on correlation between zebrafish to humans.

2.3 OBJECTIVES OF THE STUDY

1. Development, standardization and validation of the zebrafish EAE model.
2. Evaluating known drugs to validate the model as relevant models for MS.
3. To determine efficacy dose for the drugs in terms of mg/kg.

4. To correlate the data between zebrafish and higher mammals including humans based on published literature.

2.4 MATERIALS AND METHODS

2.4.1 Animal care and maintenance

Zebrafish were maintained as following general care and maintenance details mentioned in Chapter I. Four to six months old male fish were used for these experiments.

2.4.2 Chemicals, drugs and drug administration

Test drugs and Complete Freund's adjuvant (CFA) (Cat. No. F5881) were procured from Sigma Aldrich, USA. Routine laboratory chemicals were purchased from Sisco Research Laboratories, Hyderabad, India. MOG (Sequence: MEVGWYRSPFSRVVHLYRNGK) was procured from GenScript HK Limited, Hong Kong. The drugs were administered either orally (Kulkarni et al., 2014) or intra-peritoneally (Chaudhari et al., 2013). The drugs were administered daily for the duration of treatment in the morning hours between 9.00 – 10.00 AM.

2.4.3 Optimization of immunization dose

Experimental autoimmune encephalomyelitis (EAE) was induced using myelin oligodendrocyte glycoprotein – 35-55 (MOG). MOG in CFA was injected subcutaneously (s.c.) in the mid spine regions near the end of the precaudal vertebrae (Figure 1) using 10µl bevel-tipped Hamilton syringe with a volume of 5µl/fish.

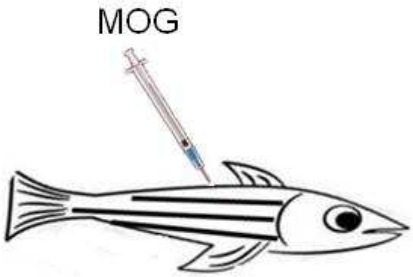


Figure 2.1: Depiction of Site of Injection for Disease Induction. Adult zebrafish are immunized with myelin oligodendrocyte glycoprotein – 35-55 (MOG) in Complete Freund's adjuvant (CFA) by subcutaneous (s.c.) injection in the mid spine regions near the end of the precaudal vertebrae to induce experimental autoimmune encephalomyelitis (EAE).

Three concentrations; 0.3, 0.6 and 1 mg/ml of MOG; were tested to standardize the dose. The criteria for selection of immunization dose were efficiency for induction of clinical symptoms, body weight reduction and low mortality. The different groups were the following: vehicle control (CFA s.c.), MOG in CFA 0.3 mg/ml s.c., MOG in CFA 0.6 mg/ml s.c and MOG in CFA 1 mg/ml s.c. The clinical scores were assigned as: 1: Normal, 2: Loss of Gait, 3: Mild Paralysis, 4: Total Paralysis. Each of the clinical signs can be seen in Video 1 of the publication of this model (Kulkarni et al., 2017). All fish were observed for clinical scores, body weight and mortality for 7 days post treatment.

2.4.4 Validation with the fingolimod hydrochloride (hereafter referred as fingolimod)

Zebrafish were immunized on day 1 with standardized concentration i.e. 0.6 mg/ml s.c. of MOG. Fingolimod hydrochloride (Trade Name: Gilenya) is a sphingosine 1-phosphate receptor modulator, is a marketed MS drug used for relapsing MS (Brinkmann, 2009; Chiba & Adachi, 2012). Fingolimod was administered orally using

two regimens; treatment starting immediately after immunization called the progressive regimen (P); and; treatment starting after disease development on day 3 called therapeutic regimen (T). The doses of 0.1, 0.3 and 1 mg/kg of fingolimod using dose volumes of 5µl p.o. were administered orally (p.o.) for 7 days in both the regimens. All fish were observed daily for clinical symptoms and mortality for 7 days of treatment. Body weights were recorded on days 1 and 7 of treatment. Qualitative scoring of EAE signs were done in blinded fashion using video recordings of 3-7 minutes. In the therapeutic regimen (T), the treatment was starting day 3, hence observations made on day 7 of treatment were day 9 post immunization, and therefore, the data reported is with respect to the days of treatment. Statistical analysis for clinical scores was performed using GraphPad Prism® software using Kruskal-Wallis analysis followed by Dunn's multiple comparison test. Statistical analysis for body weight loss was performed using One-way ANOVA followed by Dunnet's Post-hoc test.

The study for each regimen was conducted separately with twelve fish per group assigned in six treatment groups at the beginning of treatment. The different groups were: vehicle control (CFA s.c. + water p.o.), MOG control (MOG 0.6 mg/ml s.c. + water p.o.), Fingolimod 0.1 mg/kg (MOG 0.6 mg/ml s.c.+ Fingolimod 0.1 mg/kg p.o.), Fingolimod 0.3 mg/kg (MOG 0.6 mg/ml s.c.+ Fingolimod 0.3 mg/kg p.o.), Fingolimod 1 mg/kg (MOG 0.6 mg/ml s.c.+ Fingolimod 1 mg/kg p.o.).

The spinal sections (representative samples) of zebrafish were assessed for histopathological evaluation to study the extent of inflammation, neurodegeneration and demyelination with and without fingolimod treatment. Four groups were studied: vehicle control, MOG, Fingolimod 1 mg/kg from progressive regimen (P) and

Fingolimod 1 mg/kg from therapeutic regimen (T). Standardized protocols were used for issue processing and staining and various parameters were analyzed as follows:

(a) Infiltration cells in the hematoxylin and eosin stained sections were counted by using ImageJ Analysis Software, according to method suggested by Skundric et al., 2008.

(b) Glial cell in the crystal violet stained sections were counted using ImageJ Analysis Software, as per the method suggested by Kluver & Barrera, 1954.

(c) Myelination intensity of luxol fast blue stained region was measured using RGB Plug-in in ImageJ Analysis Software Kiernan, 2007.

Statistical analysis was performed using GraphPad Prism® software using One-way ANOVA followed by Dunnet's Post-hoc test.

2.4.5 Detailed validation with additional drugs

Detailed validation of model was conducted in the prophylactic regimen (P) using three drugs: dimethyl fumarate, dexamethasone and SR1001 in following manner:

(a) Dimethyl fumarate is an approved drug for MS (Chen et al., 2014); and was tested orally at doses of 15, 30 and 60 mg/kg.

(b) Dexamethasone has been reported to be efficacious in rodent EAE mdoels (Donia et al., 2010); and was tested after intra-peritoneal injection at 0.3, 1 and 3 mg/kg doses.

(c) SR1001 is a RAR-related orphan receptor (ROR) ligand, being developed by The Scripps Research Institute, and has proven efficacious in rodent EAE (Solt et al., 2011). It was tested after intra-peritoneal injection at 25, 50 and 75 mg/kg doses.

Statistical analysis for clinical scores was performed using GraphPad Prism® software using Kruskal-Wallis analysis followed by Dunn's multiple comparison test.

Statistical analysis for body weight loss was performed using One-way ANOVA followed by Dunnet's Post-hoc test.

2.4.5 Correlation of data with higher mammals and humans

Genetic correlation of was conducted using gene sequence comparison for particular genes using NCBI, USA and Ensembl, UK databases by selecting homologue option. Pair wise alignments were generated using BLAST. The percentage homology was checked for zebrafish v/s mouse & zebrafish v/s human. The efficacy data in rodents and humans for dose correlation was obtained from published literature and USFDA pharmacology reviews. Furthermore, zebrafish minimal efficacy dose (MED) was correlated with human minimal efficacy dose (MED) using Linear Regression analysis of GraphPad Prism® Software.

2.5 RESULTS

2.5.1 Optimization of immunization dose

Acute onset of EAE was observed after MOG immunization and clinical signs stated appearing in 3 – 4 days. The mean clinical scores increased gradually (Figure 2.2.(a)) at various doses of MOG and at day 7 post immunization the mean clinical scores in the groups of Vehicle Control, MOG (0.3mg/ml), MOG (0.6mg/ml) and MOG (1mg/ml) were 1.0 + 0.0, 2.0 + 0.2, 3.9 + 0.1 and 1.3 + 0.2 respectively (Figure 2.2. (b)). The survival data (Figure 2.2 (c)) showed that there was 100% survival in vehicle control and MOG (0.3mg/ml) groups whereas the survival in MOG (0.6mg/ml) and MOG (1mg/ml) groups was 70% and 60% respectively (Figure 2.2. (c)). A dose of 0.6 mg/kg MOG in CFA was standardized based on parameters of efficiency to induce clinical symptoms, body weight reduction and low. An unexpected lower mean clinical score was observed at 1 mg/kg dose of MOG, which could be attributed to local accumulation or spillage at higher concentration. This aspect was not probed

further as 0.6 mg/kg dose satisfied the conditions for reasonable induction of disease. We also performed Kaplan-Meier survival analysis to know survival probability after administration of MOG 0.6 mg/kg in 36 fish and observed that there was a constant 70% survival over a 7 days period post immunization with no increase in mortality after day 3 (Figure 2.2. (d)). With respect to the perspective of animal ethics, mortality observed was similar to those observed in mouse models of EAE (Thell et al., 2016).

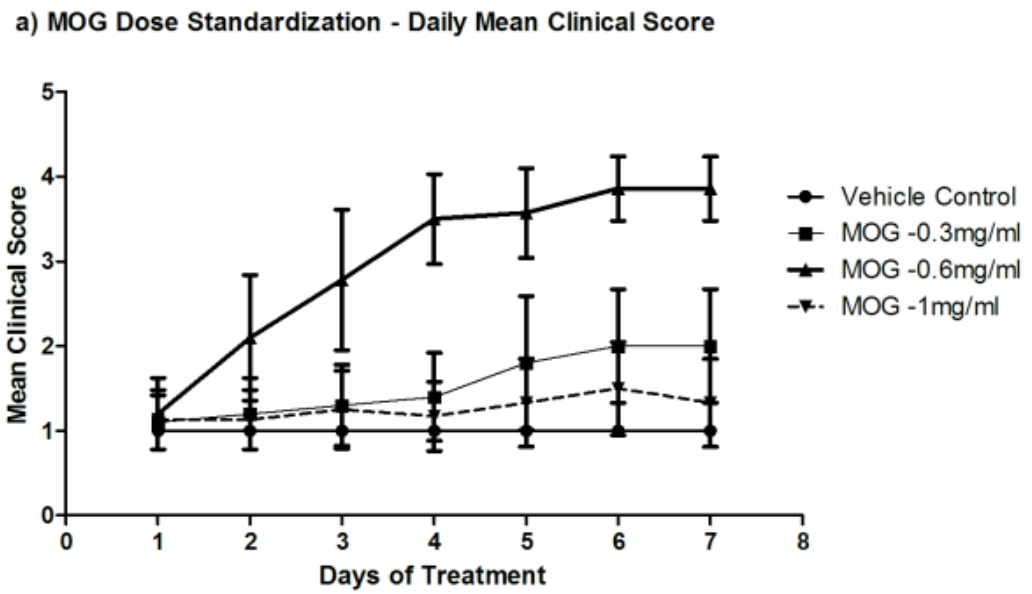


Figure 2.2: Standardization of immunization dose with myelin oligodendrocyte glycoprotein (MOG): (a) Effects of MOG (0.3 mg/kg, 0.6 mg/kg and 1 mg/kg) as mean clinical score of paralysis like activity seen every day from day 1 to day 7.

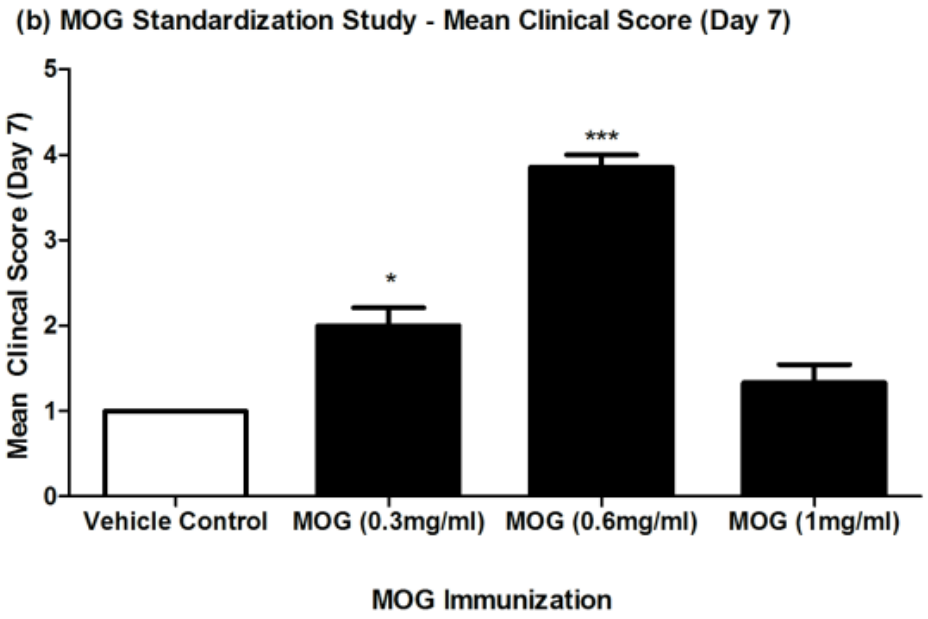


Figure 2.2: Standardization of immunization dose with myelin oligodendrocyte glycoprotein (MOG): (b) Mean clinical score on Day 7. (*p<0.05, **p<0.01 and ***p<0.001) (n=10 at the beginning of treatment).

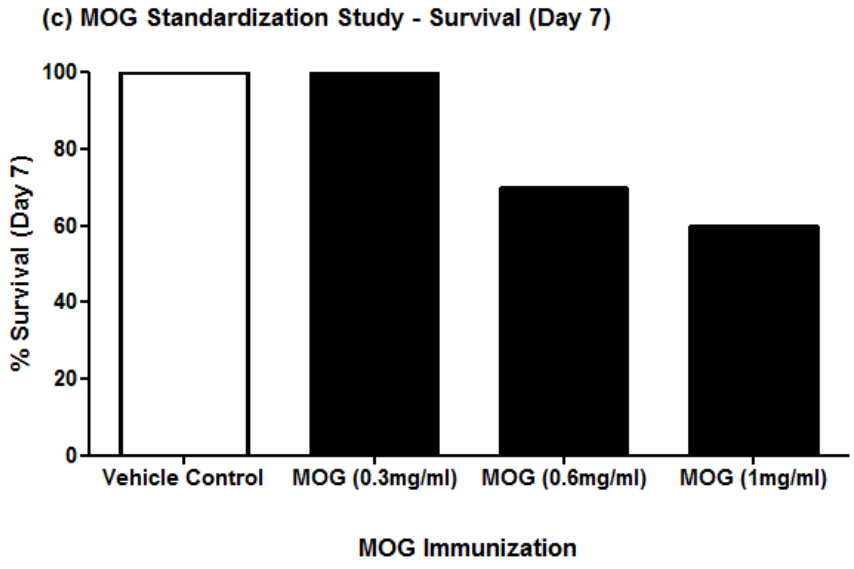


Figure 2.2: Standardization of immunization dose with myelin oligodendrocyte glycoprotein (MOG): (c) Effects of MOG on survival. Data are represented as Percentage Survival on day 7 post immunization (n=10).

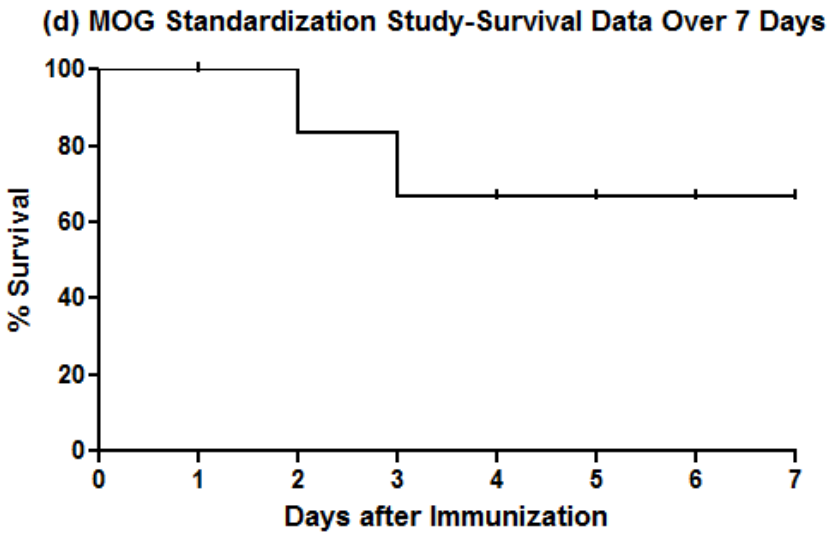


Figure 2.2: Standardization of immunization dose with myelin oligodendrocyte glycoprotein (MOG): (d) Kaplan-Meier survival analysis performed to know survival probability after administration of MOG 0.6 mg/kg (n=36 at the beginning of treatment).

The figures and legends have been adapted from Kulkarni et al., Multiple Sclerosis and Related Disorders 11 (2017) 32–39.

2.5.2 Validation with fingolimod:

The results of study with fingolimod are presented in Figure 3. The survival data for the groups vehicle control, MOG control, fingolimod 0.1 mg/kg, fingolimod 0.3 mg/kg and fingolimod 1 mg/kg was 100%, 60%, 60%, 70% and 80% respectively in prophylactic regimen and 100%, 70%, 70%, 80% and 90% in the therapeutic regimen (Figure 2.3. (a) and (b)). Daily clinical scores are depicted in Figure 2.3 ((c) and (d)) and the mean clinical scores on day 7 post initiation of treatment were 1.0 ± 0.0, 3.9 ± 0.1, 3.0 ± 0.4, 1.3 ± 0.2 and 1.0 ± 0.0 in the prophylactic regimen and 1.0 ± 0.0, 3.2 ± 0.2, 2.9 ± 0.3, 2.4 ± 0.3 and 1.6 ± 0.2 in the therapeutic regimen respectively for the groups of vehicle control, MOG control, fingolimod 0.1 mg/kg, fingolimod 0.3 mg/kg and fingolimod 1 mg/kg (Figure 2.3 (e) and (f)). In terms of percent body weight

reduction the data for groups of vehicle control, MOG control, fingolimod 0.1 mg/kg, fingolimod 0.3 mg/kg and fingolimod 1 mg/kg was 0.0 ± 2.3 %, 34.7 ± 4.0 %, 23.4 ± 2.1 %, 19.6 ± 2.0 % and 5.2 ± 2.3% for prophylactic regimen and -0.8 ± 2.3 %, 42.2 ± 2.2 %, 33.5 ± 2.0 %,16.0 ± 2.8% and 4.7 ± 2.4% for therapeutic regimen respectively (Figure 2.3 (g) and (g)).

Doses of 0.3 and 1 mg/kg showed improvement in percentage survival of ~ 10% and 20% respectively, in comparison to MOG group on day 7. Fingolimod treatment showed daily improvement in mean clinical scores as compared to the MOG group. At doses of 0.3 and 1 mg/kg of fingolimod, there was statistically significant improvement in body weight loss data. At 1 mg/kg group the fingolimod, the mean clinical score and body weight loss data was similar to the vehicle control group on day 7 demonstrating it as dose successful in rescuing disease symptoms and demonstrating absolute efficacy.

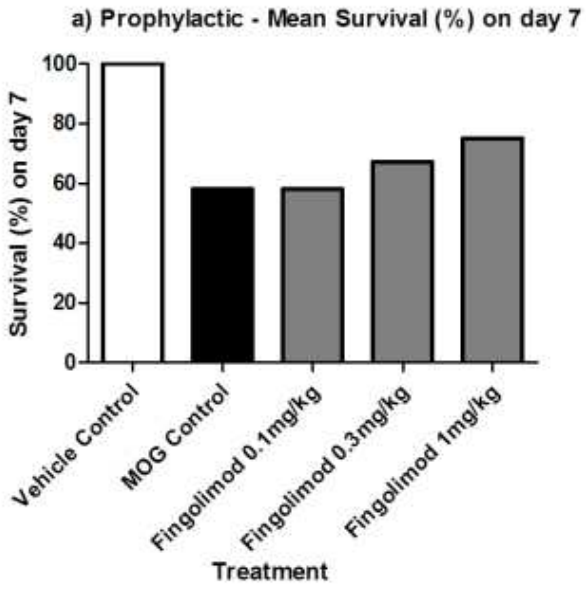


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (a) Percent Survival on Day 7 in Prophylactic Regimen.

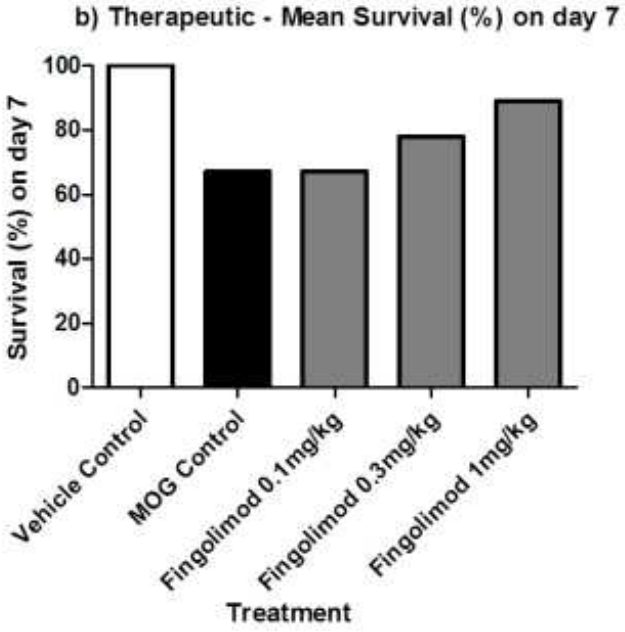


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (b) Percent Survival on Day 7 in Therapeutic Regimen.

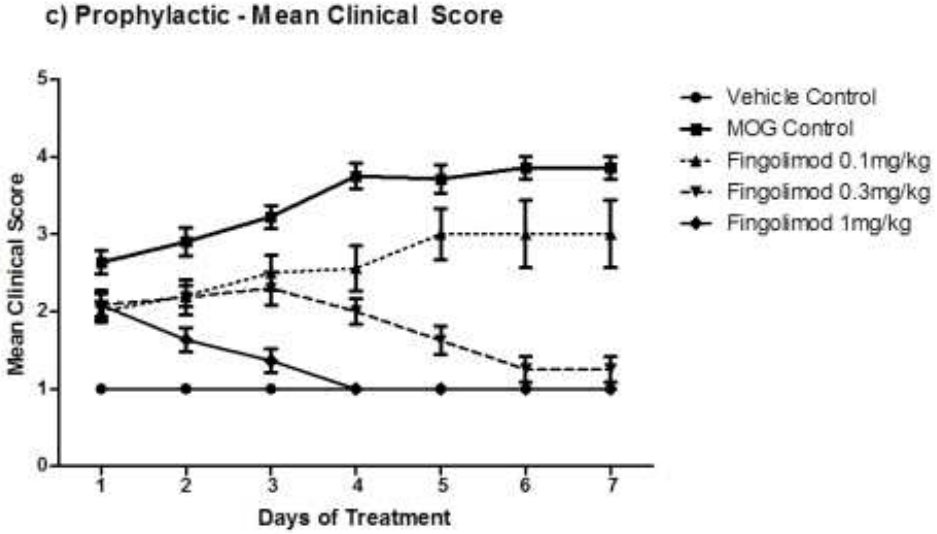


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (c) Mean Clinical Score over 7 Days in Prophylactic Regimen. (Mean ± S.E.M.) (*p<0.05, **p<0.01 and ***p<0.001) (n=12 at the beginning of treatment)

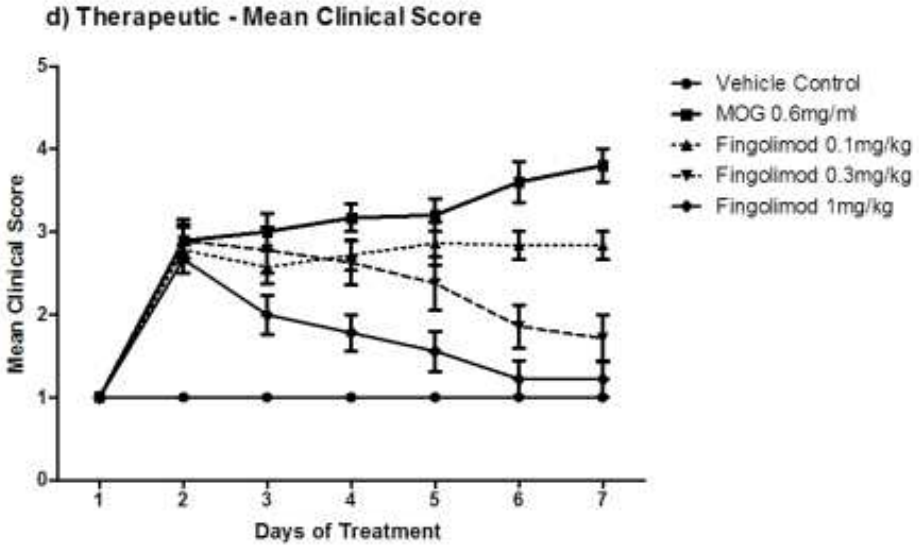


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (d) Mean Clinical Score over 7 Days in Therapeutic Regimen. (Mean ± S.E.M.) (*p<0.05, **p<0.01 and ***p<0.001) (n=12 at the beginning of treatment)

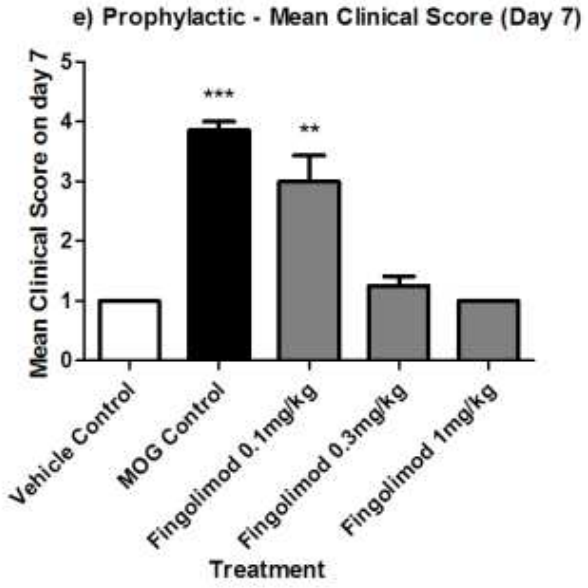


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (e) Mean Clinical Score on Day 7 in Prophylactic Regimen. (Mean ± S.E.M.) (*p<0.05, **p<0.01 and ***p<0.001) (n=12 at the beginning of treatment)

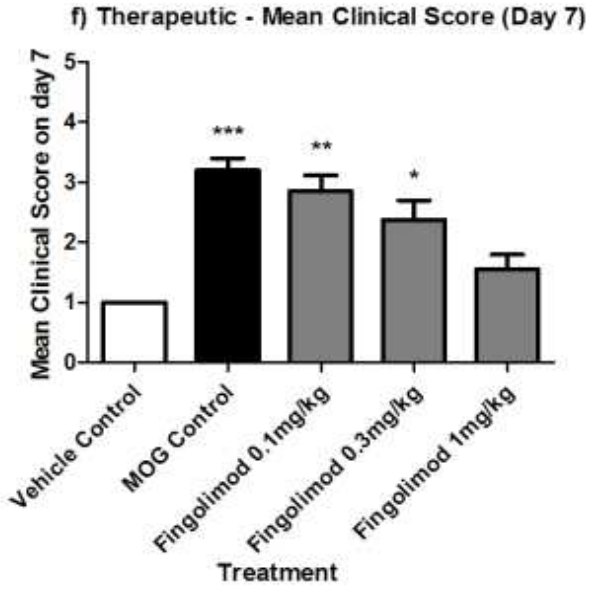


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (f) Mean Clinical Score on Day 7 in Therapeutic Regimen. (Mean ± S.E.M.) (*p<0.05, **p<0.01 and ***p<0.001) (n=12 at the beginning of treatment)

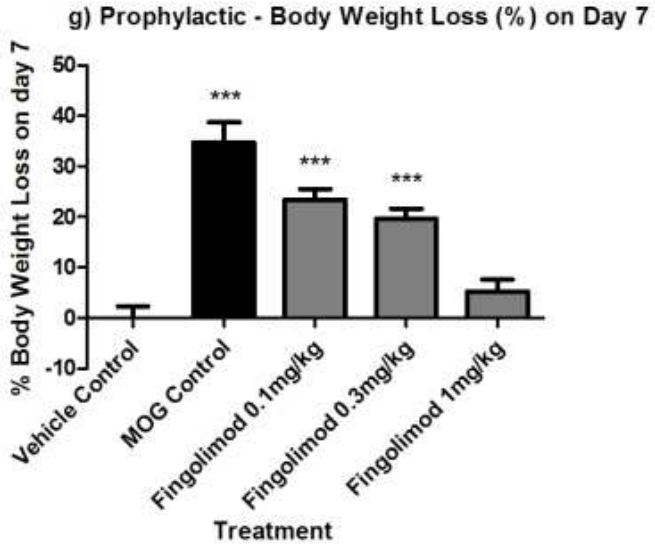


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (g) Percent Body Weight Loss on Day 7 in Prophylactic Regimen. (Mean ± S.E.M.) (*p<0.05, **p<0.01 and ***p<0.001) (n=12 at the beginning of treatment)

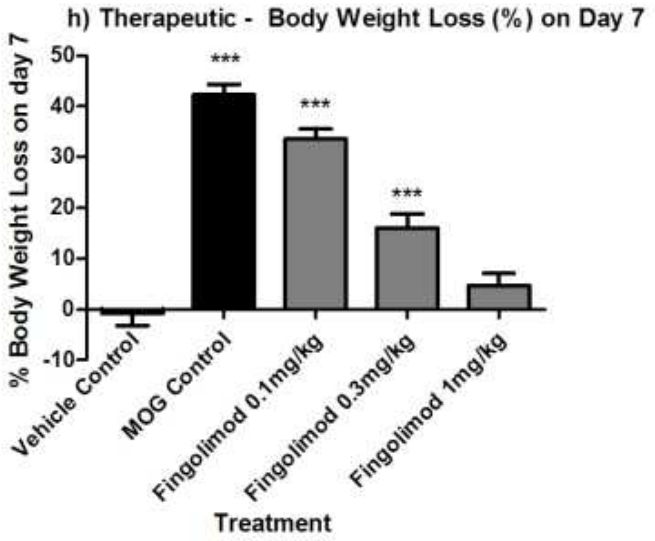


Figure 2. 3: Phenotypic effects seen in validation with fingolimod. (h) Percent Body Weight Loss on Day 7 in Therapeutic Regimen. (Mean ± S.E.M.) (*p<0.05, **p<0.01 and ***p<0.001) (n=12 at the beginning of treatment)

The figures and legends have been adapted from Kulkarni et al., Multiple Sclerosis and Related Disorders 11 (2017) 32–39.

The results of histopathological evaluation of spinal sections (performed in four groups viz. vehicle control, MOG control, fingolimod 1 mg/kg (P) and fingolimod 1 mg/kg (T), where P = prophylactic regimen and T = therapeutic regimen) are depicted in figure 2.5 and representative sections have been depicted in figure 2.4. The infiltration cell/section numbers for groups vehicle control, MOG control, fingolimod 1 mg/kg (P) and fingolimod 1 mg/kg (T) were 97.0 ± 4.7,121.0 ± 6.7, 97.7 ± 3.9 and 96.3 ± 3.2 respectively (Figure 2.5. (a)). This clearly indicates that MOG immunized fish had higher extent of inflammation that was statistically significant when compared to vehicle control whereas fingolimod treatment (in both regimens) showed similar number of infiltration cells as controls. There was statistically significant decrease in glial cell count per section of MOG immunized fish which was not the case in vehicle and fingolimod groups. The number of glial cells per section

was 46.7 ± 4.1, 31.7 ± 2.6, 46.0 ± 1.5 and 45.3 ± 1.5 for vehicle control, MOG control, fingolimod 1 mg/kg (P) and fingolimod 1 mg/kg (T) respectively. Loss of myelin indicated by luxol fast staining indicated reduction in intensity in MOG group when compared to vehicle control and fingolimod treatments. The staining intensity for the groups vehicle control, MOG control, fingolimod 1 mg/kg (P) and fingolimod 1 mg/kg (T) was 197.0 ± 2.5, 185.0 ± 6.5,194.7 ± 2.3 and 193.7 ± 3.2 respectively.

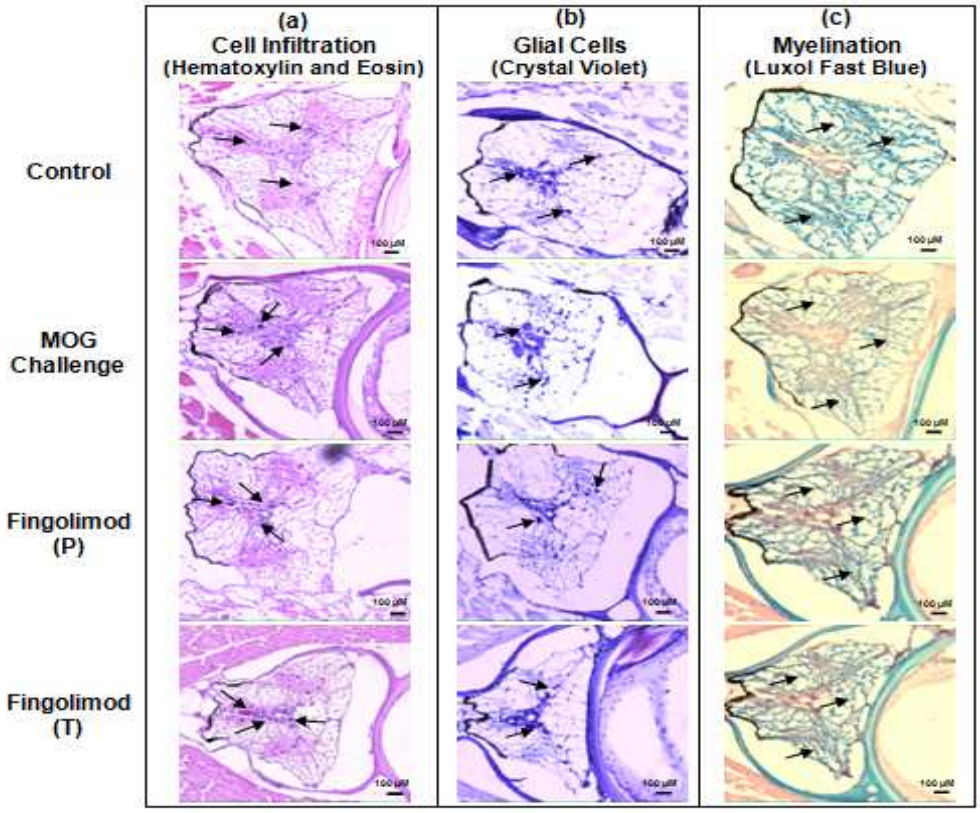


Figure 2.4: Black and white images of representative spinal cord histopathological sections (40X magnification). Groups: vehicle control, MOG control, Fingolimod 1 mg/kg (P) and Fingolimod 1 mg/kg (T) seen on day 7 of treatment in validation study with Fingolimod. The arrows (→) point towards examples of cells counted or blue intensity measured using ImageJ. *The figures and legends have been adapted from Kulkarni et al., Multiple Sclerosis and Related Disorders 11 (2017) 32–39.*

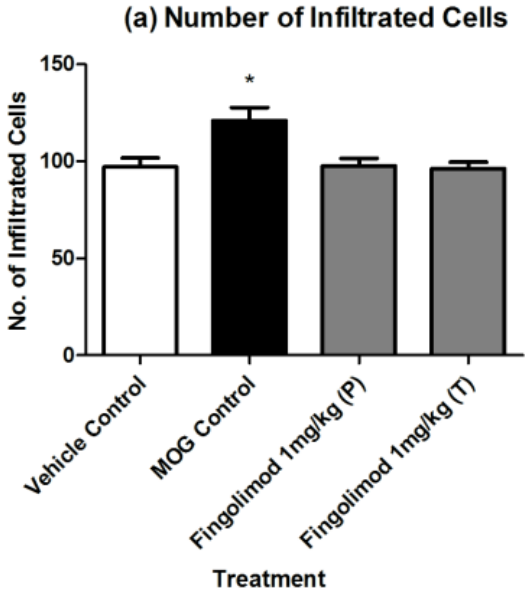


Figure 2.5: Histopathological effects seen on day 7 of treatment in validation with fingolimod. (a) Number of Infiltrated Cells

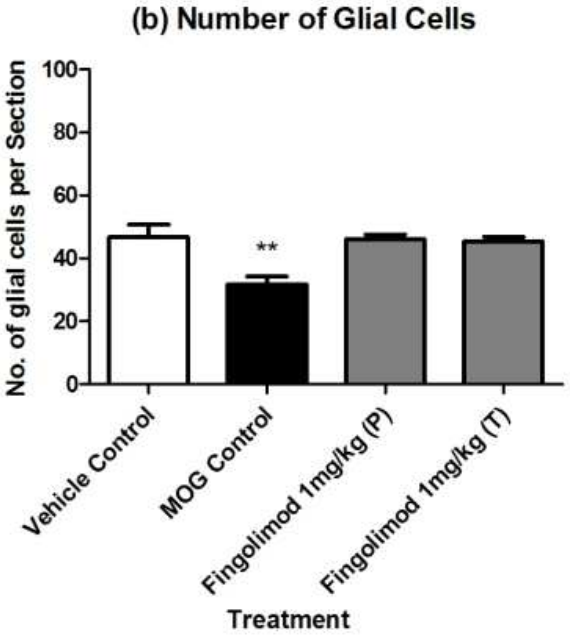


Figure 2.5: Histopathological effects seen on day 7 of treatment in validation with fingolimod. (b) Number of Glial Cells

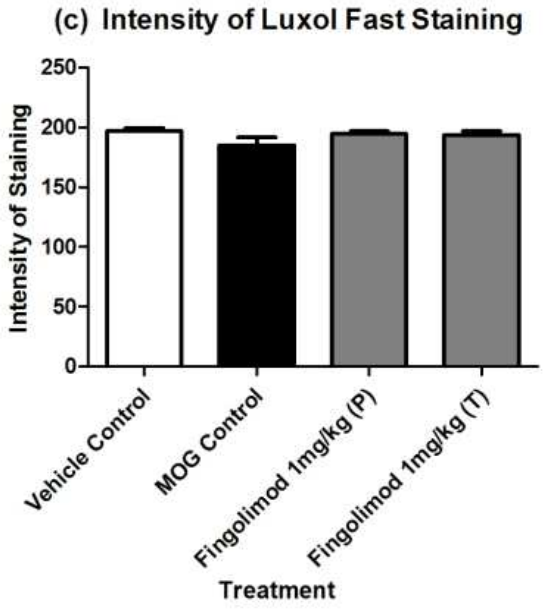


Figure 2.5: Histopathological effects seen on day 7 of treatment in validation with fingolimod. (c) Intensity of Luxol Fast Staining.

Data are represented using mean and standard error of the mean (± S.E.M.). GraphPad Prism® software was used for conducting One-way ANOVA followed by Dunnet's Post-hoc test comparing all other groups with Vehicle Control (*p<0.05, **p<0.01 and ***p<0.001; n=3).

The figures and legends have been adapted from Kulkarni et al.2017.

All the observations suggest that fingolimod was able to reverse the effects of EAE in zebrafish model. These observations concur with those reported in conventional rodent models.

2.5.3 Detailed validation with additional drugs:

The data for detailed validation is represented in Table 1. Major observations were as follows:

Table 2.1: Validation Study with Known Drugs: Phenotypic effects seen on day 7. (*p<0.05, **p<0.01 and ***p<0.001). *The table has been adapted from Kulkarni et al., Multiple Sclerosis and Related Disorders 11 (2017) 32–39.*

Groups	Drugs	No. of fish at the start of study (n)	Drug Doses (mg/kg of body weight)	Major Parameters on Day 7 Post Immunization		
				Survival (%)	Clinical Score (Mean ± S.E.M.)	Body Weight Loss (%) (Mean ± S.E.M.)
Vehicle Control	**-**	**36**	-	100	1.00 ± 0.0	0.49 ± 1.19
MOG Control	**-**	**36**	-	68	3.7 ± 0.2 ***	31.66 ± 3.21***
Positive Control	**Fingolimod**	**36**	1	82	1.0 ± 0.0	5.22 ± 2.33
Test Drugs	**Dimethyl Fumerate**	**12**	15	40	2.0 ± 0.6*	2.77 ± 1.06
			30	64	2.7 ± 0.6 ***	1.98 ± 2.32
			60	70	2.5 ± 0.7 ***	4.67 ± 1.85
	Dexamethasone	**12**	0.3	67	2.4 ± 0.2 **	1.98 ± 2.32
			1	67	1.4 ± 0.2	4.67 ± 1.85
			3	75	3.8 ± 0.3 **	6.68 ± 1.40 *
	SR1001	**12**	25	50	2.1 ± 0.2 *	6.68 ± 1.40 *
			50	67	1.2 ± 0.1	2.41 ± 1.10
			75	33	2.4 ± 0.3 *	10.57 ± 2.31 ***

(a) Dimethyl fumerate treatment showed dose dependent improvement in survival rates. There was improvement in clinical scores and body weight parameters and the data looked like a saturated effect at the doses tested as the clinical scores though better than MOG group, were similar at all doses.

(b) Dexamethasone treatment also showed improvement in survival rates, clinical scores and body weight loss when compared to MOG groups. At highest dose, however, the clinical score and body weight loss increased, which could be due to immunosuppression.

(c) SR1001, showed improvement in survival rate at one dose i.e. 50 mg/kg and improvement in clinical score and body weight loss at all doses. However, severe mortality at the highest dose was seen.

In summary, chemical classes of drugs with different mechanisms, and different administration routes at different doses have been tested to validate the model.

2.5.4 Correlation of data with respect to mechanism of action:

When correlating the data with conventional models the genetic correlation is depicted in Table 2. It is clear that zebrafish express orthologues of spingosine-1-phosphate receptors that are targets for fingolimod (Tobia et al., 2012). Similarly, glucocorticosteroid receptors which mediate the effect of dexamethasone (Schaaf et al., 2012), Nrf2 which is the major target for dimethyl fumerate (Mukaigasa et al., 2012) and ROR genes that are modulated by SR1001 (Katsuyama et al., 2007), are all expressed in zebrafish. Thus suggesting that all major pathways for disease modulation of MS and associated disorders are present in zebrafish and drugs acting though those mechanisms show efficacy in the EAE model.

Table 2.2: Genetic correlation (percentage homology) of zebrafish with mouse and humans. This analysis was conducted using gene sequence comparison for particular genes using NCBI, USA and Ensembl, UK databases by selecting homologue option. Pair wise alignments were generated using BLAST.

Drug	Drug Target	Genetic Homology	
		Zebrafish v/s Mouse	Zebrafish v/s Human
Fingolimod	SPHK2	65%	67%
	S1PR1	84%	84%
	S1PR3	79%	82%
	S1PR4	68%	65%
	S1PR5	69%	70%
Dimethyl fumarate	NRF2	59%	60%
Dexamethazone	NR3C1	88%	88%
	NR0B1	65%	65%
	ANXA1	88%	89%
	NOS2	81%	80%
SR1001	ROR ALPHA	95%	95%
	ROR GAMA	64%	64%

2.5.5 Correlation of data with respect to potency and dose:

The correlation of efficacy dose of these drugs has been depicted in Table 2.3. The data suggests that the rank order of potency of various drugs is similar in zebrafish as compared to rodents and humans. The minimum effective dose (MED) was identified as the dose that showed rescue of symptoms and statistical significance. Furthermore, linear regression analysis (Figure 2.6) demonstrated that there was a correlation between the zebrafish and human MED and the coefficient of

determination of 0.98 for efficacious drugs suggests a 98% data correlation between data on zebrafish and human doses. This suggests that effect of various candidate drugs candidates zebrafish EAE model can be useful in prioritizing the candidates for further evaluation.

Table 2.3: Minimum efficacious dose (MED) in zebrafish, mouse and humans.

Drug	Zebrafish (mg/kg)	Rodents (mg/kg)	Human (mg/kg/day)*
Gilenya	1	0.1 [a]	0.2 [a]
Dimethyl Fumarate	60	15 [b]	2 [b]
Dexamethasone	1	4 [c]	0.03 [c]
SR1001	50	25 [d]	2 [e]

[a] USFDA, 2010; [b] USFDA, 2014; [c] Donia et al., 2010; [d] Solt et al., 2011; [e] Dose extrapolation from mouse to humans based USFDA, 2005 guidelines.

2.6 DISCUSSION

2.6.1 Major advantages of the model:

The novel model for MS that has been developed and validated here has the following advantages: (i) Quick: in a span of 7 days the efficacy of a candidate drug can be evaluated as against 6-8 weeks required for conventional rodent models; (ii) Low Compound Requirement: the quantity of test compound required for efficacy evaluation is very low, for example, in rodent studies, for a 10 mice having average weight of 25g with an average dose of 10 mg/kg/day dose for 4 weeks, the total test compound required will be 70g. Whereas, for similar experimental design in the zebrafish model the requirement will be 350 µg for the 7 day study. (iii) Inexpensive In vivo Data: the low cost of maintaining zebrafish, compound usage, labor required and time saved makes this model suitable for screening higher number of compounds in-vivo.

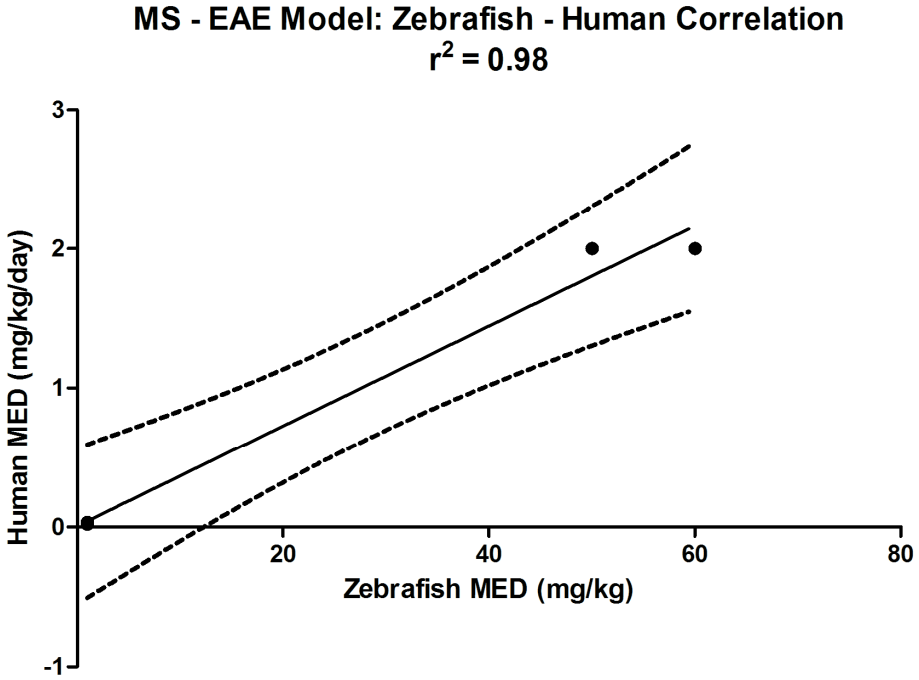


Figure 2.6: Linear regression analysis of MED for zebrafish with respect to humans. Straight (–) lines represent the linear regression, whereas, dotted (….) lines represent the 95% confidence interval with respect to liner regression. r2 = coefficient of determination.

2.6.2. Relevance in drug discovery:

The EAE model has been under question with respect of translational potential of the animal data in MS patients. However, EAE is the most widely used in vivo model and almost all approved MS drugs have been tested in this model (Constantinescu et al., 2011). The zebrafish model for EAE can be used preceding rodent models as a filter at lead optimization stage and most promising compounds selected using this screen can be further tested in conventional systems.

Zebrafish MOG has not been reported thus far. However, it has previously been reported that antibodies to MOG cross react with other butyrophilin (BTN) family

proteins in mammals and this has been implicated in MS (Guggenmos et al., 2004). The fact that zebrafish have butyrophilin-like proteins (e.g. NCBI Sequence ID: NP_001103953.1), justifies use of MOG to elicit immune response in zebrafish. Furthermore, it has been observed that clinical signs of MS using MOG induction and have seen the effect of known drugs in reversing them.

There is scope for further refinement in this model in the aspects of larger set of drugs, detailed histo and immune pathology, biomarkers, follow-up MOG injections, etc. will need to be further investigated to refine this model for optimal utilization.

2.7 CONCLUSIONS

This is the first report, suggesting an in vivo adult zebrafish EAE model. In these studies, the model has been developed, standardized and validated. The data has been correlated with data from other conventional models including humans. This model will undergo a wider validation and there is scope to for improvement. However, this work will be the starting point of all such efforts.

CHAPTER 3:

PENTYLENETETRATZOLE INDUCED ZEVBRAFISH EPILEPSY MODEL

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3.1 INTRODUCTION

3.1.1 Background:

Epilepsy is a neurological disorder outlined by transient occurrence of seizures as a consequence of aberrant neuronal activity (Fisher et al., 2014), and due to variance in excitatory/inhibitory neurotransmission (White & Smith, 2009). Based upon current statistics 50 million people worldwide have epilepsy and it accounts for 0.75% of the global essence disease (Ngugi et al., 2010; WHO, 2004). Inspite of advances in epilepsy treatment ample seizure restraint has not been acquired in certain portion of populations due to limited knowledge on genetic & neurobiology mechanisms of epilepsy (Goldsmith, 2004; Romanelli, 2012). Even though animal models like rodents are being used as universal seizure model due to their homology with humans there is need for reliable animal models that are easy to use, can help understanding of underlying molecular mechanisms (Kupferberg, 2001; Loscher & Schmdit, 2011; Kwan, 2010) and which will be helpful for discovery and subsequent development of novel anti-epileptic drugs (AEDs). Furthermore, there is a need to have animal models that can act as quick in-vivo screen in AED drug discovery due to the constrains of rodent models in terms of fulfilling the 3R requirements, cost, time and labor intensiveness.

3.1.2 Animal Models of Epilepsy:

The two most regularly used and extensively studied model of epilepsy in rodent models that are well established are as follows:

(1) Maximal electroshock (MES) model: In this model the electric shock generates brainstem mediated tonic–clonic seizures. At molecular level, in this model, there are changes observed in immediate early genes, however, one major limitation of

this model is that electrophysiological data cannot be recorded due to high seizure intensity seen in this model (Cavarsan et al., 2015).

(2) Pentylenetetrazole (PTZ) induced model: PTZ is a chemo-convulsant acts by binding to the picrtoxin site of $GABA_A$ which leads to the increase in glutamate levels to induce seizures. PTZ induced seizure model of rodents is extensively used for development of several AEDs (Mandhane et al., 2007; Loscher, 2011). In rodent model, behavioral analysis of seizure stage scoring has been correlated to abnormal electric activity in brain through EEG (Sarkisian, 2011; Watanabe et al., 2010) but due to the drawbacks described above, there is an urge for developing promising small vertebrate models.

3.1.3 Zebrafish Models of Epilepsy:

Zebrafish are becoming a powerful tool in AED research because of its high genetic homology with rodents & humans (Shin & Fishman, 2002; Dooley & Zon, 2000; Brittijin et al., 2009; Egan et al., 2009; Barbazuk et al., 2000). Recent reports emphasize the scope of zebrafish larvae in neurological disorders research such as epilepsy-like behaviour and for development of novel AEDs (Bergmans et al., 2007; Goldsmith, 2004; Langheinrich, 2003). Because of limitations like small and underdeveloped brain, BBB functioning only after 7 days post fertilization and difficulty in validating the larval model due to non-correlation to EEG; adult zebrafish epilepsy prototypes have emerged as popular beneficial approach for evaluation of pro-convulsants drugs over the embryo-larval models (Stewart et al., 2010; Friedrich et al., 2010; Goldsmith & Fleming, 2007; Eliceiri & Baird, 2011; Jeong et al., 2008).

In this study we investigate the usefulness of adult zebrafish as promising seizure model. PTZ is well known conventional chemo-convulsant inducer in all animal models like rodents, other species (Akula et al., 2009; Carmody & Brennan, 2009)

including larval (Baraban et al., 2007; Tiedeken & Ramsdell, 2009; Hortopan et al., 2010) and adult zebrafish (Wong et al., 2010; Braida et al., 2012; Desmond et al., 2012; Siebel et al., 2011). This is the study to report the effect of various anti-convulsants when administrated orally against intra-peritoneally injected PTZ induced seizures in adult zebrafish. Furthermore, we have attempted to correlate the data in zebrafish to those in higher mammals including humans for understanding the predictive value of this model in human AED drug discovery.

We had attempted to create a MES model in zebrafish, however, due to the fact that zebrafish is water borne organism and the electric conduction in water creates severe effects on entire body leading to difficulty and inconsistencies in scoring seizures and avoiding conditions like paralysis and death. This could be the reason that there have been no reports of zebrafish MES models published, to the best of our knowledge.

3.2 GAP IN EXISTING RESEARCH

PTZ induced epilepsy model has been well established and validated including molecular and mechanistic validation (Grone & Baraban, 2015), however, there are the following gaps in the existing research:

1. There is only one report; Banote et al., 2013 from our own laboratory, which has reported efficacy doses in terms of mg/kg for one drug i.e. gabapentine.
2. There is very limited of data correlation between zebrafish to humans.

3.3 OBJECTIVES OF THE STUDY

1. Evaluating known drugs to validate the PTZ model.
2. To determine efficacy dose for the drugs in terms of mg/kg.

3. To correlate the data between zebrafish and higher mammals including humans based on published literature.

3.4 MATERIALS AND METHODS

3.4.1 Animal care and maintenance:

Zebrafish were maintained as following general care and maintenance details mentioned in Chapter I. Four to six months old male fish were used for these experiments.

3.4.2 Chemicals, drugs and drug administration:

Pentylenetetrazol (PTZ) for disease induction and AEDs viz. carbamazepine (CBZ), ethosuximide (ETS), felbamate (FBM), lamotrigine (LTG), levetiracetam (LVT), phenytoin (PHT) tiagabine (TGB) and Valproic acid (VPA) were purchased from Sigma Aldrich, USA. PTZ, at concentration of 220mg/kg, was administered intraperitoneal (i.p.) injection (dose volume: 15µL) for disease induction (Banote etl., al., 2013). Anti-covulsants were administered orally (p.o.) route using reported method (Kulkarni et al., 2014). ETS, LEV, PHT, TGB and VPA were dissolved in nanopure water. CBZ, FBM and LTG were prepared as suspension in 0.5 % methylcellulose. Zebrafish were randomly divided into groups (n=8/group) for study.

3.4.3 Validation and evaluation of antiepileptic drugs in zebrafish PTZ seizure model:

Adult zebrafish PTZ seizure model was validated using wide spectrum of conventional antiepileptic drugs (AEDs) which act via various mechanisms. Anti-covulsants like ETS, FBM, LTG, TGB, VPA act effectively in PTZ induced epilepsy pathway where as CBZ, LEV, PHT show their potency in MES induced epilepsy (Mandhane et al., 2007; Löscher, 2011). Drug doses were selected based upon

literature review from various sources and preliminary experiments for safety dose assessment of each candidate. Efficacy was determined by rescue of symptoms and statistically significant reduction of the seizure scores in a treatment group as compared to PTZ group. All behavioral tests done between 11 a.m. to 4 p.m. as explained (Banote et al., 2013) by recording videos using a digital camera attached to a dial-gauge stand looking down at the observation tank. Qualitative seizure scoring was conducted by observers in a blinded fashion. Evaluation of seizure like behavior for each fish was done using 8 minutes recorded video. Score was assigned as follows for each fish according to scale:

Stage1: intermittent immobility and hyperventilation
Stage 2: rotational swimming
Stage 3: side-to-side movements
Stage 4: visible muscular spasms and contractions
Stage 5: quick convulsions of the entire body
Stage 6: spasms and high frequency convulsions including sinking of fish
Stage 7: complete immobility and death

This method of scoring has been validated and its correlation to zebrafish cephalic field potential has been well established (Afrikanova et al., 2013; Banote et al., 2013). As the main objective of this work was to provide methodologies for data correlation other parameters were considered necessary for the purpose of establishment of efficacy. Statistical analysis of this data was performed using GraphPad Prism® Software using Kruskal-Wallis analysis followed by Dunn's multiple comparison tests. The drugs were administered half an hour before PTZ and recording were carried out half an hour after PTZ administration.

3.4.4 Correlation with rats for confirming the mechanism of action:

Zebrafish data was correlated for efficacy with respect to rodent PTZ data especially comparing the performance of molecules based on their mechanism of action. The

rodent data was retrieved from published literature (Löscher & Schmidt, 2011); wherein the authors provide a compilation of drugs based on the mechanism of action and whether they were efficacious in the rat PTZ model or the rat MES model. Furthermore, gene homology for the genes encoding these mechanistic pathways was also correlated to establish the mechanistic validity of this model. The gene sequences encoding the principle drug targets were retrieved from the national center for the biotechnology information data base (NCBI) and the gene homology or the percentage identity between rat, human and zebrafish was calculated using the BLAST option of NCBI.

3.4.4 Data correlation with including humans:

Zebrafish data was correlated for efficacy with respect to rodent PTZ data especially comparing the performance of molecules based on their mechanism of action. Furthermore, zebrafish minimum effective dose (MED) (mg/kg) was correlated with human dose (mg/kg/day) by Linear Regression analysis using GraphPad Prism® Software. The data on human MED was based on clinical label data as compiled and reported by Rosati et al. (2015). Correlation of optimal dose between zebrafish and rodents was not possible due to variability of rodent data in literature.

3.5 RESULTS

3.5.1 Validation and evaluation of antiepileptic drugs in zebrafish PTZ seizure model:

The results of seizure score assessment of various AEDs in zebrafish PTZ model have been presented in Figure 4.1. The mean seizer scores for various treatment groups were as follows:

For untreated control group it was 0 + 0, whereas for the positive control group of PTZ (220 mg/kg i.p.) was 5.2 ± 0.1. For CBZ (PTZ + CBZ) treatment the seizure scores for dose groups of 200 mg/kg, 400 mg/kg and 600 mg/kg were 4.5 ± 0.2, 3.3 ± 0.2 and 1.7 ± 0.2 respectively. In case of ETS (PTZ + ETS); for 150 mg/kg, 300 mg/kg and 500 mg/kg the scores were 3.6 ± 0.2, 2.4 ± 0.4 and 0.1 ± 0.1 respectively. The doses for FBM (PTZ + FBM) were 500 mg/kg, 750 mg/kg and 1000 mg/kg and scores were 3.8 ± 0.4, 2.3 ± 0.3 and 0.1 ± 0.1. LEV (PTZ + LEV) was tested at 100 mg/kg, 250 mg/kg and 500 mg/kg and the mean seizure scores observed were 3.5 ± 0.3, 2.4 ± 0.3 and 1.2 ± 0.1 respectively. LTG (PTZ + LTG) was tested at 50 mg/kg, 100 mg/kg and 200 mg/kg and the mean seizure scores observed were 3.5 ± 0.1, 2.5 ± 0.2 and 0.0 ± 0.0 respectively. For PHT (PTZ + PHT) treatment the seizure scores for dose groups of 250 mg/kg, 500 mg/kg and 750 mg/kg were 3.0 ± 0.2, 2.4 ± 0.2 and 1.3 ± 0.1 respectively. In case of TGB (PTZ + TGB); for 5 mg/kg, 10 mg/kg and 20 mg/kg the scores were 4.0 ± 0.4, 3.3 ± 0.2 and 0.3 ± 0.1 respectively. Finally, for the widely used AED, VPA (PTZ + VPA) the test doses were 150 mg/kg, 300 mg/kg and 600 mg/kg and the observed seizure scores were 2.4 ± 0.3, 1.9 ± 0.1 and 0.0 ± 0.0 respectively.

Ethosuximide (ETS), felbamate (FBM), lamotrigine (LTG), tiagabine (TGB) and valproic acid (VPA) were found to be efficacious based on total rescue of symptoms and statistically significant reduction of the seizure scores as compared to PTZ group. Carbamazepine (CBZ) and phenytoin (PHT) did reduce the seizure scores, however, were considered non efficacious due to non-rescue and statistical non-significance. Levetiracetam (LVT) was considered to be partially efficacious due to relatively lower statistical and symptomatic significance.

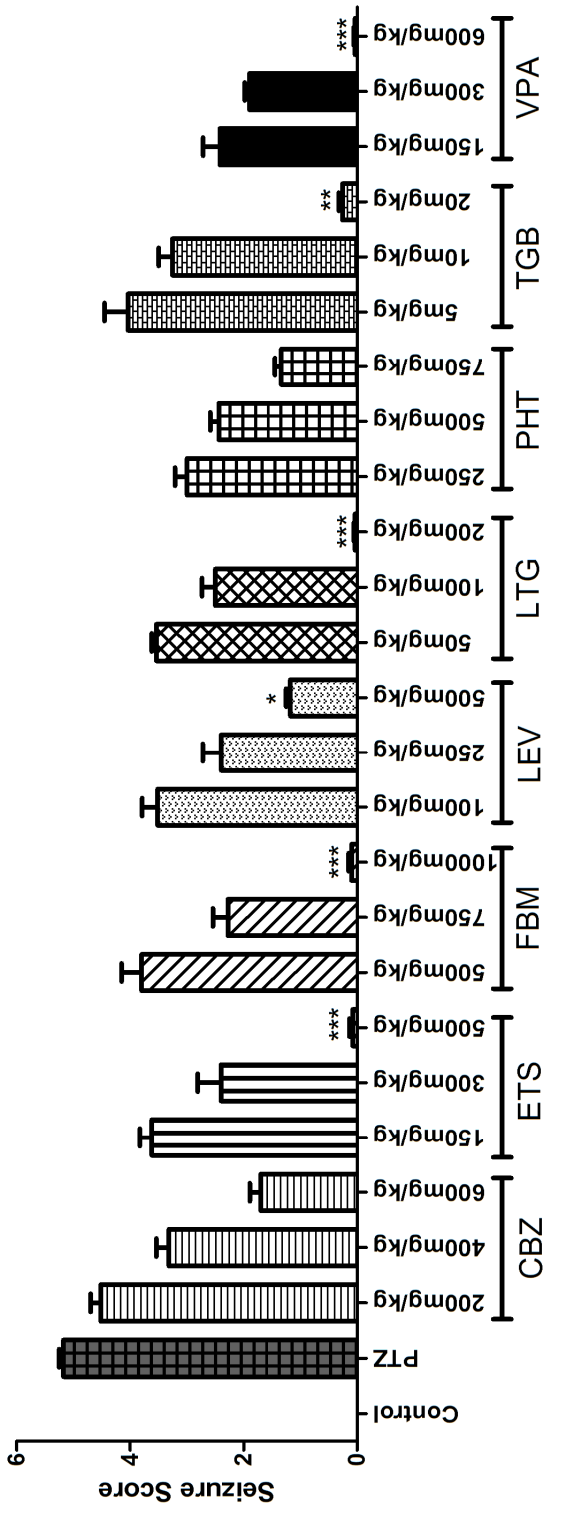

Figure 4.1: Seizure score of orally administered AEDs viz. carbamazepine (CBZ), ethosuximide (ETS), felbamate (FBM), lamotrigine (LTG), levetiracetam (LVT), phenytoin (PHT), tiagabine (TGB) and valproic acid (VPA) on seizure-like activity induced by intraperitoneally injected pentylenetetrazole PTZ (220 mg/kg). Statistical significance was analyzed as PTZ group vs all AED groups. (*p<0.05, **p<0.01, and ***p<0.001). The sample size for this study for each group was n = 8.

3.5.2 Correlation with rats for confirming the mechanism of action:

The correlation of data for between zebrafish and rats has been presented in Table 3.1. It can be interpreted that the compounds that were efficacious in rat PTZ model were also efficacious in zebrafish PTZ model and the same correlation applied to non efficacious compounds except for LEV which was partially efficacious in zebrafish although its shown to be non efficacious in both the rat models. This confirmed the mechanistic correlation of zebrafish to mammals (Table 3.1. (A)). Similarly, data on gene homology suggested a high degree of genetic conservation between zebrafish and rats, humans with respect to genes encoding the major AED targets (Table 3.1. (B)).

Table 3.1: Correlation of mechanism of action between rats and zebrafish is presented in this table. (A) The correlation pathway based efficacy of AEDs in Rat PTZ, Rat MES and Zebrafish PTZ models has been shown.

Compound	Models		
	Rat PTZ*	**Rat MES***	**Zebrafish PTZ**
CBZ	N.E.	E	N.E.
ETS	E	N.E.	E
FBM	E	E	E
LEV	N.E.	N.E.	P.E.
LTG	E	E	E
PHT	N.E.	E	N.E.
TGB	E	N.E.	E
VPA	E	E	E

* Löscher et al., 2011; E = efficacious; P.E. = partially efficacious; N.E. = not efficacious.

Table 3.1: Correlation of mechanism of action between rats and zebrafish is presented in this table.. (B) Correlation of zebrafish with rats based on gene homology of genes encoding these mechanisms.

Compound	**Mechanism of Action**	**Encoding genes**	**Zebrafish v/s rat % ***	**Zebrafish v/s humans % ***
CBZ	Na+ & Ca2+ channel activity	Scn1a,2a,3a,4a,5a,8a,9a, α1G, α1H, α1I	58 – 82 %	59 – 82 %
ETS	Ca2+ channel activity	α1G, α1H, α1I	58 – 78%	59 – 79 %
FBM	Mixed activity (Na+, Ca2+, GABA)	Scn1a,2a,3a,4a,5a,8a,9a, α1G, α1H, α1I, $SLC6A_1$	58 – 85 %	59 – 85 %
LEV	Presynaptic Ca2+ channel inhibition	SV2A	78 %	79 %
LTG	Na+ & Ca2+ channel activity	Scn1a,2a,3a,4a,5a,8a,9a, α1G, α1H, α1I	58 – 82 %	59 – 82 %
PHT	Na+ & Ca2+ channel activity	Scn1a,2a,3a,4a,5a,8a,9a, α1G, α1H, α1I	58 – 82 %	59 – 82 %
TGB	GABA activity	$SLC6A_1$	85 %	85 %
VPA	Mixed activity (Na+, Ca2+, GABA)	Scn1a,2a,3a,4a,5a,8a,9a, α1G, α1H, α1I, $SLC6A_1$	58 – 85 %	59 – 85 %

*Gene homology has been presented as % identity as a range to cover all encoding genes.

3.5.3 Correlation with humans and formulae for extrapolation:

The dose at which the seizures were completely rescued with statistical significance under the experimental conditions was designated as the MED. The zebrafish MEDs for absolutely efficacious drugs i.e. ETS, FBM, LTG, TGB and VPA were 750, 1000, 200, 20 and 600 mg/kg body weight respectively. Similarly, human MEDs was calculated for absolutely efficacious drugs as the arithmetic mean of the human daily dose and compiled based on clinical label data (Rosati et al., 2015). The human MEDs were 25, 27.5, 10, 1.25 and 27.5 mg/kg/day for ETS, FBM, LTG, TGB and VPA respectively. For the purpose of dose correlation, linear regression analysis was conducted. The results of the analysis suggest that there was a correlation between the zebrafish and human MED (Figure 3.2). The coefficient of determination (r^2) of 0.87 for efficacious drugs suggests an 87% data correlation between data on zebrafish and human doses. It was also observed that the potency ranking for all the AEDs were similar between zebrafish and humans.

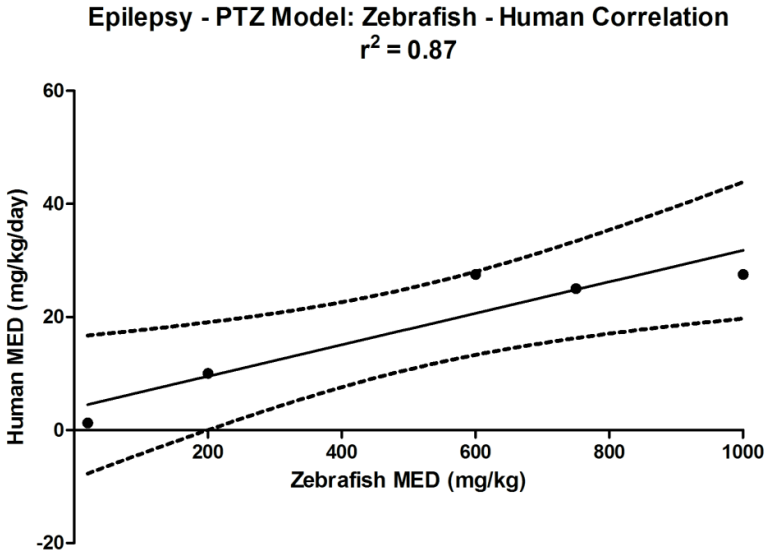

Figure 3.2.: Linear regression analysis of MED between zebrafish and humans.

r^2 = coefficient of determination. Straight (–) lines represent the linear regression, whereas, dotted (….) lines represent the 95% confidence interval with respect to liner regression.

3.6 DISCUSSION AND CONCLUSIONS

The PTZ model in zebrafish along with its phenotypic and molecular validity, has been well established for quite some time (Grone & Baraban, 2015). The present study illustrates the validation and evaluation of wide spectrum of anti-epileptic drugs (AEDs) in adult zebrafish PTZ seizure model via oral drug administration. There have been several reports on adult zebrafish PTZ model, however, effect of oral dosing and correlation of data between zebrafish and higher mammals has not been elucidated. This is the first study, to the best of our knowledge, to report correlation in terms of drug doses with respect to rats and humans respectively. Similarly, we have tried to indirectly correlate the mechanism of action comparing the efficaciousness and non-efficaciousness of drugs acting through different mechanisms between zebrafish and rats. We have also tried to use the gene homology data for correlation of mechanism of action; however, demonstration of the molecular changes post drug administration in zebrafish will be required for a definitive validation of this model.

We have generated zebrafish pharmacological data in terms of milligrams per kilograms for various AEDs in adult zebrafish. The availability of the data on dosage in an in vivo model helps the drug discovery process in terms of ranking of test compounds and taking decisions for drug discovery. We have also demonstrated a good correlation with other higher mammals including humans. The logical next steps for research community working on zebrafish epilepsy models would be further experiments such as molecular analysis and pharmacokinetic analysis of AEDs in zebrafish. These studies can provide information about metabolism and pharmacokinetic – pharmacokinetic modeling to establish zebrafish seizure models for in-vivo drug discovery.

CHAPTER 4:

ZEBRAFISH LIGHT/DARK MODEL OF ANXIETY

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4.1 INTRODUCTION

4.1.1 Background:

Anxiety disorder is a state of apprehension and/or fear resulting from the anticipation of events or situation. Anxiety disorders have been estimated to have approximately 29% prevalence amongst mental health problems and thus can be called as the most the most common mental health problem in the recent times (Kessler et al., 2005). Anxiety disorders include various conditions such as Generalized Anxiety Disorder, Panic Disorder, Social Phobia, Post-Traumatic Stress Disorder, Adult Separation Disorder, Agoraphobia, and Specific Phobia. WHO World Mental Health (WMH) survey suggests that approximately one in every four human beings suffers from some form of mental disorders (Kessler et al., 2009). The data from WMH combined with the data on anxiety suggests that that anxiety disorders amount to approximately 7-8% of the global disease burden. Furthermore, anxiety disorders not only impact daily lives of the individuals affected but also the impact family members, colleagues and other interacting individuals leading to significant socio-economic burden (Whiteford et al., 2013). Cognitive Behaviour Therapy (CBT) along with symptomatic anxiolytic drugs has shown improvement in therapy outcomes, however, there is significant scope to improve treatments and thus an unmet need to find novel therapeutic solutions (Osmanağaoğlu et al., 2017).

In terms of pathophysiology, almost all neurotransmitters pathways have been shown to have been associated with anxiety disorders. The neurotransmitters pathways involved are the GABA-ergic system, opioidergic system, serotonergic system, cholinergic system, histaminergic system, glutamatergic system and adenosine and its receptors (Stewart et al., 2011). In most cases there is no single system that is involved in symptoms shown by any particular patient, therefore, making therapeutic

choices difficult. Although, various drugs targeting these systems are available in market and are prescribed by physicians, however, all the therapies are symptomatic and short term with patients needing to be treated for long term. This emboldens the quest for disease modifying therapies for anxiety disorders (Leichsenring et al., 2017).

4.1.2 Animal Models of Anxiety:

The classical models of anxiety in rodent models are the open field test, elevated plus maze, elevated T-maze, holeboards, dark/light transition, active and passive avoidance tests, separation-induced vocalizations (pups), stress-induced vocalization (adults), defensive (probe or prod) burying, startle response test and many tests that are similar to or versions of these tests. These tests can be categorized as unconditioned response tests and conditioned response tests. Tests wherein no training is required are called unconditioned response tests and considered to have high eco/ethological validity. Tests that require extensive training are conditioned response tests and are questioned for validity they do not represent the spontaneous or uncertainty aspect of human anxiety (Steimer, 2011). All marketed anxiolytics have been tested in one or more of these models and have shown efficacy in these models. Therefore, the rationale for use of zebrafish models for anxiety despite the availability of several rodent models are as follows (Stewart et al., 2012):

(a) Zebrafish anxiety models have ability to test of large number of candidate drugs at very low cost in terms of maintenance, breeding and low compound requirement.

(b) The possibility of correlating the mechanism of action by creating follow-up mechanistic models (through genetic/chemical manipulations or by tracking mechanisms in transparent larval or adult zebrafish) with comparatively quick turnaround time.

4.1.3 Zebrafish Models of Anxiety:

Commonly used paradigms for assessing anxiety related behavior in adult zebrafish include open field test, light-dark box test, social preference test; shoaling, boldness and novel object approaching, predator avoidance. It has been demonstrated though various studies that zebrafish anxiety-like behaviors can be modulated (in both anxiogenic and anxiolytic direction) by drugs affecting almost all the neurotransmitter pathways involved in human anxiety disorders (Stewart et al., 2011).

4.1.4 Zebrafish Light/Dark Model of Anxiety:

The dark/light preference test, also, known as scototaxis, is a behavioral model wherein animal (rodent, fish, etc.) is placed in a central compartment of a half-black, half-white box/tank. The behaviour is observed for a period of time (specifically standardized for the species) and the important parameters assessed are the number and duration of entries in each compartment (white or black) (Bourin & Hascöett, 2003). Zebrafish, due to the uncertainty arising from sudden introduction to a non-familiar environment, show a preference for the dark compartment that reflects anxiety behavior. An increase in activity in the white compartment thus reflects anxiolytic behavior (Maximino et al., 2010). This test is one of the most well validated zebrafish tests and a defined protocol published in reputed journals including Nature Protocols.

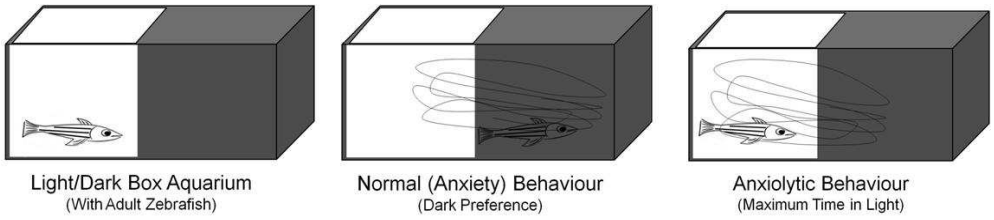

Figure 4.1: Depiction of the principle of the light/dark preference test in zebrafish.

4.2 GAP IN EXISTING RESEARCH

Zebrafish models for anxiety and especially the light/dark preference test have been well established and validated, however, there are the following gaps in the existing research:

1. All the reported pharmacological data is based on drug exposure through aquarium water and there is no report, to the best of our knowledge, which has reported efficacy doses in terms of mg/kg.
2. There is a paucity of data correlation between zebrafish to humans limiting the use of this model in drug discovery decision making.

4.3 OBJECTIVES OF THE STUDY

1. Evaluating known drugs to validate the model as relevant models for anxiety.
2. To determine efficacy dose for the drugs in these models in terms of mg/kg.
3. To correlate the data between zebrafish and higher mammals including humans based on published literature.

4.4 MATERIALS AND METHODS

4.4.1. Animal care and maintenance:

Zebrafish were maintained as following general care and maintenance details mentioned in Chapter I. Four to six months old male fish were used for these experiments.

4.4.2. Chemicals, drugs and drug administration:

Anxiolytics viz. Busporin, Clonidine, Fluoxetine, Hyroxazine, Imipramine and a negative control anxiogenic Capsaicin were purchased from Sigma Aldrich, USA. All drugs were dissolved in water and administered orally (p.o.) using reported method

(Kulkarni et al., 2014). Zebrafish were randomly divided into groups (n=8/group) for study.

4.4.3. Validation and evaluation of anxiolytics in zebrafish light/dark model:

Adult zebrafish light/dark model was validated using known anxiolytics and Capsaisin (10 mg/kg) as a negative control. The negative control and its dose has been standardized in our laboratory and the data on the same has been published by us in a report by Dulla et al. (2014). The study was conducted by introducing the fish to the light/dark aquarium and recording videos for 15 minutes duration. Animals were placed in the centre of white and dark compartment using an intersection compartment which was removed after an acclimatization period of 5 minutes. The fish were then allowed to explore for 15 min. The test was conducted between 11 a.m. to 4 p.m. as explained before (Dulla et al., 2014) by recording videos using a digital camera. Drug doses were selected based upon literature review from various sources and preliminary experiments for safety dose assessment of each candidate. The drugs were administered an hour before the video recordings. Statistical analysis of this data was performed using GraphPad Prism® Software using One-way ANOVA followed by Dunnet's Post-hoc test.

4.4.4 Correlation for the mechanism of action:

Zebrafish data for most of these molecules by dissolving the drugs in water is already reported through different publications and the mechanistic validity of zebrafish model is well established in a recent review (Stewart et al., 2014). We have further established the mechanistic validity by gene homology analysis. The gene sequences encoding the principle drug targets were retrieved from the national center for the biotechnology information data base (NCBI) and the gene homology or

the percentage identity between rat, human and zebrafish was calculated using the BLAST option of NCBI.

4.4.5 Data correlation with including humans:

Zebrafish minimum effective dose (MED) (mg/kg) was correlated with human dose (mg/kg/day) by Linear Regression analysis using GraphPad Prism® Software. The data on human MED was difficult to establish as most of the drugs have different therapeutic regimens for different patients based on their clinical condition of acute anxiety, chronic anxiety, depression, chronic depression and other such disorders. Therefore, we considered the minimum starting dose for acute forms of anxiety behaviors (acute anxiety, panic, etc.) based on the drug data base at Drugs.com accessed in August 2017. The dose in terms of mg/kg/day was calculated considering 60 kg body weight for average human.

4.5 RESULTS

4.5.1 Validation and evaluation of anxiolytic drugs in light/dark model:

The results of percentage time spent in light have been presented in Figure 4.1. The percent time spent in light by the untreated control fish was 19.9 ± 4.1 % whereas in the positive control group administered Capsaicin 10 mg/kg was 9.5 ± 4.2 %. The percentage time spent in light for Busporine at doses of 1 mg/kg, 5 mg/kg and 10 mg/kg was 29.9 ± 3.3, 49.7 ± 2.9 and 44.1 ± 4.2 %; Clonidine at doses of 2.5 µg/kg, 5 µg/kg and 10 µg/kg was 16.3 ± 1.6, 44.9 ± 5.1 and 91.0 ± 5.9 %; Fluoxictine at doses of 15 mg/kg, 30 mg/kg and 45 mg/kg was 56.7 ± 13.2, 80.5 ± 3.9 and 65.0 ± 5.6 %; Hydroxazine at doses of 25 mg/kg, 50 mg/kg and 75 mg/kg was 44.8 ± 7.3, 45.2 ± 4.0 and 69.5 ± 8.7 %; and; Imipramine at doses 1 mg/kg, 5 mg/kg and 10 mg/kg was 48.3 ± 5.3, 29.9 ± 3.3 and 33.1 ± 4.4 % respectively.

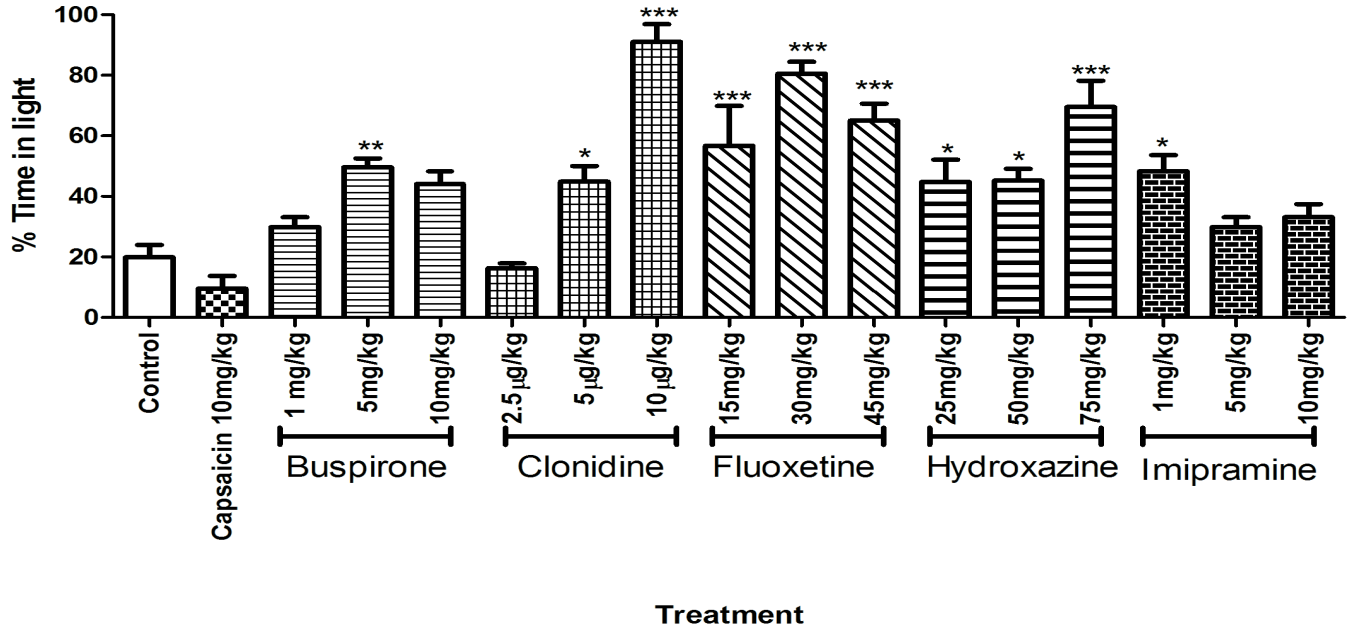

Figure 4.1: Efficacy data of known anxiolytics in light/dark test for anxiety viz. capsaicin (negative control), busporine, clonidine, fluoxetine, hydroxazine and imipramine in terms of percentage time spent by zebrafish in light. Statistical significance was analyzed as control group vs all other groups. (*p<0.05, **p<0.01, and ***p<0.001). The sample size for this study for each group was n = 8.

All known anxiolytic drugs showed efficacy in this model and the negative control Capsaicin showed anxiogenic activity. A negative control for this model was felt necessary as there was no disease induction per say and the fact that control zebrafish in new/uncertain situation naturally prefer the dark compartment; therefore, a negative control data validated the experimental conditions and assessment by blinded observers. Minimum Effective Dose (MED) dose was assigned as the minimum dose at which the fish spent atleast 50% time in the light compartment of the tank and statistical significance. Thus the MEDs for busporine, clonidine, fluoxetine, hydroxazine and imipramine were 5, 0.005, 15, 25 and 1 mg/kg respectively.

4.5.2 Correlation for the mechanism of action:

The gene homology data presented in Table 4.1 suggested a high degree of genetic conservation between zebrafish and rats, humans with respect to genes encoding the major targets.

Table 4.1: Correlation of zebrafish with rats and humans based on gene homology of genes encoding these mechanisms.

Compound	Mechanism of Action	Encoding genes	Zebrafish v/s rat % *	Zebrafish v/s humans % *
Buspiron	5-HT1A agonist	HTR1A	75%	75%
Clonidine	α2 agonist	α2A, α2C	72-91%	56-73%
Fluoxetine	Selective serotonin reuptake inhibitor	SLC6A4	68%	70%
Hydroxazine	H1 inverse agonist	HRH1	44%	42%
Imipramine	Non selective inhibitor of monoamine uptake.	SLC6A4,	68%	70%

*Gene homology has been presented as % identity as a range of encoding genes.

3.5.3 Correlation of doses with humans:

As mentioned above zebrafish MED dose was the statistically significant minimum dose at which the fish spent atleast 50% time in the light compartment. Thus the MEDs for busporine, clonidine, fluoxetine, hydroxazine and imipramine were 5, 0.005, 15, 25 and 1 mg/kg respectively. The human MEDs based on the minimum starting dose for acute forms of anxiety based on the drug data base at Drugs.com were 0.25, 0.003, 0.16, 3.33 and 0.400 mg/kg/day for busporine, clonidine, fluoxetine, hydroxazine and imipramine respectively. The results of the liner regression (Figure 4.2) show that the coefficient of determination (r^2) was 0.68 suggesting an 68% data correlation between data on zebrafish and human doses. It was also observed that the potency rankings were similar between zebrafish and humans.

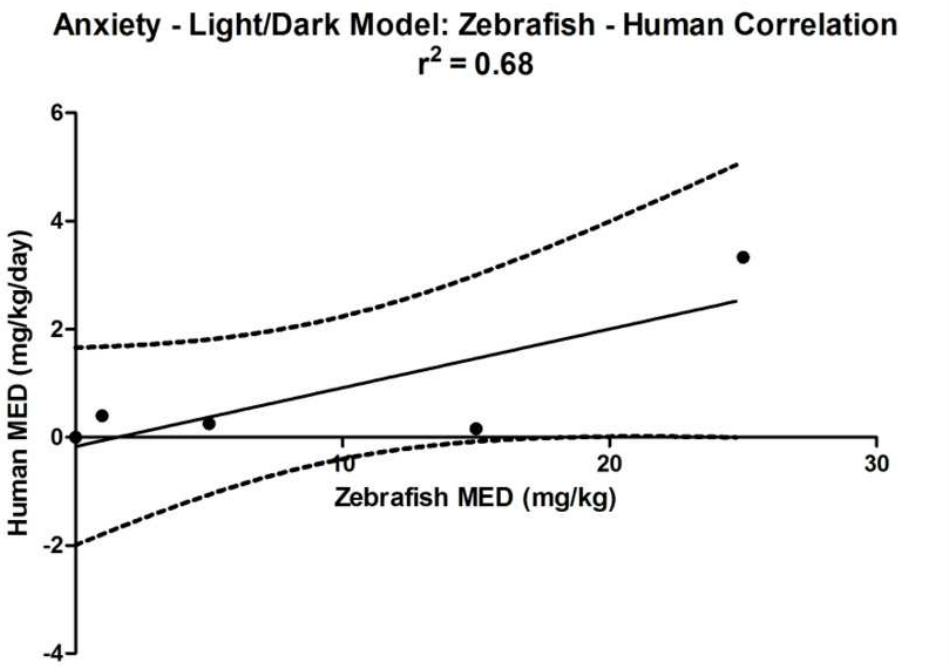

Figure 4.2.: Linear regression analysis of MEDs for anxiolytics between zebrafish and humans. r^2 = coefficient of determination. Straight (–) lines represent the linear regression, whereas, dotted (….) lines represent the 95% confidence interval with respect to liner regression.

4.6 DISCUSSION AND CONCLUSIONS

The present study was conducted to evaluate of a set of known anxiolytic drugs in adult zebrafish light/dark model via oral drug administration. There have been several reports on adult zebrafish anxiety models; however, this is the first study, to report efficacious oral dose and data correlation with humans. The correlation of many of the pathways between zebrafish and other species including humans has been validated before and most of the pathways through which the drugs tested here act have been shown to be conserved and active in zebrafish (Stewart et al., 2012).

The data on dosage in an in-vivo model helps the drug discovery process in terms of ranking of test compounds and taking decisions for lead optimization. We have also demonstrated a good correlation with other higher mammals including humans. The logical next steps for research community working on zebrafish anxiety models would be further experiments towards pharmacokinetic – pharmacokinetic modeling.

CHAPTER 5:

ZEBRAFISH PHARMACOKINETICS & BRAIN PENETRATION

CHAPTER 5: ZEBRAFISH PHARMACOKINETICS & BRAIN PENETRATION

5.1 INTRODUCTION

5.1.1 Background

Pharmacokinetics (PK) is a time course study of drug absorption, distribution, metabolism and excretion (ADME) and how these processes affect the time course and intensity of therapeutic or toxic effects of drugs. PK data in an in-vivo system has always considered more valuable and useful, over in-vitro or in-silico predictions, with most drug discovery scientists considering it as a necessary step before detailed pharmacological characterization (Austel et. al., 1989, Panchagnula et. al., 2000; Jang et. al., 2001). For drugs that are especially targeting the central nervous system need PK evaluation to ascertain their brain penetration as in absence of this property the drugs cannot be moved forward in drug discovery process, however potent they may be towards their molecular target.

An important development in PK research in the last couple of decades has been the development of biopharmaceutical classification system (BCS) to classify drug substances according to their solubility, permeability, dissolution properties and for the prediction of in-vivo PK (Amidon et al., 1995; Karalis et. al., 2010; Reddy et. al., 2011). In another important development, Dried Blood Spot (DBS) technique has been introduced for PK as it helps analysis of test drugs by using small volumes of blood or tissue samples (Beaudette et. al., 2004; Li et. al., 2010). We have used DBS technique in the experiments described here.

5.1.2 Zebrafish pharmacokinetics

Adult zebrafish are being used for various pharmacological and safety evaluation including neuropharmacology, infectious diseases, cancer models, cardiovascular safety, seizure liability, etc. (MacRae & Peterson; 2015; Sridevi et al., 2014; Khan et

al., 2017). However, understanding pharmacokinetics (PK) and tissue distribution is essential to carry out pharmacokinetic – pharmacodynamic (PK-PD) correlation and optimize the utilization of zebrafish (Kulkarni et al., 2014; Kalueff et al., 2015; Kim et al., 2017). An understanding of PK-PD correlation in an in vivo data has multiple benefits over theoretical predictions of drug design as it helps in not just determining the drugability of a candidate molecules, but also provides information about dosage, probable dosing intervals and designing of experiments in conventional animal models with minimizing repetitions, following 3Rs of animal ethics and reduced costs of experimentation (Jang et al., 2001).

There have been plenty of experimental reports on adult zebrafish pharmacology and toxicology; however, there have been very few PK reports with our laboratory being the first to report oral drug administration method and detailed PK procedure using Dried Blood Spots (DBS) (Kulkarni et al., 2014). The present manuscript is in continuation with our effort to study the predictive value of zebrafish for human drug discovery. In the present study we have attempted to correlate PK and brain penetration data of adult zebrafish with higher mammals including humans. For the purpose of this study, two compounds with distinct PK properties were selected; viz Irinotecan, and Lorcaserin. Irinotecan was selected as a compound that was metabolised to SN-38 and the brain penetration of both these compounds was below the level of detection. Lorcaserin was selected as compound that had very high brain penetration and retention.

5.2 GAP IN EXTSING RESEARCH

1. There have been very few reports in PK and brain penetration studies in zebrafish, necessitating a need to generate more data with probe molecules having distinctive PK characteristics.

2. The PK data needs to be correlated with higher mammals to understand if zebrafish can be predictive of the trend of the data so as to take decisions in drug discovery.

5.3 OBJECTIVES

1. Evaluating drugs with distinct PK properties like irinotecan and lorcaserin,in zebrafish for PK and brain penetration.
2. To determine the key PK parameters.
3. To correlate the data between zebrafish and higher mammals including humans based on published literature.

5.4 METHODS

5.4.1. Animal care and maintenance

Zebrafish were maintained as following general care and maintenance details mentioned in Chapter I. Four to six months old male fish were used for these experiments.

5.4.2 Drugs, Chemicals, Instruments and Materials

All drugs were purchased from Sigma Aldrich, USA. Heparin and other routine chemicals were purchased from Sisco Research Laboratories, Hyderabad, India. The drugs were administered using either oral (p.o.) (Kulkarni et al., 2014) or intra-peritoneal (i.p.) drug administration (Chaudhari et al., 2013) routes. These methods ensured the delivery of exact doses of the drugs in terms of milligrams per kilograms (mg/kg) of body weight.

The HPLC system consisted of an Agilent 1200 quaternary pump, auto sampler with thermostat, column oven, and online degasser , triple quadrupole mass spectrometer

(Mass hunter software version B.03.01) with multimode source (Agilent Technologies, Inc. 2850 Centerville Rd. Willington, DE 19808-1644, USA). FTA® Elute blood spot cards (DMPK type-B cards) were supplied by Whatman (Sanford, USA), Ultrasonic bath from Bandelin sonorex sonicator, centrifuge from Eppendorf (model# Centrifuge 5810), and Milli Q Water system from Millipore (model #Gradient A10).

5.4.3 Pharmacokinetic studies

We conducted zebrafish PK and brain penetration studies on two known compounds with distinct PK properties. The details of the methodology for extraction and measurement of Irinotecan and Loracserin has been described in Table 5.1. Irinotecan was administered i.p. at a dose of 100 mg/kg and blood and brain samples were collected at 0, 0.08, 0.17, 0.5, 1, 2, 4 and 8 h. Lorcaserin was administered by two routes i.p. and p.o. at a dose of 10 mg/kg and blood and brain samples were collected at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h.

5.4.4 Preparation and analysis of blood and brain samples

At every time point, three adult male fish were sacrificed using an aesthetic tricaine (MS-222) for blood and brain sample collection. Blood and brain were collected and processed using reported methods (Jagadeeswaran & Sheehan 1999; De´glonet al., 2012; Kulkarni et al., 2014). In brief, blood (7 – 10µl/fish) was collected by cardiac puncture using heparin rinsed insulin syringes and collected in heparin containing tubes. Brain was collected by incising the head portion followed by removal of eyes and skull with forceps. Thereafter, the brain homogenate was prepared in Dulbecco's Phosphate Buffer Saline (DPBS) using Qiagen's Tissue homogenizer

Whatman FTA® DMPK Cards were used for drug analysis using a method described before (De´glonet al., 2012; Kulkarni et al., 2014). In brief, 7µL aliquots of sample from blood and homogenized brain were spotted on the DMPK type-B cards, followed by, punching out of a 3 mm diameter disc from the centre of each DBS, followed by extraction using a protocol described for each drug in Table 5.1.

Table 5.1: Details methodology for PK and brain penetration studies. Dose, route of administration, sampling time points, methodology for extraction and LCMS/MS protocol for measurement of Irinotecan and Loracserin have been described in this table. *The table has been adapted from Kulkarni et al., Journal of Pharmacological and Toxicological Methods 88 (2017) 147–152.*

Drug	Dose; Route	Time Points (h)	Extraction Method	LC-MS/MS Protocol
Irinotecan (SN – 38 by chromatographic assessment)	100 mg/kg; i.p.	0, 0.08, 0.17, 0.5, 1, 2, 4, 8	**Extracting Solvent**: Acetonitile **Centrifugation:** 14000rpm, 10min. **Volume Separation**: 5 µL	**Column:** Zorbax Bonus RP 50x4.6mm, i.d-3.5µm. **Run Time:** 6min **Mobile Phase:** A: 5 mM Ammonium Acetate B: Acetonitile **Volume of Injection in LCMS/MS:** 20µl **Ion Mode:** MRM mixed mode. **Internal Standard:** Indinavir

Lorcaserin	10 mg/kg; i.p., p.o.	0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 24	**Extracting Solvent**: Acetonitile **Centrifugation:** 14000rpm, 10min. **Volume Separation**: 5 µL	**Column:** X Bride ™ C-18, 46x5.0mm, i.d-3.5µm. **Run Time:** 8min **Mobile phase:** A: 0.1% Formic Acid in Water B: Acetonitile **Volume of injection in LCMS/MS:** 5µl **Ion Mode:** ESI positive MRM. **Internal Standard:** Rolipram

5.4.5 Pharmacokinetic parameter calculation and correlation with other species

PK parameters of blood and brain were calculated using PKSolver; an add-in program for in Microsoft Excel using non-compartmental analysis (Zhang et al., 2010). Key PK parameters for each drug were correlated with literature data about rats, primates and humans. Key parameters for each drug were as follows: Irinotecan: percent metabolism to SN-38, plasma half life and brain to blood ratio; and; Lorcaserin: plasma and brain half life, brain to blood ratio and relative bioavailability.

5.5 RESULTS

The results of analysis of blood and brain for levels of the test drugs at various time points have been presented in Figure 5.1 and the PK parameters in Table 5.2:

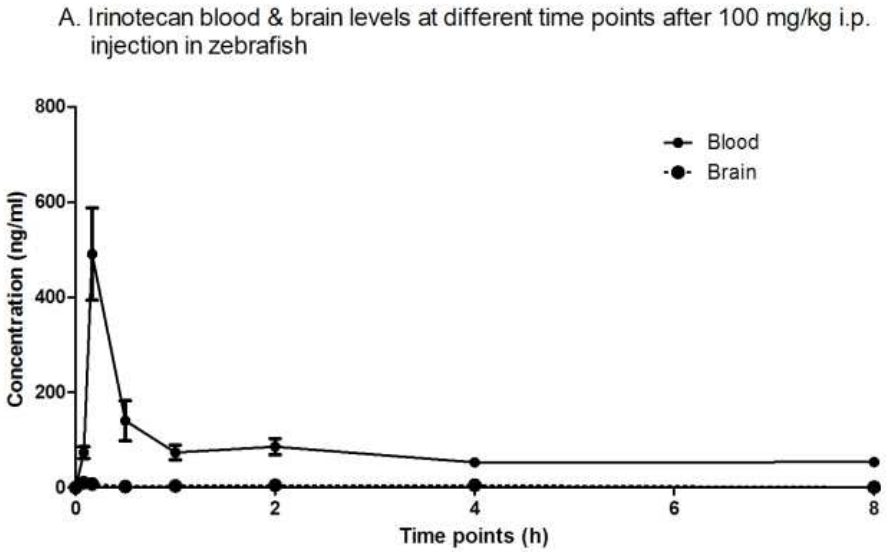

Figure 5.1.: Zebrafish PK of Irinotecan and Lorcaserin. (A) Blood and brain for levels of Irinotecan at various time points have been presented as Mean ± S.E.M. with the line graph on kinetics in blood and brain.

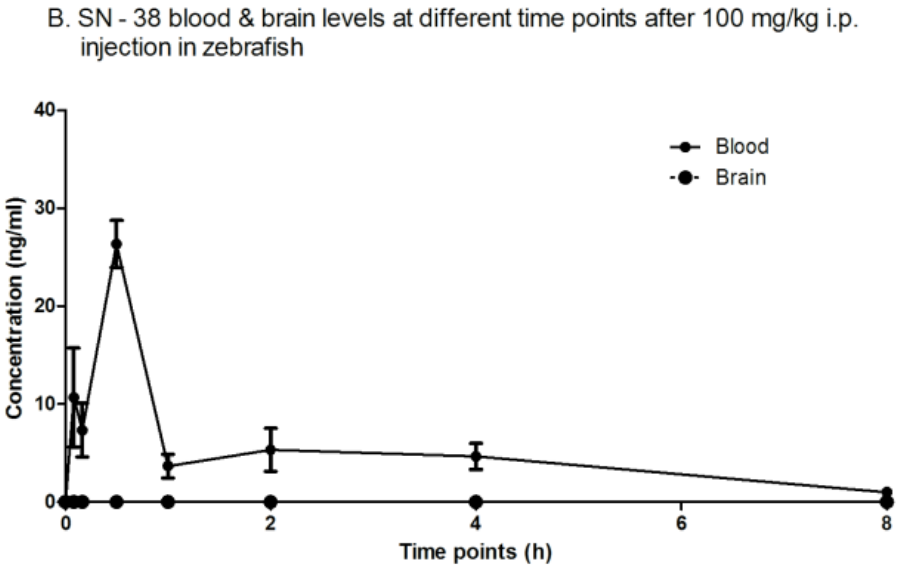

Figure 5.1.: Zebrafish PK of Irinotecan and Lorcaserin. (B) Blood and brain for levels of SN-38 at various time points have been presented as Mean ± S.E.M. with the line graph on kinetics in blood and brain.

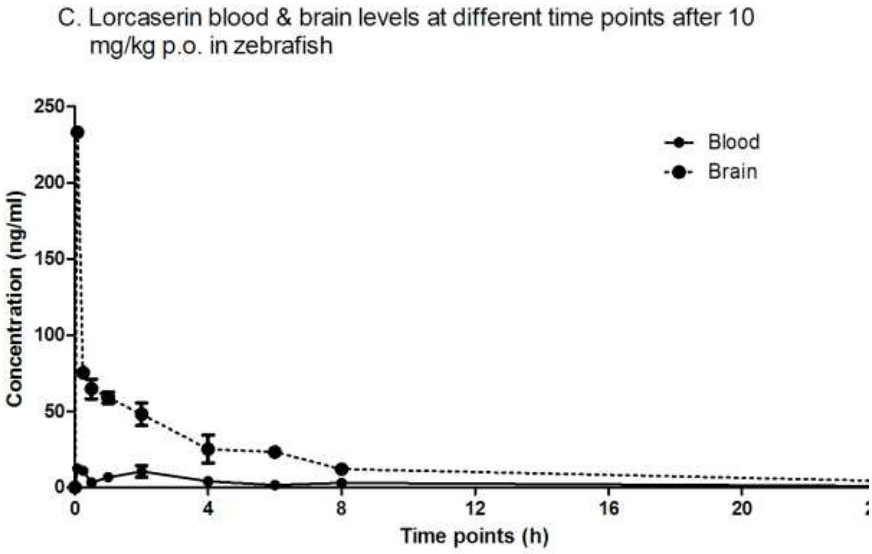

Figure 5.1.: Zebrafish PK of Irinotecan and Lorcaserin. (C) Blood and brain for levels of Loracserin (p.o.) at various time points have been presented as Mean ± S.E.M. with the line graph on kinetics in blood and brain.

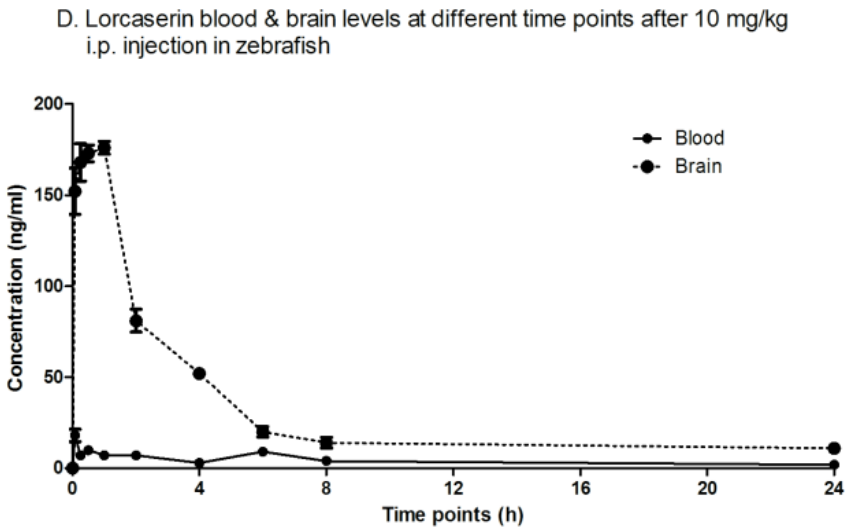

Figure 5.1.: Zebrafish PK of Irinotecan and Lorcaserin. (C) Blood and brain for levels of Loracserin (i.p.) at various time points have been presented as Mean ± S.E.M. with the line graph on kinetics in blood and brain.

The figures and legends have been adapted from Kulkarni et al., Journal of Pharmacological and Toxicological Methods 88 (2017) 147–152.

5.5.1 Irinotecan

Irinotecan was administered to zebrafish i.p. and blood and brain concentrations were measured at different time points. The peak blood concentration (C_{max}) of 10.67 ng/ml was observed at 10 minutes (T_{max}) time point with a half life (t½) of 6.9 h and exposure (AUC $_{(0-t)}$) of 618.45 ng/ml*h. Similarly, peak brain concentration (C_{max}) of 491 ng/ml was observed at 5 minutes time point (T_{max}) with a half life (t½) of 1.89 h and exposure (AUC $_{(0-t)}$) of 29.61 ng/ml*h.

Table 5. 2: Pharmacokinetic parameters derived from zebrafish blood and brain. *The table has been adapted from Kulkarni et al., Journal of Pharmacological and Toxicological Methods 88 (2017) 147–152.*

(A) Irinotecan and its metabolite (SN – 38)

Parameter	Unit	Irinotecan		SN - 38	
		Blood	Brain	Blood	Brain
Lambda_z	1/h	0.10	0.37	0.29	-
t1/2	h	6.94	1.89	2.36	-
Tmax	h	0.17	0.08	0.50	-
Cmax	ng/ml	491.00	10.67	26.33	-
Tlag	h	0.00	0.00	0.00	-
Clast_obs/Cmax		0.11	0.06	0.04	-
AUC 0-t	ng/ml*h	618.45	29.61	40.14	-
AUC 0-inf_obs	ng/ml*h	1155.94	31.43	43.54	-
AUC 0-t/0-inf_obs		0.54	0.94	0.92	-
AUMC 0-inf_obs	ng/ml*h^2	11539.33	105.81	135.32	-
MRT 0-inf_obs	h	9.98	3.37	3.11	-
Vz/F_obs	(mg/kg)/(ng/ml)	0.87	8.68	7.81	-
Cl/F_obs	(mg/kg)/(ng/ml)/h	0.09	3.18	2.30	-

Irinotecan has a major active metabolite SN – 38 and thus is an important analyte to study (Ramesh et al., 2010). The important blood kinetic parameters of SN – 38 were: C_{max} = 10.67 ng/ml; T_{max} = 0.5 h; t½ = 2.36 h; AUC $_{(0-t)}$= 40.14 ng/ml*h. The levels in brain were below the quantification threshold that could not be analysed using our method.

Zebrafish data was correlated with rats, primates and humans (based on literature data in case of the later three species) for the key parameters of plasma half life and % blood : brain ratio. The data in primates and humans was after intravenous infusion of 30 minutes and 90 minutes respectively (USFDA, 2015). The data has been presented in Table 5.3 and correlation has been discussed in the discussion section.

5.5.2 Lorcaserin

Lorcaserin was administered to zebrafish by two routes, p.o. and i.p.; blood and brain concentration were measured at different time points. The i.p. administration and PK evaluation was conducted with a purpose to assess bioavailability after oral administration with respect to parenteral administration.

The important blood kinetic parameters of lorcaserin after oral administration were: C_{max} = 12.24 & 233.16 ng/ml for blood and brain respectively; T_{max} = 0.08 h for both blood and brain; t½ = 7.73 & 7.95 hfor blood and brain respectively; AUC $_{(0-t)}$ = 71.29 & 424.03 ng/ml*h for blood and brain. All the PK parameters in blood and brain after intraperitoneal administration of lorcaserin are presented in Table 5.2, however, the key parameter to report here is an AUC $_{(0-t)}$of 99.22 ng/ml*h.

Data correlation in case of lorcaserin was conducted for the key parameters of plasma and brain half life, % blood: brain ratio and % bioavailability. The data has been presented in Table 5.3 and correlation has been discussed in the discussion section.

Table 5. 2: Pharmacokinetic parameters derived from zebrafish blood and brain. *The table has been adapted from Kulkarni et al., Journal of Pharmacological and Toxicological Methods 88 (2017) 147–152.*

(B) Locarserin by both routes oral and intraperitoneal

Parameter	Unit	Locarserin - Oral		Loracserin - Intraperitoneal	
		Blood	Brain	Blood	Brain
Lambda_z	1/h	0.09	0.09	0.07	0.03
t1/2	h	7.73	7.95	10.17	26.40
Tmax	h	0.08	0.08	0.08	1.00
Cmax	ng/ml	12.24	233.16	18.00	176.00
Tlag	h	0.00	0.00	0.00	0.00
Clast_obs/Cmax		0.07	0.02	0.11	0.06
AUC 0-t	ng/ml*h	71.29	424.03	99.22	730.66
AUC 0-inf_obs	ng/ml*h	81.22	472.43	128.58	1149.62
AUC 0-t/0-inf_obs		0.88	0.90	0.77	0.64
AUMC 0-inf_obs	ng/ml*h^2	825.35	4077.66	1968.18	30206.40
MRT 0-inf_obs	h	10.16	8.63	15.31	26.28
Vz/F_obs	(mg/kg)/(ng/ml)	1.37	0.24	1.14	0.33
Cl/F_obs	(mg/kg)/(ng/ml)/h	0.12	0.02	0.08	0.01

5.6 DISCUSSION AND CONCLUSIONS

Adult zebrafish pharmacokinetics has been largely a neglected aspect of zebrafish research despite a large number of pharmacological models being developed and reported using this model. There is a need to generate more data and publish it in for the purpose of understanding the validity and predictive ability of zebrafish data by scientific community. The first pharmacokinetic report, to the best of our knowledge was by Zang et al. (2011), wherein, the efficiency of oral delivery of felbinac through gluten granules was confirmed using a HPLC -based method. Thereafter, we published the first report describing oral drug administration, use of DBS cards for extraction of analyte and measurement using LCMS/MS method (Kulkarni et al., 2014). The most recent report on this subject was by Kim et al. (2017). This study was conducted to investigate the possibility of using zebrafish as a screening tool to estimate partition coefficient ($K_{p,brain}$) to predict drug brain penetration in humans. All these studies are suggestive of the fact that zebrafish can act as model organism for studying PK and brain penetration aspects which are essential for taking decisions during the process of drug discovery. In the present study, we choose compounds that show distinct PK properties, especially properties that led to their pharmaceutical development and therapeutic regimen.

In case of irinotecan (Table 5.3. (A)); zebrafish data was correlated for the key parameters of plasma half life, and % blood:brain ratio. The parameters of plasma half life and % blood : brain ratio were correlated as these determine the dosing frequency and tissue distribution to an organ which is separated by blood brain barrier. The plasma half life of irinotecan in zebrafish suggested a profile of a once a day drug. This data, if it had been generated in zebrafish during the process of drug discovery, correlated with primates and human but not with rats. Despite the fact that the data in primates and humans is based on intravenous infusion of 30 and 90

minutes, the data in zebrafish is predictive of a once a day administration regimen. Similarly, the data on brain penetration suggests zebrafish to be better predictor for primates and humans when compared to rats. Though there is high inter-species variability, the irinotecan data in rats is suggestive of negligible brain penetration whereas zebrafish data suggests the brain penetration being low but not negligible, a trend observed in primates too. This result has been vindicated by the clinical reports (that do not clearly provide brain concentrations) of use of irinotecan in glioma patients wherein, efficacy has been attributed to irinotecan permeability across the blood brain barrier and conversion to SN – 38 in brain (Vredenburgh et al., 2009).

Conversion of irinotecan to SN – 38 was higher in zebrafish as compared to other species (zebrafish: 7%; rats: 0.8% (USFDA, 2015); primates: 2.4% (Blaney et al., 1998); and; humans: 2.3% (USFDA, 2014)); however, the data still indicates a low metabolism of irinotecan, which is consistent with other spices. Also, it re-emphasises conservation of metabolic pathways and blood brain barrier permeability in zebrafish with respect to higher mammals (Alderton et al.; 2010; Jeong et al., 2008; Kim et al., 2017).

Table 5.3: Correlation of key (distinct) pharmacokinetic parameters. (A) Irinotecan and (B) Lorcaserin in zebrafish, rats, primates and humans.

(A) Irinotecan

Species	**Dose/ Route**	**Plasma half life (t½)**	**% Brain : Blood Ratio (based on AUC_{0-t})**
Zebrafish	100 mg/kg i.p.	6.9	4.78
Rat	130 mg/kg i.v. [a]	2.1 [a]	> 1% [a]
Primate	225 mg/m^2 i.v. inf. [b]	4.9 [b]	13 [b]
Human	125 mg/m^2 i.v. inf. [c]	5.8 [c]	n.a.

[a] USFDA, 2015; [b] Blaney et al., 1998; [c] USFDA 2014. n.a. – not accessible

(B) Lorcaserin

Species	Dose/ Route	Plasma half life (t½)	Brain half life (t½)	% Brain : Blood Ratio (based on AUC_{0-t})	% Bioavailability p.o.: i.p./i.v. (based on AUC_{0-t})
Zebrafish	10 mg/kg p.o.	7.7	8.0	595	72
Rat	10 mg/kg p.o. [d,e]	4.9 [d]	4.7 [d]	1374 [d]	93 [e]
Primate	10 mg/kg [d,e] p.o.	n.a.	n.a.	1010 [e]	51 [e]
Human	10 mg/kg p.o. [e]	11 [e]	n.a.	170 [e]	n.a.

[d] Thomsen et al., 2008; [e] USFDA, 2011. n.a. – not accessible

The table has been adapted from Kulkarni et al., Journal of Pharmacological and Toxicological Methods 88 (2017) 147–152.

Lorcaserin, a selective 5-hydroxytryptamine (5-HT_{2C}) receptor agonist, an appetite lowering drug; has distinguishing PK properties of high brain penetration, long half life in both blood an brain and good bioavailability (Thomsen et al., 2008). As the drug acts on the central nervous system, these parameters are essential drugable properties of this drug. The data in zebrafish shows that these parameters correlate with all mammalian species including humans. Even though the actual ratios of these parameters have high inter species variability, the trend demonstrates that, if zebrafish data was available during the process of drug discovery, it would have helped in decision making regarding drugability of the candidate.

In conclusion, zebrafish PK has been an understated and understudied aspect in zebrafish research. It needs to be explored in details across various laboratories for optimal utilization of zebrafish model organism for pharmaceutical and disease biology research. The experiments reported in this manuscript demonstrate the correlation of zebrafish PK data to higher mammalian species including humans. Despite the variations in the actual values, this study suggests the utility of zebrafish in understanding the trend of pharmacokinetic data of candidate drugs in early drug discovery process.

CHAPTER 6:

SUMMARY, DATA EXTRAPOLATION AND CONCLUSIONS

CHAPTER 6: SUMMARY, DATA EXTRAPOLATION AND CONCLUSIONS

6.1 SUMMARY

6.1.1 Introduction

Zebrafish models are becoming increasing popular in academic and pharmaceutical research. There are a few thousand zebrafish publications published every year and zebrafish research publications in last many years the statistics for annual increase in zebrafish publications have topped the number of publications published using other experimental animal models. Furthermore, recent decisions by regulatory agencies have emboldened the belief of their use for drug discovery research. The sequencing of zebrafish genome has further improved the scope for utilization of zebrafish in disease biology research, drug target identification & validation, creation of genetically modified models and many other research avenues.

Neuroscience research in zebrafish has been a fertile area due to the fact that zebrafish have complex brain capable of performing context specific functions with conserved neurotransmitters and other molecular mechanisms when compared to higher mammalian species. Therefore, many models have been developed over last few years that are analogues to the conventional mammalian models and have measurable and definable phenotypes.

The major gap in zebrafish research is the ability to use zebrafish data for decision making in human drug discovery research. Even though the decision to use a preclinical model for screening in “target based drug discovery” is on considerations of molecular and genetic validity of the model, however, the decisions to filter molecules for development is based largely on considerations of potency and therapeutic index (taking in account toxicity). In case of “phenotypic drug discovery”, the molecular mechanisms are given consideration much later in the development

process. The important parameters of potency and therapeutic index are solely related to the drug dose. Therefore, accurate identification of the drug dose in an in-vivo model is the fundamental parameter for the fate of drug candidates in the process. The paucity of data on this fundamental parameter and its use in making decisions, we believe, has been a major gap that has deterred the more visible use of zebrafish by pharmaceutical industry.

The relevance of the dose (efficacy as well as safety) has to complement with the pharmacokinetic parameters that qualify the accuracy of the dose. A drug candidate that is highly potent in in-vitro assays might not be suitable for development over a drug that has comparative low potency in that assay due to the fact that its pharmacokinetic parameters are unknown. Moreover, in neuropharmacology the key parameter of brain permeability might deter such a highly potent (in-vitro) drug candidate from actually being useful in in-vivo situation. The adult zebrafish model could help in overcoming this challenge wherein the efficacy dose will have factored in the bioavailability and brain penetration potential of the candidate drug, thus making the decision based on potency easier. However, strong experimental evidence is required to conclude that metabolism and drug distribution can get factored in a zebrafish efficacy models.

The above arguments have been vindicated by a very recent report by Brock et al., 2017; published during the process of preparation of this thesis manuscript. The study involved scientists from major pharmaceutical companies like Pfizer and Astrazeneca and they studied the value of the zebrafish conditioned place preference model for predicting human abuse potential. The study was conducted in adult zebrafish and the drug administration was performed by solubilizing the test drugs in aquarium water with the data obtained as mg/L. the authors concluded that zebrafish

model for abuse liability had high concordance with respect to specificity and negative prediction values but had low sensitivity. The parameter for sensitivity is directly related to dose. The authors discuss the possibility of lower and uneven absorption as possible reasons for the low positive predictions and sensitivity. These results strengthen our arguments of using oral or injection routes of administration, generating data in terms of mg/kg and correlating it humans.

The summary of each of the models studied is as follows:

6.1.2 Zebrafish Multiple Sclerosis Model

Pre-clinical drug discovery for multiple sclerosis (MS) in rodent models is a cumbersome and highly laborious activity to perform. The time required for disease induction is long and thereafter, treatment effects need to be observed for many days, thus required about 4-8 weeks to study a drug candidate. We have developed a novel experimental autoimmune encephalomyelitis (EAE) model in adult zebrafish based which is a simple protocol and offers to be a quick screen between in-vitro and conventional in-vivo experiments. The disease induction to create the EAE model was using myelin oligodendrocyte glycoprotein, 35-55 (MOG). The observation conducted were survival, clinical signs and body weight changes. We first validated the model using the most widely used known drug fingolimod followed by a detailed validation using dimethyl fumarate, dexamethasone and SR1001. We demonstrated the validation of disease symptoms with histopathological evaluation in the study conducted for fingolimod, and the detailed validation with other known drugs showed that our results were in agreement with those in rodent models. The data on genetic correlation based on gene homology analysis as well as literature reports related to mechanism of actions of the tested drugs, suggested a good mechanistic correlation between zebrafish and other higher mammals. Furthermore, the correlation of the

minimum effective dose between zebrafish and humans suggested at 98% coefficient of determination. In summary, we have developed a new model, standardized and validated it and correlated the data with rodents and humans.

6.1.3 Pentylenetetratzole Induced Zebrafish Epilepsy Model

Adult zebrafish model of epilepsy using pentylenetetrazole (PTZ) is gaining acceptance in scientific community and by regulators. This model in both larvae and adults has been very well characterized and validated from both phenotypic as well as molecular perspectives. We have evaluated known antiepileptic drugs (AEDs) in adult zebrafish PTZ model after oral dosing to arrive at a minimum efficacy dose in terms of milligram per kilogram (mg/kg) of body weight. Thereafter, we have tried to correlate the data on dose with humans (based on published literature) using linear regression. To validate that mechanism of action of AEDs in zebrafish is similar to that in mammals we correlated the efficacy data of underlying pathways between zebrafish to rats. The data on dose correlation between zebrafish and humans suggested at 87% coefficient of determination. The correlation data proposed here can be useful in ranking of test compounds and selection of doses for further in-vivo characterization. In case of rare disease “aquarium to bedside” approach it could also be helpful in arriving at starting dose for human studies for new drugs as well as repurposed candidates.

6.1.4 Zebrafish Light/Dark Model of Anxiety

The study was conducted to evaluate of a set of known anxiolytic drugs in adult zebrafish light/dark model via oral drug administration. The dark/light test, or scototaxis, is one in which a behavioral of the animal (in this case zebrafish) is observed in half-black, half-white box/tank. A normal zebrafish will tend to spend more time in dark light after introduction to such a tank owing to fear of new,

unknown and uncertain environment. This display of anxiety can be rescued using anxiolytics wherein treated fish show light exploration over dark preference. All known anxiolytics were found to be efficacious in this model, whereas, the negative control Capsaicin showed anxiogenic activity. There have been several reports on adult zebrafish anxiety models; and the molecular mechanisms have also been well established. However, we believe this to be the first study, to report efficacious oral dose and data correlation (68% coefficient of determination) with humans. The data on dosage in an in-vivo model helps the drug discovery process in terms of ranking of test compounds and taking decisions for drug discovery.

6.1.5 Zebrafish Pharmacokinetics & Brain Penetration

Adult zebrafish neuropharmacology is evolving rapidly; however, there is very limited research in understanding pharmacokinetics (PK) and brain penetration in adult zebrafish. We conducted adult zebrafish PK and brain penetration studies on two known compounds (irinotecan and lorcaserin) with distinct PK and brain penetration properties using validated LCMS/MS method. Irinotecan was studied at a dose of 100 mg/kg i.p. and levels of the parent drug and active metabolite SN - 38 were measured. Loracserin was studies at a dose of 10 mg/kg by two routes i.p. and p.o. Zebrafish PK and brain penetration profiles for both compounds were very similar to that of higher mammals including humans. Irinotecan was metabolised to SN-38 and the compound had long half life with very low brain penetration in our studies. Loracasin was highly permeable in brain as compared to the exposure in blood, with long half life and high relative bioavailability, similar to other mammalian species including humans. The zebrafish data for key parameters of irinotecan and loracserin shows a high correlation to the data from higher species, including human. This report validates the use of adult zebrafish as a predictive PK tool for higher animal studies.

6.2 EXTRAPOLATION

6.2.1 Zebrafish to Human Dose Extrapolation

Data extrapolation is a routine exercise during drug discovery and development process. The data on dose is extrapolated from a lower species to higher for the purpose of selecting doses for the second stage pharmacological and toxicological characterization including in clinical trials. The relevance of data extrapolation from zebrafish to humans has been discussed in the subsequent sections, however, we have made an attempt to extrapolate data based on the experiments reported in this manuscript wherein minimum effective doses (MEDs) 14 drugs that were efficacious in various zebrafish models were correlated with the human MEDs (established using various publications reported earlier).

The graph in Figure 6.1 shows represents the liner regression analysis of data of MEDs between zebrafish and humans. Coefficient of determination (r^2) and slope of the line have been calculated and presented in the title of the graph. The coefficient of determination (r^2) of 0.94 for suggests a high (94 %) data correlation between data on zebrafish and human doses across different models and different classes of compounds. The slope of the regression line (with 95% confidence interval) was used to propose a formula of extrapolation between zebrafish and humans. We here propose the formula as follows:

Human dose (mg/kg/day) = (0.026 ± 0.04) x Zebrafish dose (mg/kg)

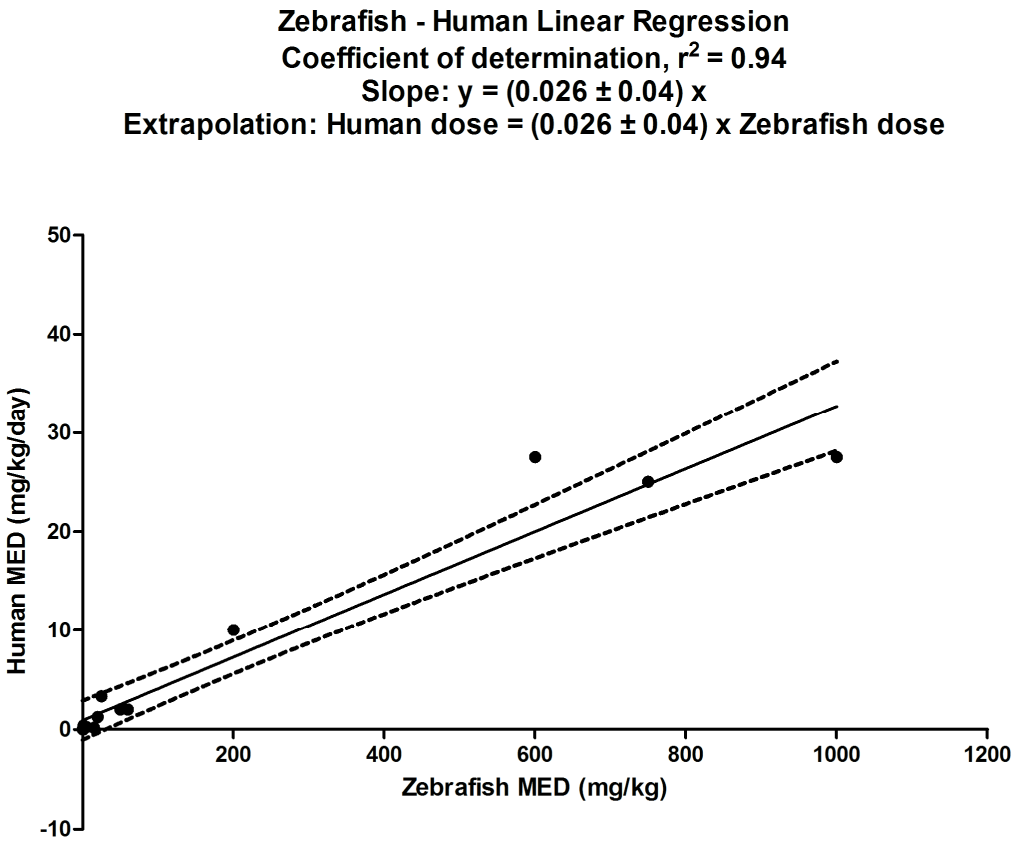

Figure 4.2.: Linear regression analysis of MEDs between zebrafish and humans.

Coefficient of determination (r^2) and Slope of the line have been calculated and presented in the title of the graph. Straight (–) lines represent the linear regression, whereas, dotted (….) lines represent the 95% confidence interval with respect to liner regression.

6.2.2 Dose Conversion using the Formula for Extrapolation

This formula for extrapolation suggested by us is very similar to the body surface area (BSA) conversion suggested by USFDA, 2005. A comparison of the formulae is presented below

Formula derived from this thesis work:

Human dose (mg/kg/day) = (0.026 ± 0.04) x Zebrafish dose (mg/kg)

Formula derived based on body surface area calculations (USFDA, 2005):

Human dose (mg/kg/day) = 0.022 x Zebrafish dose (mg/kg)

(calculated as HED = animal dose in mg/kg x (animal weight in kg/human weight in kg) $^{0.33}$, for 0.006 kg (600 mg) zebrafish and 60 kg human).

Combining the two suggest that zebrafish dose is approximately 1/40th the human dose or human dose can be obtained by diving the zebrafish dose by an extrapolation factor of 40.

6.2.3 Relevance of Dose Extrapolation

The process of data extrapolation from animals to humans is mainly to give direction of the starting dose for "first-in-humans" clinical trials. The USFDA has provided Guidance for Industry for Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers (USFDA, 2005). The main objectives of the guidance, in brief, are to provide common conversion factors from animals to humans for deriving a human equivalent dose (HED). USFDA in this guidance suggests the use of an algorithm or formulae based approach as scientific simulation for each drug might vary based on multiple factors influencing efficacy and pharmacokinetic parameters across species. Therefore, a formula based approach can help reviews, regulators and developers with a framework wherein a clinical trial can be initiated keeping in mind minimum toxicity as well as attainment of the human assessment objectives.

Although the formula for extrapolation suggested by USFDA is based on the No Observed Adverse Effect Level (NOAEL) dose, we have tried to use this approach for pharmacological data based on the following rationale:

The first rationale is based on a recent development in regulatory acceptance of zebrafish. Zebrafish model of Dravet's syndrome, a rare epileptic disease, has recently being recognized by the regulators to grant approval for efficacy clinical trials for repurposing of an already marketed drug, Lorcaserin (NIH, 2017). Considering the limitations of recruiting patients for rare diseases for Phase II clinical trials, the availability of dose extrapolation can be useful in determining a starting dose for such clinical trials as the safety dose for such repurposed doses is known and risk will already have been factored.

Secondly, the zebrafish models are being proposed as bridging steps between in-vitro and in-vivo experiments. A formula for extrapolation and conversion of zebrafish dose to other animals will save time and costs involved in conducting range finding studies. A recent publication calculates the cost/day of using zebrafish to be ~ $0.01 in comparison to ~$0.20 for rats and $27.30 and $19.75 for dogs and monkeys respectively (Grone & Baraban, 2015). This indirectly means that 20 in-vitro hits can be screened in zebrafish in the cost required to screen one drug in rats. With the ability to obtain and extrapolate data in terms of mg/kg, further time and costs can be saved as well as the 3R principle of animal use can be practiced. The fact that the zebrafish data would have factored in bioavailability and brain penetration, the dosing regimen can also be determined using this approach.

Finally, we are for the proposing a framework for using zebrafish data optimally. A larger validation with large set of compounds across different laboratories will help in arriving at a more methodology for correlating and extrapolating zebrafish data to various species including humans. A question can be asked on the ideal sample size for conducting such extrapolations and proposing such formulae. The USFDA evolved the guidance and the algorithms over a period; however, the guidance

document mentions that the first attempt for such correlation was conducted by Freireich et al., 1966; wherein they compared the toxicity of 18 anticancer agents and found that doses could be correlated based on body surface area of the species. Our extrapolation based on 14 neuroactive drugs can thus be a valid beginning of such work related to zebrafish.

6.3 CONCLUSIONS

This thesis work aims to address gaps in existing research that hamper the utilization of zebrafish optimally in neuropharmacology and drug discovery research. We have attempted to address two major aspects though this work, viz., robust protocols and methodology to obtain drug dosage data in terms of mg/kg; correlation and extrapolation of data that can be used for drug discovery decisions making. We believe this to be the first such concentrated effort and will lead to more such efforts by research community to optimize the use of zebrafish research in general and neuropharmacology research in particular.

FUTURE DIRECTIONS

Future directions for the research conducted in this thesis work are suggested as follows:

- The novel Experimental Autoimmune Encephalomyelitis (EAE) of Multiple Sclerosis (MS) can be improved for disease induction and validation with zebrafish native proteins other than Myelin Oligodendrocyte Glycoprotein (MOG35-55); and; histopathological and molecular characterization of the same.

- Similarly, for the pentalenetetrazole (PTZ) induced epilepsy model and light/dark model of anxiety, more molecular characterization can help in optimizing these models and correlating them to higher species including human beings.

- In terms of pharmacokinetics (PK) and blood-brain-barrier (BBB) penetration, conducting PK evaluation of various drugs screened in the different models and correlating the pharmacokinetic-pharmacodynamic (PK-PD) paradigm to higher mammals including humans is required to provide assurance to scientists about the predictive ability of these models in drug discovery research.

- There is also scope for utilizing these models for identifying newer targets and validating them using genomic and proteomic approaches to make them useful for academic as well as industrial research.

LIST OF PUBLICATIONS AND PRESENTATIONS

Publications from Thesis Work

1. Kulkarni P, Yellanki S, Medishetti R, Sriram D, Saxena U, Yogeeswari P. Novel Zebrafish EAE model: A quick in vivo screen for multiple sclerosis. Mult Scler Relat Disord. 2017; 11: 32-39.
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BRIEF BIOGRAPHY OF THE SUPERVISOR

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ANNEXURE: PUBLICATIONS FROM THESIS WORK

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Novel Zebrafish EAE model: A quick *in vivo* screen for multiple sclerosis

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ABSTRACT

Introduction: Pre-clinical drug discovery for multiple sclerosis (MS) is a labor intensive activity to perform in rodent models. This is owing to the long duration of disease induction and observation of treatment effects in an experimental autoimmune encephalomyelitis (EAE) model. We propose a novel adult zebrafish based model which offers a quick and simple protocol that can used to screen candidates as a step between *in vitro* experiments and rodent studies. The experiments conducted for this manuscript were to standardize a suitable model of EAE in adult zebrafish and validate it using known modulators.

Methods: The EAE model was developed by disease induction with myelin oligodendrocyte glycoprotein – 35–55 (MOG) and observation of survival, clinical signs and body weight changes. We have validated this model using fingolimod. We have further performed detailed validation using dimethyl fumarate, dexamethasone and SR1001, which are known modulators of rodent EAE.

Results: The immunization dose for the disease induction was observed to be 0.6 mg/ml of MOG in CFA (Complete Freund's adjuvant), injected subcutaneously (s.c.) near spinal vertebrae. In the validation study with fingolimod, we have demonstrated the modulation of disease symptoms, which were also confirmed by histopathological evaluation. Furthermore, detailed validation with three other known drugs showed that our observations concur with those reported in conventional rodent models.

Discussion: We have standardized and validated the adult zebrafish EAE model. This model can help get a quick idea of *in vivo* activity of drugs in a week using very low quantities of candidate compounds. Further work will be required to define this model particularly as it is found that zebrafish may not express a MOG homologue.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is the condition in which interaction between neurological and immune pathological pathways result in features such as axon loss, inflammation and demyelination similar to that of multiple sclerosis (MS) (Prineas et al., 1984; Raine and Wu, 1993; Constantinescu et al., 2011). Experimental autoimmune encephalomyelitis can be induced by immunization with self antigens derived from central nervous system (CNS) myelin components, such as myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP) (Tuohy et al., 1988; Amor et al., 1994; Johns et al., 1995). Most of the current drugs that are used for MS have been efficacious in EAE models (Constantinescu et al., 2011).

Mouse models of EAE typically develop within 1–3 weeks following induction, but are often monitored for 4–8 weeks to monitor drug responses, which may require significant quantities of test drug following long duration studies (Merrill, 2009; Getts et al., 2012). This makes these studies labor, time and cost intensive.

Zebrafish (*Danio rerio*), have emerged as a promising model to study autoimmune demyelination and neuro-degeneration (Buckley et al., 2008; Fang et al., 2015). The rationale for the experiments conducted in this manuscript was to develop a model that will be useful as a quick whole organism screen for drugs being developed for multiple sclerosis and associated disorders. We believe that a zebrafish based model can act as a preliminary *in vivo* model which can help in

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selecting molecules for further *in vivo* evaluation in conventional mouse models. A step between *in vitro* and in conventional mammalian models will help reduce late stage attrition of drug candidates and help select better candidates for detailed *in vivo* experimentation. Thus, we believe that the proposed model will exhibit 3 R benefits.

Myelin oligodendrocyte glycoprotein peptide residues 35–55 is a myelin component that activates T cells in mice and humans (Koehler et al., 2002; Weir et al., 2002). This results in T-cell trafficking to the brain and spinal cord, where these cells initiate CNS tissue destruction (Weir et al., 2002). Experimental autoimmune encephalomyelitis is known to be a T-cell dependent disorder because adoptive transfer of *in vitro* activated myelin-reactive CD4+ T cells have shown to induce the disease (Olsson, 1995; Weir et al., 2002). MOG is a commonly used protein to induce EAE in animal experiments.

Fingolimod hydrochloride (Trade Name: Gilenya) is a marketed multiple sclerosis drug used for treatment of relapsing multiple sclerosis to reduce the frequency of relapses. Fingolimod is a sphingosine 1-phosphate receptor modulator, and is a first in class orally administered drug (Brinkmann, 2009; Chiba and Adachi, 2012).

In this manuscript, we have standardized the regimen of MOG for disease induction; we have monitored the clinical scores, body weight and histopathology changes in the fish. We have assessed the rescue of the clinical symptoms seen due to induction of the disease by using an approved and marketed drug fingolimod hydrochloride. We have further validated this model by testing a group of drugs marketed or under development for the treatment of MS which have been shown to be efficacious in the EAE model.

2. Animal ethics statement

All zebrafish experiments were performed following animal ethics guidelines of the institution as per the animal ethics laws of India. A licensed veterinarian supervised all the experimentation.

3. Materials and Methods

3.1. Animal care and maintenance

Wild type zebrafish (*Danio rerio*) were procured from local vendor (Vikrant Aquaculture, Mumbai, India) and maintained in re-circulatory system with controlled environment conditions with a temperature of 28 °C, and a light/dark cycle of ~14/10 h. They were fed thrice with live hatched brine shrimp and dry food (supplied by Vikrant Aquaculture, Mumbai, India) and were maintained as previously described (Banote et al., 2013). Four to six months old fish were used for these experiments.

3.2. Chemicals, drugs and drug administration

All drugs were purchased from Sigma Aldrich, USA. All other routine chemicals were purchased from Sisco Research Laboratories, Hyderabad, India. Complete Freund's adjuvant (CFA) was also procured from Sigma Aldrich, USA (Cat. No. F5881). MOG (Sequence: MEVGWYRSPFSRVVHLYRNGK) was purchased from GenScript HK Limited, Hong Kong. The drugs were administered using either oral (Kulkarni et al., 2014) or intra-peritoneal drug administration (Chaudhari et al., 2013) routes. These methods ensured the delivery of exact doses of the drugs in terms of milligrams per kilograms (mg/kg) of body weight.

3.3. Optimization of immunization dose

Experimental autoimmune encephalomyelitis (EAE) was induced by immunization with myelin oligodendrocyte glycoprotein – 35–55 (MOG). MOG in CFA was injected subcutaneously (s.c.) in the mid spine regions (near the end of the precaudal vertebrae as depicted in the Graphical Abstract) using 10 µl bevel-tipped Hamilton syringe with a volume of 5 µl/fish. Three concentrations of MOG: 0.3, 0.6 and 1 mg/ml were tested to standardize the dose that showed maximum efficiency for induction of clinical symptoms, body weight reduction with low mortality. The vehicle control was injected CFA at the same site as MOG injection. The different groups were the following: vehicle control (CFA s.c.), MOG in CFA 0.3 mg/ml s.c., MOG in CFA 0.6 mg/ml s.c and MOG in CFA 1 mg/ml s.c. The clinical signs were assigned scores to the surviving fish in the following order: 1: Normal, 2: Loss of Gait, 3: Mild Paralysis, 4: Total Paralysis. Each of the clinical signs can be seen in Video 1. All fish were observed for 7 days post treatment for clinical scores, body weight and mortality.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.msard.2016.11.010.

3.4. Validation with the fingolimod hydrochloride (hereafter referred as fingolimod)

In this study immunization of zebrafish was done on the 1st day with standardized concentration of MOG. Fingolimod treatment was performed using two regimens (I) progressing regimen wherein the treatment started immediately after immunization; and, (II) therapeutic regimen wherein treatment started after disease development on day 3. The doses of 0.1, 0.3 and 1 mg/kg of fingolimod were administered orally (p.o.) for 7 days in both the regimens (Dose volume used: MOG – 10 µl s.c.; fingolimod – 5 µl p.o.). Control group was injected with CFA s.c. and water p.o.. All fish were observed daily for 7 days of treatment for clinical symptoms, mortality and body weights were recorded on days 1 and 7 of treatment. Qualitative scoring of EAE signs were done in blinded fashion. To ensure that the scoring is conducted in blinded fashion, video recordings (3–7 min) were performed each day by the personnel performing drug administration, the videos was coded by the supervisor, and the qualitative scoring was performed by trained personnel not involved in experimental design or immunization or drug administration. In the therapeutic regimen, the treatment started on day 3, hence all observations that were made on day 7 of treatment were day 9 post immunization, however, the data reported has been with respect to the days of treatment. Statistical analysis for clinical scores was performed using GraphPad Prism® software using Kruskal-Wallis analysis followed by Dunn's multiple comparison test. Statistical analysis for body weight loss was performed using One-way ANOVA followed by Dunnet's Post-hoc test.

The study for each regimen was conducted separately wherein fish were assigned in six treatment groups with twelve fish per group at the beginning of treatment. The different groups were the following: vehicle control (CFA s.c. + water p.o.), MOG control (MOG 0.6 mg/ml s.c. + water p.o.), Fingolimod 0.1 mg/kg (MOG 0.6 mg/ml s.c.+ Fingolimod 0.1 mg/kg p.o.), Fingolimod 0.3 mg/kg (MOG 0.6 mg/ml s.c.+ Fingolimod 0.3 mg/kg p.o.), Fingolimod 1 mg/kg (MOG 0.6 mg/ml s.c.+ Fingolimod 1 mg/kg p.o.).

The spinal sections from four groups of zebrafish: control, MOG, Fingolimod 1 mg/kg (from prophylactic regimen) and Fingolimod 1 mg/kg (from therapeutic regimen) were analyzed for histopathological assessment at the end of the study to know the extent of inflammation, neurodegeneration and demyelination and also the effect of fingolimod treatment. The histological evaluation was performed on representative sample of spinal tissue taken from fixed tissue. Tissue processing and staining was performed as per standard sequential staining protocols. Infiltration cells were counted by using ImageJ Analysis Software in the hematoxylin and eosin stained sections. The region of interest was converted to 8 bit type to clearly visualize infiltrated cells as intense dark spots and the cells in each section were counted accordingly (Skundric et al., 2008). Glial cell count per section was also performed using ImageJ Analysis Software, in the sections stained using crystal violet stain. In this case, back-

ground subtraction of the selected area was performed and the cyton and nucleus in neuron which appear as dark purple spots were counted (Kluver and Barrera, 1954)). For myelination, the intensity of blue stained region of spinal cord section, in the luxol fast stained sections, was measured using RGB Plug-in in ImageJ Analysis Software (Kiernan, 2007). Statistical analysis was performed using GraphPad Prism® software using One-way ANOVA followed by Dunnet's Post-hoc test.

3.5. Detailed validation with additional drugs

Further validation of model was conducted in the prophylactic regimen of this model. Three drugs: dimethyl fumarate, dexamethasone and SR1001; were selected for the validation. Dimethyl fumarate is a drug approved and marketed for MS and related disorders (Chen et al., 2014); it was administered orally at doses of 15, 30 and 60 mg/kg and effects were evaluated for a period of seven days. Dexamethasone is a corticosteroid and has been shown to be effective in prevention and treatment of EAE in rodents (Donia et al., 2010); it was tested after intra-peritoneal administration at 0.3, 1 and 3 mg/kg doses. SR1001 is a ligand of RAR-related orphan receptors (ROR), which has been demonstrated to suppress Th17 cell differentiation and cytokine expressions. It is being developed by The Scripps Research Institute, and has been proven efficacious in rat model of EAE (Solt et al., 2011). It was tested in the zebrafish EAE model through intra-peritoneal administration at 25, 50 and 75 mg/kg doses.

4. Results

4.1. Optimization of immunization dose

Acute onset of EAE in zebrafish after immunization with mylein oligodendrocyte glycopeptide (MOG), known to develop progressive paralysis, began to show clinical signs 3 – 4 days from the day of immunization. The dose of MOG in CFA was standardized as 0.6 mg/kg, based upon efficiency to induce clinical symptoms, a significant reduction in percent body weight reduction with low incidence of mortality (See Fig. 1). The lower mean clinical score to 1 mg/kg dose of MOG could be attributed to local accumulation or spillage at higher concentration; however, we did not probe it further as 0.6 mg/kg dose satisfied the conditions for reasonable disease induction. The vehicle control group (CFA s.c.) did not show any clinical signs. From the animal ethics perspective, disease induction in zebrafish resulted in mortality numbers similar to those observed in conventional mouse models of EAE published in recent studies (Thell et al., 2016).

4.2. Validation with fingolimod

The phenotypic results of validation study with fingolimod in the prophylactic and therapeutic regimens have been depicted in Fig. 2 and can be appreciated in Video 1. We would reiterate here that, the treatment started on day 3 in the therapeutic regimen, thus, observations made on day 7 of treatment were day 9 post immunization, and, data reported has been with respect to the days of treatment. fingolimod at doses of 0.3 and 1 mg/kg showed marked improvement of ~10% and 20% respectively, in percentage survival as compared to MOG immunized group on day 7 post immunization (Fig. 2: (a) & (b)). Mean clinical scores showed daily improvement with fingolimod treatment (Fig. 2: (c) & (d)) and on day 7 (Fig. 2: (e) & (f)) showed statistically significant dose dependent improvement in the clinical score as compared to the MOG immunized group. The body weight loss data (Fig. 2: (g) & (h)) suggests dose dependent and statistically significant improvement in this parameter at doses 0.3 and 1 mg/kg of fingolimod as compared to MOG group. In the fingolimod 1 mg/kg group, on day 7, the mean clinical score and body weight loss data was similar to the vehicle control group suggesting the efficacy of fingolimod upon clinical progression of disease. The phenotypic effects, recorded on Day 7 of treatment in the prophylactic regimen of the

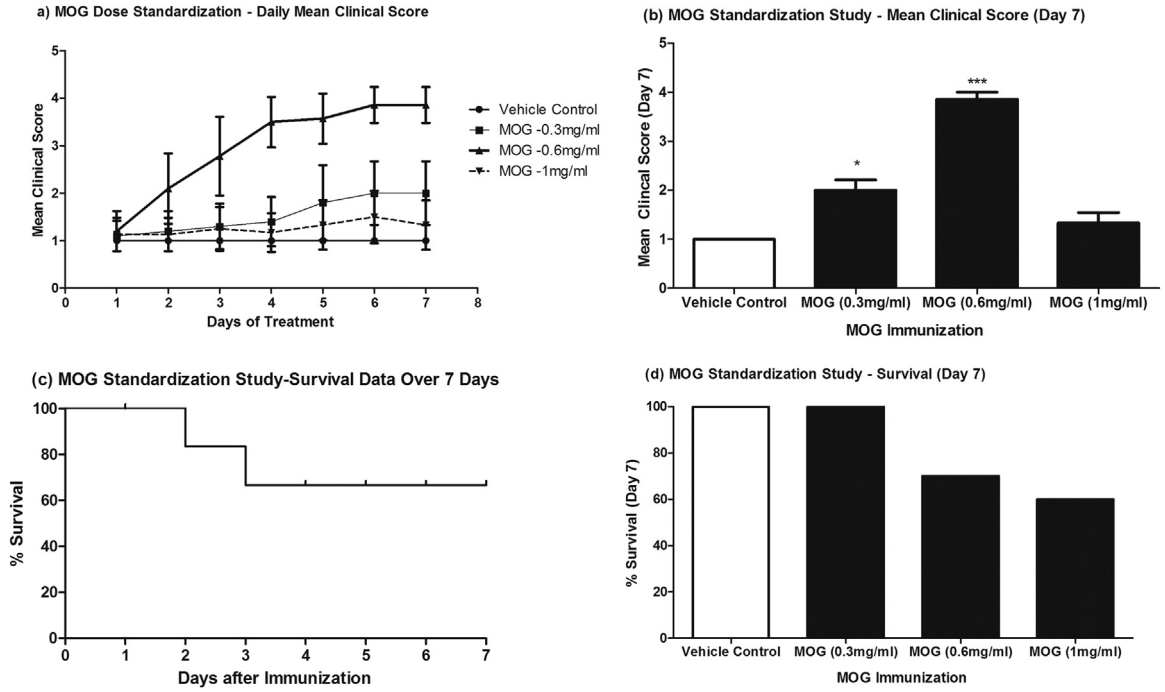

Fig. 1. Standardization of immunization dose with myelin oligodendrocyte glycoprotein (MOG): (a) Effects of MOG (0.3 mg/kg, 0.6 mg/kg and 1 mg/kg) as mean clinical score of paralysis like activity seen every day from day 1 to day 7. (b) Mean clinical score on Day 7. Statistical analysis for clinical scores was performed using GraphPad Prism® software using Kruskal-Wallis analysis followed by Dunn's multiple comparison test comparing all other groups with Vehicle Control. Data are represented using mean and standard error of the mean (± S.E.M.) clinical score on day 7 post immunization (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) (n=10 at the beginning of treatment). (c) Kaplan-Meier survival analysis performed to know survival probability after administration of MOG 0.6 mg/kg (n=36 at the beginning of treatment). (d) Effects of MOG on survival. Data are represented as Percentage Survival on day 7 post immunization (n=10).

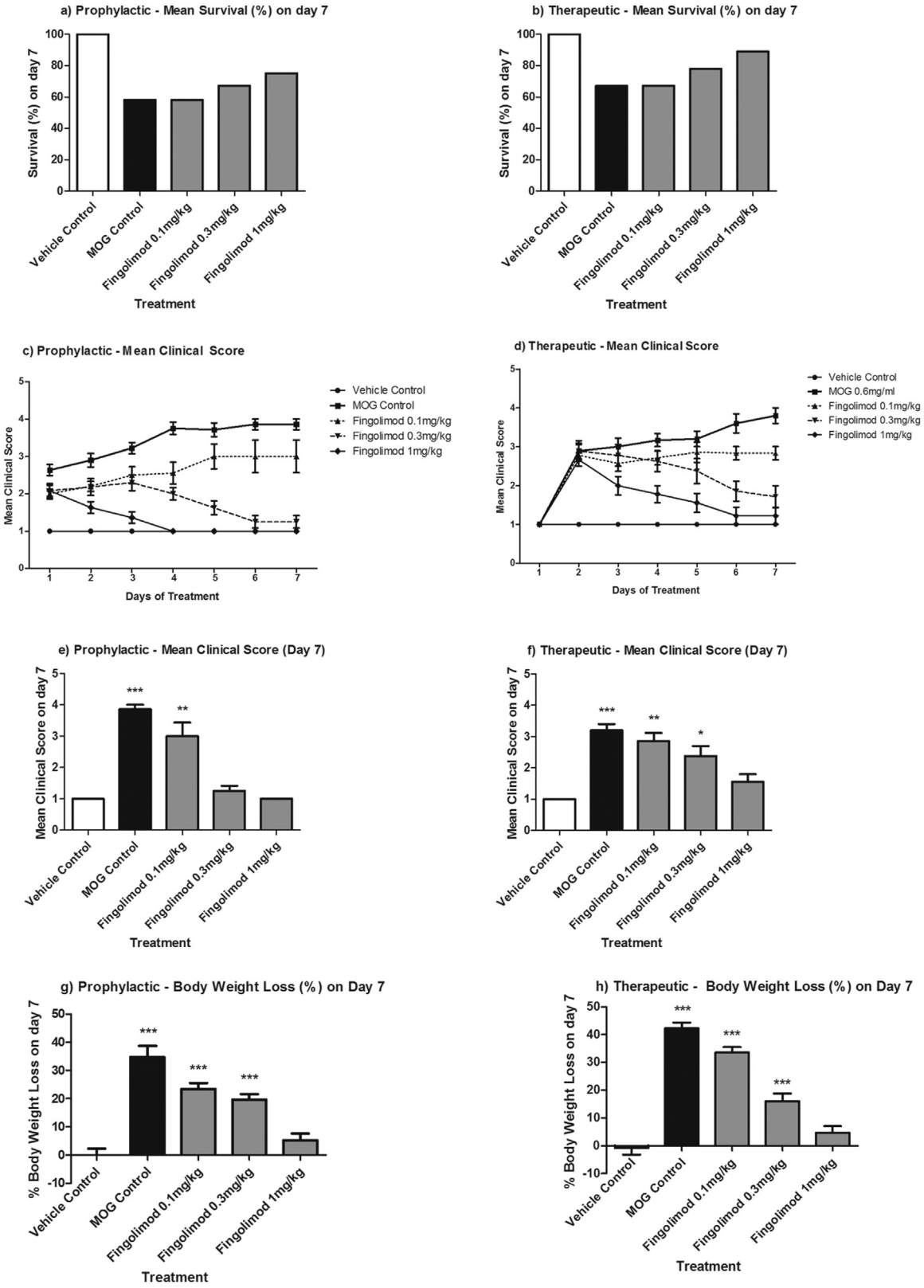

Fig. 2. Phenotypic effects seen in validation with fingolimod. Graph labels are: Percent Survival on Day 7, Mean Clinical Score over 7 Days, Mean Clinical Score on Day 7 and Percent Body Weight Loss on Day 7 in Prophylactic Regimen (a, c, e, g) and Therapeutic Regimen (b, d, f, h). Survival data are represented as Percentage Survival. Clinical score and body weight loss are represented using mean and standard error of the mean (± S.E.M.). For Mean Clinical Score on Day 7 statistical analysis for clinical scores was performed using GraphPad Prism® software using Kruskal-Wallis analysis followed by Dunn's multiple comparison test comparing all other groups with Vehicle Control. For Percent Body Weight Loss on Day 7; GraphPad Prism® software was used for conducting One-way ANOVA followed by Dunnet's Post-hoc test comparing all other groups with Vehicle Control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) (n=12 at the beginning of treatment).

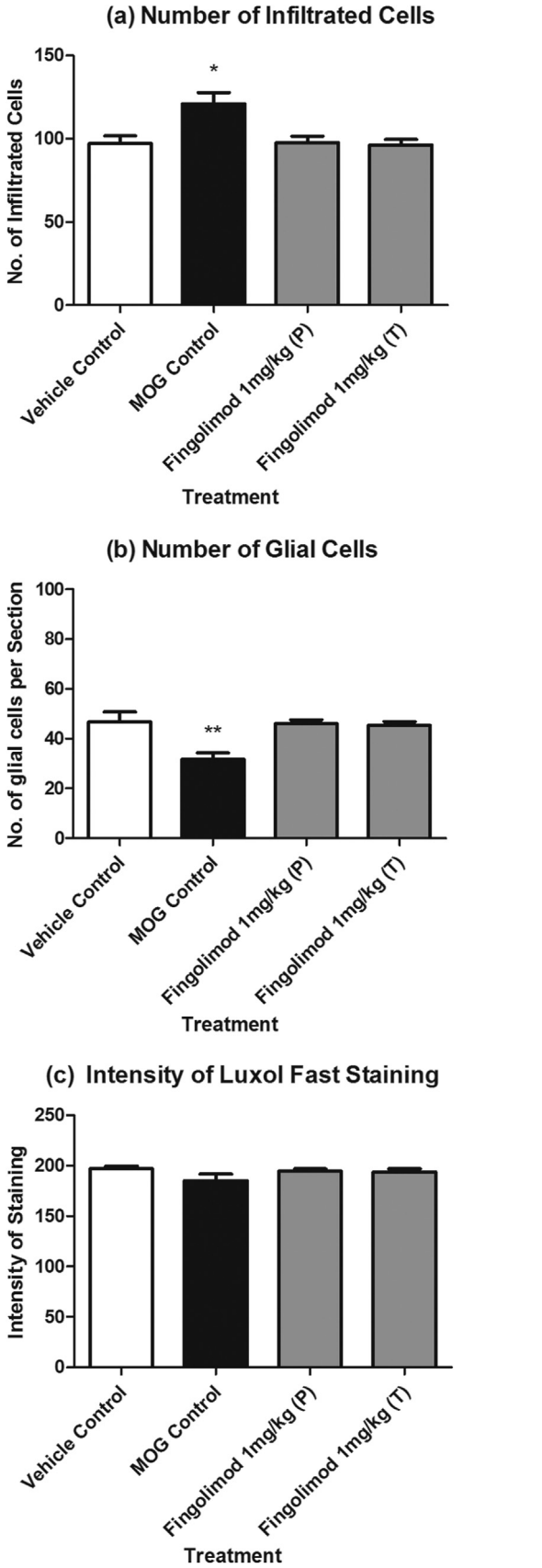

Fig. 3. Histopathological effects seen on day 7 of treatment in validation with fingolimod with four groups: Vehicle control, MOG control, Fingolimod 1 mg/kg (from prophylactic regimen abbreviated as P) and Fingolimod 1 mg/kg (from therapeutic regimen abbreviated as T). Graph labels are: (a) Number of Infiltrated Cells (b) Number of Glial cells (c) Intensity of Luxol Fast Staining. Data are represented using mean and standard error of the mean (± S.E.M.). GraphPad Prism® software was used for conducting One-way ANOVA followed by Dunnet's Post-hoc test comparing all other groups with Vehicle Control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; n=3).

model, of Control (untreated), MOG induced EAE and Fingolimod 1 mg/kg body weight treatment in adult zebrafish can be seen in Video 1.

The histopathological evaluation of spinal sections was performed on four groups: control, MOG, Fingolimod 1 mg/kg (from prophylactic regimen) and Fingolimod 1 mg/kg (from therapeutic regimen). The infiltration cell number (Figs. 3(a) and 4(a)) clearly indicates that the extent of inflammation was more and statistically significant in MOG immunized fish when compared to vehicle control and fingolimod treatment at 1 mg/kg (in both prophylactic and therapeutic regimens). The histopathology of spinal cord region of MOG immunized fish also showed statistically significant decrease in glial cell count per section (Figs. 3(b) and 4(b)) when compared to vehicle control and the glial cell density was found to be within normal limit in 1 mg/kg fingolimod treated. Luxol fast staining indicates the extent of loss of myelin based on reduction in intensity of staining (Figs. 3(c) and 4(c)). MOG immunized fish were found to show slightly low intensity, though statistically not significant, of myelination when compared to vehicle control and fingolimod treatments in both regimens. These are aspects of further refinement of this model; however, in our judgment, the phenotypic effects and other histopathological changes satisfy the conditions to consider it as a reasonable and quick screening model for further investigation.

The efficacy data on fingolimod, as reported to the USFDA suggest that it has been shown to be efficacious in various rodent models of EAE (US FDA, 2010). Thus the observations in zebrafish model concur with those reported in conventional rodent models.

4.3. Detailed validation with additional drugs

Three known drugs have been evaluated, as a part of validation of the model, and the data is represented in Table 1. Dimethyl fumerate when administered orally at doses of 5, 15 and 60 mg/kg showed dose dependent improvement in survival rates. Clinical scores and body weight loss parameters also improved when compared to the MOG group, however there was no dose dependence and the data looked like a saturated effect at the doses tested. Dexamethasone was administered intra-peritoneally at doses 0.3, 1 and 3 mg/kg. There was an improvement in survival rates, clinical scores and body weight loss when compared to MOG treated groups; however, at highest dose the clinical score and body weight loss increased, which could be because of immunosuppressant effect of dexamethosone. SR1001, a synthetic ROR ligand, showed improvement in survival rate at one dose i.e. 50 mg/kg when compared to MOG treated group. It showed improvement in clinical score and body weight loss at all doses, however, not in a dose dependent manner. This could be because the highest dose had severe mortality.

In summary we have tested wide chemical classes of drugs, which have different mechanisms of action, through different routes of administration and at different doses to validate the model.

5. Discussion and conclusions

5.1. Major advantages of the model

We have developed and validated, a novel zebrafish EAE model, that can be used to test drug candidates for MS and related disorders. The model proposed by us has the following advantages: (i) Quick: the efficacy of candidate drugs can be evaluated in a short span of 7 days; (ii) Low Compound Requirement: the efficacy evaluation can be performed using very little amounts of test compound, for example, compound requirement to test at 10 mg/kg dose (sample size of 10 fish weighing approximately 0.5 g each) for 7 days will be 350 µg. This is a minuscule quantity as compared to that required for a rodent EAE study; and; (iii) Inexpensive *In vivo* Data: the cost of maintaining zebrafish is very low as compared to rodents and *in vivo* data can be

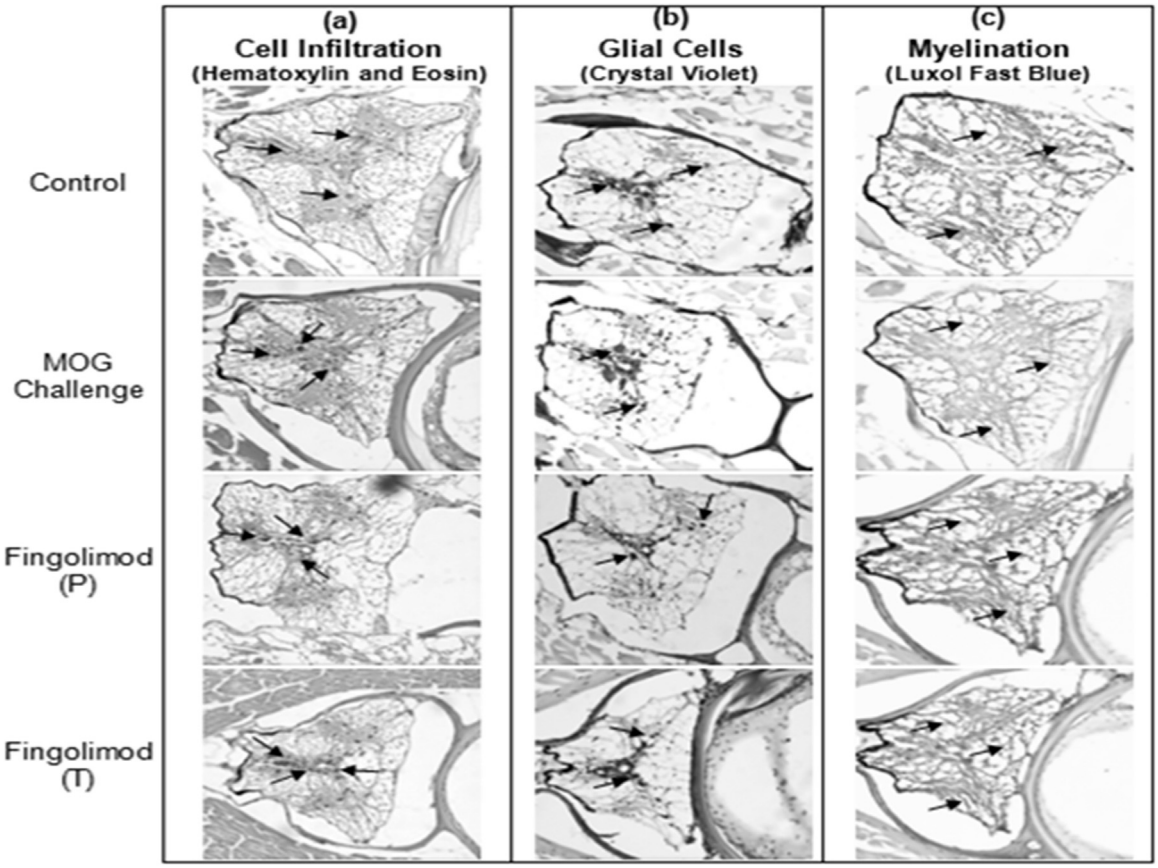

Fig. 4. Black and white images (see electronic supplementary data for colored images) of representative spinal cord histopathological sections for vehicle control, MOG control, Fingolimod 1 mg/kg (P) and Fingolimod 1 mg/kg (T) seen on day 7 of treatment in validation study with Fingolimod. The arrows (→) point towards examples of cells counted or blue intensity measured using ImageJ.

obtained quickly and with low quantities of test compound, further making it inexpensive.

5.2. Relevance in drug discovery

The EAE model has been used for pre-clinical evaluation of candidates being screened for MS and associated disorders for quite some time; however, there has been a question on the translational potential of the animal efficacy data in clinics. Despite this skepticism EAE is the most widely used model for *in vivo* efficacy evaluation and almost all drugs approved for MS were tested in this model before selecting them as clinical candidates (Constantinescu et al., 2011).

Table 1
Validation Study with Known Drugs: Phenotypic effects seen on day 7. Data are represented as mean and standard error of the mean (± S.E.M.). Statistical analysis for clinical scores was performed using GraphPad Prism® software using Kruskal-Wallis analysis of variance followed by Dunn's multiple comparison test, whereas, statistical analysis for body weight loss was performed using One-way ANOVA followed by Dunnet's Post-hoc test. Comparison is between all other groups with Vehicle Control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Groups	Drugs	Route of administration	Number of fish at the start of study	Drug doses (mg/kg of body weight)	Major parameters on Day 7 post immunization		
					Surival (%)	Clinical score (Mean ± S.E.M.)	Body weight loss (%) (Mean ± S.E.M.)
Vehicle Control	–	**Same as Drug**	**36**	–	100	1.00 ± 0.0	0.49 ± 1.19
MOG Control	–	**Subcutaneously at Mid Spine**	**36**	–	68	3.7 ± 0.2 ***	31.66 ± 3.21 ***
Positive Control	**Fingolimod**	**Per Oral**	**36**	1	82	1.0 ± 0.0	5.22 ± 2.33
Test Drugs	**Dimethyl Fumerate**	**Per Oral**	**12**	15	40	2.0 ± 0.6 *	2.77 ± 1.06
				30	64	2.7 ± 0.6 ***	1.98 ± 2.32
				60	70	2.5 ± 0.7 ***	4.67 ± 1.85
	Dexamethasone	**Per Oral**	**12**	0.3	67	2.4 ± 0.2 **	1.98 ± 2.32
				1	67	1.4 ± 0.2	4.67 ± 1.85
				3	75	3.8 ± 0.3 **	6.68 ± 1.40*
	SR1001	**Intra-peritoneal**	**12**	25	50	2.1 ± 0.2 *	6.68 ± 1.40*
				50	67	1.2 ± 0.1	2.41 ± 1.10
				75	33	2.4 ± 0.3 *	10.57 ± 2.31***

Therefore, we believe that this model will play a significant role in preclinical evaluation of drug candidates in the near future as well. The zebrafish model for EAE can be used preceding the use of the rodent EAE models. This model can be used as a filter at lead optimization stage wherein substantial number of compounds selected through *in vitro* screening can be filtered using this model. The most promising compounds thereafter can be screened in the rodent models. Using all the advantages of the zebrafish models stated above, late stage attrition of compounds and associated costs can be saved, furthermore, the possibility of oral/intra-peritoneal drug administration can indicate the their dose range for rodent testing.

There is an obvious question related to the translation of zebrafish data to conventional mammalian models. Zebrafish are being increasingly proposed as screening tools for potential remyelination therapies due to their regenerative abilities, suggesting its relevance as a screening tool for MS and related disorders (Preston and Macklin, 2015). However, all the models suggested are larval models, which even though act as alternative to animal experimentation, have limitations of (i) not having fully developed organ systems, (b) inability to test poorly soluble drugs, and, (c) inability to evaluate drug kinetics and carrying out pharmacokinetic – pharmacodynamic correlation. All these factors attribute to the questions relating to translation of zebrafish data to other mammals. The use of adult zebrafish and its ability to overcome the limitations of a larval model have been suggested in several publications before (Banote et al., 2013; Chaudhari et al., 2013; Kulkarni et al., 2014). Thus, the model suggested here promises to overcome most of these limitations.

The study was performed using a mouse MOG peptide previously shown to be encephalitogenic in mice (Bernard et al., 1997), however upon analysis of the zebrafish genome, it appears that a direct homologue of MOG has not been reported yet and is unlikely to be present (Birling and Nussbaum, 1995). Homology searches of the peptide used showed some similarity (~40%) with zebrafish butyrophilin-like molecules (NCBI Sequence ID: NP_001103953.1), involved in lipid metabolism, and cross-reactive antibodies between MOG and butyrophilin have been reported (Guggenmos et al., 2004). However, whilst this peripheral antigen could account for molecular mimicry triggering MOG-reactive autoimmunity, it is not clear how this would manifest as a CNS restricted disease. Alternatively this peptide could induce an unusual cross reactivity at the T cell level, as it has been reported that T cells specific for MOG 35–55 peptide can also react with neurofilament medium 18–30 and such that the MOG peptide can induce disease in MOG-deficient mice (Krishnamoorthy et al., 2009). Repetition of these results in this study can confirm the value of the model and further work will be needed to determine the target autoantigen driving the paralysis in the zebrafish.

Zebrafish express orthologues of glucocorticosteroid receptors (Schaaf et al., 2009), Nrf2 (Mukaigasa et al., 2012) and ROR genes (Katsuyama et al., 2007), which could mediate the effects of the immunomodulatory drugs (dexamethasone, dimethyl fumarate and SR1001 respectively) used here. In addition, although zebrafish have spingosine-1-phosphate receptors (Tobia et al., 2012), they do not have lymph nodes (Renshaw and Trede, 2012). As limiting egress of lymphocytes from lymph nodes is a main mechanism of action of fingolimod in mammals (Brinkmann, 2009), it remains to be established whether the mechanism of action of fingolimod in zebrafish is via influences of egress from the spleen or other surrogate lymphoid tissue present in zebrafish (Renshaw and Trede, 2012). Alternatively, there could be direct influences on CNS endothelial (Tobia et al., 2012), as occurs in mammals (Spampinato et al., 2015).

An argument can be made regarding the relation of paralysis observed in fish to demyelination as the phenotypes seen in fish might be due to inflammation near the site of injection. This argument can be countered by the following facts of the experiment: (a) the control group was injected with CFA s.c. at the same site as MOG injected groups and did not show the paralytic phenotypes suggesting that the paralysis was due to MOG; (b) MOG induced inflammation is an established method for creating an EAE models; and; (c) the MOG induction has shown that on day 7 post immunization the intensity of myelination was slightly reduced in the luxol fast stained sections. The aspects of detailed pathological assessment, booster injections, larger set of drugs, biomarkers, endpoints other than mortality, culling the animals before death, etc. will need to be further investigated to refine this model for optimal utilization.

We propose this model to be a potential tool for quick assessment of candidate drugs and also to study disease pathobiology. The present manuscript can be inspiring to researchers in the field to explore and refine this model further as larger network of laboratories will be required to make it robust for use of industry and academia.

6. Conclusions

This is the first report, to the best of our knowledge, suggesting an *in vivo* adult zebrafish EAE model. It is possibly the quickest and most inexpensive *in vivo* model proposed for drug discovery of MS and related disorders. This model will need to undergo a wider validation by using larger set of drugs, identification of biomarkers and through larger network of zebrafish laboratories. There is scope to further improve this model for drug screening as well as for biological research; however, this will be the starting point of all such efforts.

Conflict of interest declaration

The authors have no conflict of interest with respect to this manuscript. Authors receive monetary compensation from their affiliation; however, the present manuscript does not in part or full disclose any information pertaining to the research of authors' affiliations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.msard.2016.11.010.

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Correlation of pharmacokinetics and brain penetration data of adult zebrafish with higher mammals including humans

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ABSTRACT

Introduction: Adult zebrafish pharmacology is evolving rapidly for creating efficacy and safety models for drug discovery. However, there is very limited research in understanding pharmacokinetics (PK) in adult zebrafish. Methods for understanding PK will help in conducting pharmacokinetic – pharmacodynamic (PK-PD) correlations and improving the quality and applicability of data obtained using zebrafish.

Methods: We conducted adult zebrafish PK and brain penetration studies on two known compounds (irinotecan and lorcaserin) with distinct PK and brain penetration properties using validated LCMS/MS method. Irinotecan was studied at a dose of 100 mg/kg i.p. and levels of the parent drug and active metabolite SN-38 were measured. Loracserin was studies at a dose of 10 mg/kg by two routes i.p. and p.o.

Results: Zebrafish PK and brain penetration profiles for both compounds were very similar to that of higher mammals including humans. Irinotecan was metabolised to SN-38 in ratios similar to ratios seen in other species and the compound had long half life with very low brain penetration in our studies. Loracasin was highly permeable in brain as compared to the exposure in blood, with long half life and high relative bioavailability, similar to other mammalian species including humans.

Discussion: Adult zebrafish PK studies are relatively an unexplored area of zebrafish research. The zebrafish data for key parameters of irinotecan and loracserin shows a high correlation to the data from higher species, including human. This report explores and discusses the use of adult zebrafish as a predictive PK tool for higher animal studies.

1. Introduction

Adult zebrafish are being used for various pharmacological and safety evaluation including neuropharmacology, infectious diseases, cancer models, cardiovascular safety, seizure liability, etc. (Khan et al., 2017; MacRae & Peterson, 2015; Sridevi, Anantaraju, Kulkarni, Yogeeswari, & Sriram, 2014). However, understanding pharmacokinetics (PK) and tissue distribution is essential to carry out pharmacokinetic – pharmacodynamic (PK-PD) correlation and optimize the utilization of zebrafish (Kulkarni et al., 2014; Kalueff et al., 2016; Kim et al., 2017). Literature reports have demonstrated that factors that impact absorption, distribution, metabolism and excretion (ADME) properties of drugs are conserved in zebrafish. These factors include structural and functional aspects of intestine and kidneys, phase I and II metabolism and blood-brain-barrier structure (Alderton et al., 2010; Kim et al., 2017; MacRae & Peterson, 2015). However, an understanding of PK-PD correlation in an in vivo data has multiple benefits over theoretical predictions of drug design as it helps in not just determining the drugability of a candidate molecules, but also provides information about dosage, probable dosing intervals and designing of experiments in conventional animal models with minimizing repetitions, following 3Rs of animal ethics and reduced costs of experimentation (Jang, Harris, & Lau, 2001).

There have been plenty of experimental reports on adult zebrafish pharmacology and toxicology; however, there have been very few PK reports with our laboratory being the first to report oral drug

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Table 1
Details of dose, route of administration, sampling time points, methodology for extraction and LCMS/MS protocol for measurement of Irinotecan and Loracserin has been described in this table.

Drug	Dose & route	Time points (h)	Extraction method	LC-MS/MS protocol
Irinotecan (SN-38 by chromatographic assessment)	100 mg/kg; i.p.	0, 0.08, 0.17, 0.5, 1, 2, 4, 8	**Extracting solvent**: acetonitile **Centrifugation:** 14,000 rpm, 10 min **Volume separation**: 5 μl	**Column:** Zorbax Bonus RP 50 × 4.6 mm, i.d-3.5 μm. **Mobile phase:** A: 5 mM ammonium acetate B: acetonitile **Volume of injection in LCMS/MS:** 20 μl **Flow rate**: 0.6 ml/min **Run time:** 6 min **Ion mode:** MRM mixed mode. **Internal standard:** indinavir **T/%B (Gradient):** 0/25, 0.10/25, 0.15/95, 6.0/95 **MRM:** irinotecan 587 > 167.1 (polarity-positive) SN-38392.8 > 349.1 (polarity-positive) Indinavir 614.1 > 421.1 (polarity-negative) **LLOQ:** 5 ng/ml (irinotecan) and 2.5 ng/ml (SN-38)
Lorcaserin	10 mg/kg; i.p., p.o.	0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 24	**Extracting solvent**: acetonitile **Centrifugation:** 14,000 rpm, 10 min **Volume separation**: 5 μl	**Column:** X Bride™ C-18, 46 × 5.0 mm, i.d-3.5 μm **Run time:** 8 min **Mobile phase:** A: 0.1% formic acid in water B: acetonitile **Volume of injection in LCMS/MS:** 5 μl **Ion mode:** ESI positive MRM **Internal standard:** Rolipram **T/%B (gradient):** 0/20, 0.50/20, 2/98, 8/98 **MRM:** Lorcaserine 196 > 129.1 (polarity-positive) Rolipram 276 > 191 (polarity-negative) **LLOQ:** 5 ng/ml (loracaserin)

administration method and detailed PK procedure using Dried Blood Spots (DBS) (Kulkarni et al., 2014). The present manuscript is in continuation with our effort to study the predictive value of zebrafish for human drug discovery. In the present study we have attempted to correlate PK and brain penetration data of adult zebrafish with higher mammals including humans. For the purpose of this study, two compounds with distinct PK properties were selected; viz. Irinotecan, and Lorcaserin. Irinotecan was selected as a compound that was metabolised to SN-38 and the brain penetration of both these compounds was below the level of detection. Lorcaserin was selected as compound that had very high brain penetration and retention.

2. Methods

2.1. Animal ethics statement

All experiments were performed following animal ethics guidelines of Dr. Reddy's Institute of Life Sciences, Hyderabad, India. These guidelines are as per the animal ethics laws of India. A licensed veterinarian supervised all the experimentation.

2.2. Animal care and maintenance

Wild type zebrafish (*Danio rerio*) were procured from local vendor (Vikrant Aquaculture, Mumbai, India) and maintained in re-circulatory system with controlled environment conditions with a temperature of 28 °C, and a light/dark cycle of ~14/10 h. They were fed thrice with live hatched brine shrimp and dry food and were maintained as previously described (Banote, Koutarapu, Chennubhotla, Chatti, & Kulkarni, 2013; Kulkarni et al., 2017). Four to six months old fish were used for these experiments.

2.3. Drugs, chemicals, instruments and materials

All drugs were purchased from Sigma Aldrich, USA. Heparin and other routine chemicals were purchased from Sisco Research Laboratories, Hyderabad, India. The drugs were administered using either oral (p.o.) (Kulkarni et al., 2014) or intra-peritoneal (i.p.) drug administration (Chaudhari, Chennubhotla, Chatti, & Kulkarni, 2013) routes. These methods ensured the delivery of exact doses of the drugs in terms of milligrams per kilograms (mg/kg) of body weight.

The HPLC system consisted of an Agilent 1200 quaternary pump, auto sampler with thermostat, column oven, and online degasser, triple quadrupole mass spectrometer (Mass hunter software version B.03.01) with multimode source (Agilent Technologies, Inc. 2850 Centerville Rd. Willington, DE 19808-1644, USA). FTA® Elute blood spot cards (DMPK type-B cards) were supplied by Whatman (Sanford, USA), Ultrasonic bath from Bandelin sonorex sonicator, centrifuge from Eppendorf (model# Centrifuge 5810), and Milli Q Water system from Millipore (model #Gradient A10).

2.4. Pharmacokinetic studies

We conducted zebrafish PK and brain penetration studies on two known compounds with distinct PK properties. The details of the methodology for extraction and measurement of Irinotecan and Loracserin has been described in Table 1. Irinotecan was administered i.p. at a dose of 100 mg/kg and blood and brain samples were collected at 0, 0.08, 0.17, 0.5, 1, 2, 4 and 8 h. Lorcaserin was administered by two routes i.p. and p.o. at a dose of 10 mg/kg and blood and brain samples were collected at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h. The doses for the study were selected considering the ability to correlate data with other species based on literature reports. The doses that were closest to most of data available on humans were selected for the zebrafish studies. As compared to loracserin, irinotecan was studied for lesser

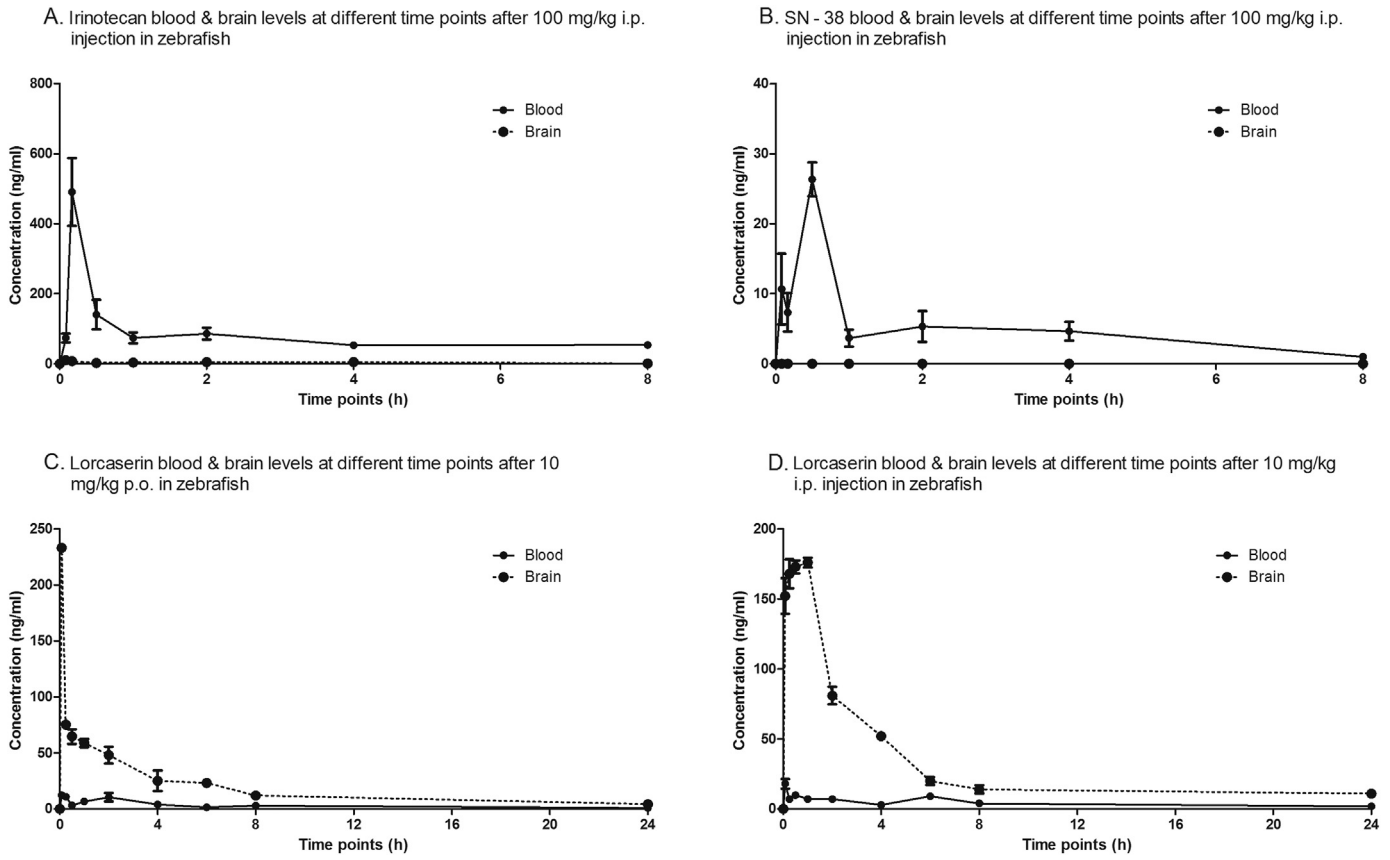

Fig. 1. Concentrations (ng/ml) of the test drugs at various time points have been presented as mean ± S.D. The graphs have been assigned alphabetical order as A. Irinotecan, B. SN-38, C. Loracserin – Oral, and, D. Lorcaserin – Intraperitoneal. Sample size; $n = 3$ fish/time point.

number of time points and shorter duration of 8 h considering the fact that irinotecan was administered by i.p. and thus its PK profile might be shorter than that of lorcaserin that was tested both p.o. and i.p.

2.5. Preparation and analysis of blood and brain samples

At every time point, three adult male fish were sacrificed using an aesthetic tricaine (MS-222) for blood and brain sample collection. Blood and brain were collected and processed using reported methods (Déglon, Thomas, Mangin, & Staub, 2012; Jagadeeswaran & Sheehan, 1999; Kulkarni et al., 2014). In brief, blood (7–10 µl/fish) was collected by cardiac puncture using heparin rinsed insulin syringes and collected in heparin containing tubes. Brain was collected by incising the head portion followed by removal of eyes and skull with forceps. Thereafter, the brain homogenate was prepared in Dulbecco's Phosphate Buffer Saline (DPBS) using Qiagen's Tissue homogenizer.

Whatman FTA® DMPK Cards were used for drug analysis using a method described before (Déglon et al., 2012; Kulkarni et al., 2014). In brief, 7 µl aliquots of sample from blood and homogenized brain were spotted on the DMPK type-B cards, followed by, punching out of a 3 mm diameter disc from the centre of each DBS, followed by extraction using a protocol described for each drug in Table 1. The details of calibration curves and representative chromatograms have been provided in Supporting information.

2.6. Pharmacokinetic parameter calculation and correlation with other species

PK parameters of blood and brain were calculated using PKSolver; an add-in program for in Microsoft Excel using non-compartmental analysis (Zhang, Huo, Zhou, & Xie, 2010). Key PK parameters for each drug were correlated with literature data about rats, primates and humans. Key parameters for each drug were as follows: Irinotecan: percent metabolism to SN-38, half life and brain to blood ratio; and; Lorcaserin: blood/plasma and brain half life, brain to blood ratio and relative oral exposure.

3. Results

The results of analysis of blood and brain for levels of the test drugs at various time points have been presented in Figs. 1 & 2 (Fig. 2 has a semi-log graph where the logarithmic concentrations have been depicted to reflect the differences in blood and brain) and the PK parameters have been presented in Table 2. The results of the correlation of data between zebrafish and other species have been presented in Table 3. Results of each of the drugs tested in this study are being described as follows:

3.1. Irinotecan

Irinotecan was administered to zebrafish i.p. and blood and brain concentrations were measured at different time points. The peak blood concentration (C_{max}) of 10.67 ng/ml was observed at 10 min (T_{max}) time point with a half life (t½) of 6.9 h and exposure ($AUC_{(0-t)}$) of 618.45 h*ng/ml. Similarly, peak brain concentration (C_{max}) of 491 ng/ml was observed at 5 min time point (T_{max}) with a half life (t½) of 1.89 h and exposure ($AUC_{(0-t)}$) of 29.61 h*ng/ml.

Irinotecan has a major active metabolite SN-38 and thus is an important analyte to study (Ramesh, Ahlawat, & Srinivas, 2010). The important blood kinetic parameters of SN-38 were: C_{max} = 26.33 ng/ml; T_{max} = 0.5 h; t½ = 2.36 h; $AUC_{(0-t)}$ = 40.14 h*ng/ml. The levels in brain were below the quantification threshold that could not be analysed using our method.

Zebrafish data was correlated with rats, primates and humans

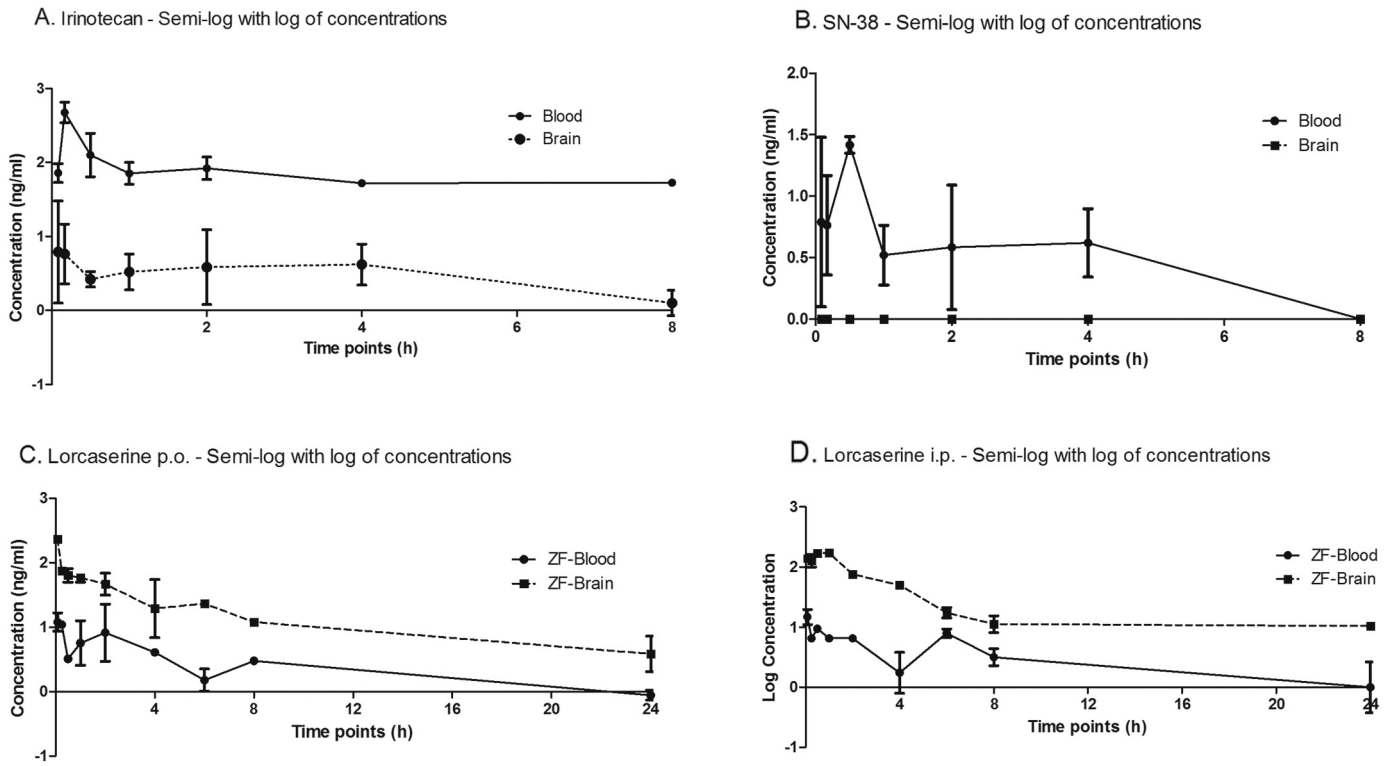

Fig. 2. Logarithmic concentrations of the test drugs at various time points have been presented as mean ± S.D. The graphs have been assigned alphabetical order as A. Irinotecan, B. SN-38, C. Loracserin – Oral, and, D. Lorcaserin – Intraperitoneal. Sample size; $n = 3$ fish/time point.

(based on literature data in case of the later three species) for the key parameters of half life, percentage metabolism to SN-38 and % blood:brain ratio. The data in primates and humans was after intravenous infusion of 30 min and 90 min respectively (USFDA, 2015). The data has been presented in Table 3 and correlation has been discussed in the discussion section.

3.2. Lorcaserin

Lorcaserin was administered to zebrafish by two routes, p.o. and i.p.; blood and brain concentration were measured at different time points. The i.p. administration and PK evaluation was conducted with a purpose to assess bioavailability after oral administration with respect to parenteral administration. The important blood kinetic parameters of lorcaserin after oral administration were: C_{max} = 12.24 & 233.16 ng/ml for blood and brain respectively; T_{max} = 0.08 h for both blood and brain; t½ = 7.73 & 7.95 h for blood and brain respectively; $AUC_{(0-t)}$ = 71.29 & 424.03 h*ng/ml for blood and brain. All the PK parameters in blood and brain after intraperitoneal administration of lorcaserin are presented in Table 2, however, the key parameter to report here is an $AUC_{(0-t)}$of 99.22 h*ng/ml.

Data correlation in case of lorcaserin was conducted for the key parameters of blood/plasma and brain half life, % blood:brain ratio and % relative oral exposure. The data has been presented in Table 3 and correlation has been discussed in the Discussion section.

4. Discussion

Adult zebrafish pharmacokinetics has been largely a neglected aspect of zebrafish research despite a large number of pharmacological models being developed and reported using this model. There is a need to generate more data and publish it in for the purpose of understanding the validity and predictive ability of zebrafish data by scientific community. The first pharmacokinetic report, to the best of our knowledge

Table 2
Pharmacokientic parameters of Irinotecan, SN-38, Locarserin – Oral, Loracserin – Intraperitoneal derived from zebrafish blood and brain samples. Sample size: $n = 3$ fish/time points.

Parameter	Unit	Irinotecan		SN-38		Locarserin – oral		Loracserin – intraperitoneal	
		Blood	Brain	Blood	Brain	Blood	Brain	Blood	Brain
Lambda_z	1/h	0.10	0.37	0.29	–	0.09	0.09	0.07	0.03
$t_{½}$	h	6.94	1.89	2.36	–	7.73	7.95	10.17	26.40
T_{max}	h	0.17	0.08	0.50	–	0.08	0.08	0.08	1.00
C_{max}	ng/ml	491.00	10.67	26.33	–	12.24	233.16	18.00	176.00
T_{lag}	h	0.00	0.00	0.00	–	0.00	0.00	0.00	0.00
C_{last_obs}/C_{max}		0.11	0.06	0.04	–	0.07	0.02	0.11	0.06
AUC_{0-t}	h*ng/ml	618.45	29.61	40.14	–	71.29	424.03	99.22	730.66
AUC_{0-inf_obs}	h*ng/ml	1155.94	31.43	43.54	–	81.22	472.43	128.58	1149.62
$AUC_{0-t/0-inf\_obs}$		0.54	0.94	0.92	–	0.88	0.90	0.77	0.64
$AUMC_{0-inf\_obs}$	ng/ml*h^2	11,539.33	105.81	135.32	–	825.35	4077.66	1968.18	30,206.40
MRT_{0-inf_obs}	h	9.98	3.37	3.11	–	10.16	8.63	15.31	26.28
Vz/F_obs	(mg/kg)/(ng/ml)	0.87	–	–	–	1.37	–	1.14	–
Cl/F_obs	(mg/kg)/(ng/ml)/h	0.09	–	–	–	0.12	–	0.08	–

Table 3
Key (distinct) pharmacokinetic parameters of (A) irinotecan and (B) lorcaserin in zebrafish, rats, primates and humans. Abbreviations: p.o. – per os (oral); i.p. – intraperitoneal injection; i.v. – intravenous, i.v. inf. – intravenous infusion, n.a. – not available/accessible.

Species	Dose/route	Key (distinct) pharmacokinetic parameters		
(A) Irinotecan		Plasma/blood half life (t½)	% Metabolism to SN-38 (based on AUC_{0-t})	% Brain:blood ratio (Kp, based on AUC_{0-t})
Zebrafish	100 mg/kg i.p.	6.9	6.4	4.78
Rat	130 mg/kg i.v.[a]	2.1[a]	0.8[a]	< 1%[a]
Primate	225 mg/m^2 i.v. inf.[b]	4.9[b]	2.4[b]	13[b]
Human	125 mg/m^2 i.v. inf.[c]	5.8[c]	2.3[c]	n.a.

(B) Lorcaserin		Plasma/blood half life (t½)	Brain half life (t½)	% Brain:blood ratio (based on AUC_{0-t})	% Relative oral exposure p.o.: i.p./i.v. (Kp, based on AUC_{0-t})
Zebrafish	10 mg/kg p.o.	7.7	8.0	595	72
Rat	10 mg/kg p.o.[d,e]	4.9[d]	4.7[d]	1374[d]	93[e]
Primate	10 mg/kg p.o.[d,e]	n.a.	n.a.	1010[e]	51[e]
Human	10 mg/kg p.o.[e]	11[e]	n.a.	170[e]	n.a.

[a] USFDA, 2015.
[b] Blaney et al., 1998.
[c] USFDA, 2014.
[d] Thomsen et al., 2008.
[e] USFDA, 2011.

was by Zang, Morikane, Shimada, Tanaka, and Nishimura (2011), wherein, the efficiency of oral delivery of felbinac through gluten granules was confirmed using a HPLC -based method. Thereafter, we published the first report describing oral drug administration, use of DBS cards for extraction of analyte and measurement using LCMS/MS method (Kulkarni et al., 2014). The most recent report on this subject was by Kim et al. (2017). This study was conducted to investigate the possibility of using zebrafish as a screening tool to estimate partition coefficient (Kp, brain) to predict drug brain penetration in humans. All these studies are suggestive of the fact that zebrafish can act as model organism for studying PK and brain penetration aspects which are essential for taking decisions during the process of drug discovery. In the present study, we choose compounds that show distinct PK properties, especially properties that led to their pharmaceutical development and therapeutic regimen.

In case of irinotecan; zebrafish data was correlated for the key parameters of half life, percentage metabolism to SN-38 and % blood:-brain ratio. The parameters of half life and % blood:brain ratio were correlated as these determine the dosing frequency and tissue distribution to an organ which is separated by blood brain barrier. Furthermore, irinotecan is also considered as a pro-drug from the clinical and therapeutic perspective due to it is metabolised to SN-38 which is 100–1000 fold more potent than irinotecan (Ramesh et al., 2010). Therefore, the percentage metabolism to SN-38 in zebrafish was essential to determine the predictive value of zebrafish for metabolic conversion. This data supplements the observations of Alderton et al. (2010); where authors had demonstrated that zebrafish perform phase I and phase II metabolism reactions similar to other mammals. The blood half life of irinotecan in zebrafish suggested a profile of a once a day drug. This data, if it had been generated in zebrafish during the process of drug discovery, correlated with primates and human but not with rats. Despite the fact that the data in primates and humans is based on intravenous infusion of 30 and 90 min, the data in zebrafish is predictive of a once a day administration regimen. We would like to put a caveat here, that, irinotecan has an active metabolite with variable pharamcokinetics and in clinical situation the dosage regimen is decided specific to patient depending on metabolism and half life of both irinotecan as well as SN-38 (Mathijssen et al., 2001). Similarly, the data on brain penetration suggests zebrafish to be better predictor for primates (which are closer to humans) when compared to rats. Though there is high inter-species variability, the irinotecan data in rats is suggestive of negligible brain penetration whereas zebrafish data suggests the brain penetration being low but not negligible, a trend observed in primates too. This result has been vindicated by the clinical reports (that do not clearly provide brain concentrations) of use of irinotecan in glioma patients wherein, efficacy has been attributed to irinotecan permeability across the blood brain barrier and conversion to SN-38 in brain (Vredenburgh, Desjardins, Reardon, & Friedman, 2009). Furthermore, the large difference in blood and brain $t_{½}$ (6.94 and 1.89 h, respectively) in zebrafish irinotecan data suggests the possible impact of P-glycoprotein efflux on irinotecan, trend known to impact brain penetration of irinotecan in higher species including humans (Adkins et al., 2015).

Conversion of irinotecan to SN-38 was higher in zebrafish as compared to other species; however, the data still indicates a low metabolism (~7%) of irinotecan, which is consistent with other spices. It re-emphasises conservation of metabolic pathways and blood brain barrier permeability in zebrafish with respect to higher mammals (Alderton et al., 2010; Jeong et al., 2008; Kim et al., 2017). It is important to note that the metabolism, elimination and distribution of irinotecan are highly complicated though various enzymes like carboxylesterases UGT1A1 mediating glucuronidation, CYP3A4, P-glycoprotein, etc. and vary from patient to patient (Mathijssen et al., 2001). Therefore, one of the important next steps of this manuscript may be evaluating these mechanisms in zebrafish.

Lorcaserin, a selective 5-hydroxytryptamine (5-HT_{2C}) receptor agonist, an appetite lowering drug; has distinguishing PK properties of high brain penetration, long half life in both blood an brain and good bioavailability (Thomsen et al., 2008). As the drug acts on the central nervous system, these parameters are essential drugable properties of this drug. The data in zebrafish shows that these parameters correlate with all mammalian species including humans. Even though the actual ratios of these parameters have high inter species variability, the trend demonstrates that, if zebrafish data was available during the process of drug discovery, it would have helped in decision making regarding drugability of the candidate. In terms of metabolism, multiple pathways including involvement of almost all cytochrome P450 enzymes (CYP) (Gustafson, King, & Rey, 2013) are involved in Loracaserin and study of drug interactions through these pathways could be a logical next step to this manuscript.

The limitations of using DBS cards for studying tissue penetration especially brain penetration is that the data obtained for apparent tissue distribution (Kp) is a surrogate representation of unbound brain-to-

plasma concentration (Kp, uu). The acceptability of surrogate data is dependent on the variability in the unbound faction between tissues. Therefore, in case on new chemical entities it has been advised to carry out in vitro assessment of this variability and then use appropriate conversion to calculate tissue distribution (Rowland & Emmons, 2010).

A major limitation of using adult zebrafish for PK studies is the difficulty to conduct intravenous (i.v.) drug administration and thus inability to directly evaluate the PK parameters after administration in the blood vessel/s. There has been one report by Pugach, Li, White, & Zon, 2009; wherein retro-orbital injection has been attempted to deliver cells directly in the blood vessels, however, there have been no reports on using this technique to deliver drugs. While the retro-orbital route could be a subject of further study, i.p. route has been considered as a preferable parenteral route of administration in zebrafish for various pharmacological studies. Intrapertinoeal (i.p.) route is not an intravascular route and first pass metabolism may be similar to oral, yet relative analysis of p.o. v/s i.p. does provide confidence about the gut absorption (as compared to parenteral) and resulting relative oral drug exposure during discovery of orally administered drugs. This information can be helpful as it is a major decision criterion for establishing the drugability of oral drug candidate. Similarly the half-life data obtained after i.p. administration for a drugs meant to be administered i.v. might not reflect the correct trend due the quicker onset of metabolism. In such cases in silico or in vitro information about metabolism could be used along with zebrafish data for taking decisions in drug discovery. Experimentation and correlation of such compounds will be required to further improve the utility of this model. While these limitations are overcome, the adult zebrafish model for PK can definitely help in PK-PD correlation where the efficacy has been studied in adult zebrafish, a field that is rapidly developing especially for neuropharmacology.

In conclusion, zebrafish PK has been an understated and understudied aspect in zebrafish research. It needs to be explored in details across various laboratories for optimal utilization of zebrafish model organism for pharmaceutical and disease biology research. The experiments reported in this manuscript demonstrate the correlation of zebrafish PK data to higher mammalian species including humans. Despite the variations in the actual values, this study suggests the utility of zebrafish in understanding the trend of pharmacokinetic data of candidate drugs in early drug discovery process.

Contributors

PK, RM, NN, SY and VS performed all experiments. PK also conducted data interpretation and analysis and prepared the manuscript. PR, DS, US, SO and PY contributed towards the ideas, design and supervised all the experiments and manuscript preparation. US, SO, PY also made necessary arrangements for resources either from the institute or various grants.

Conflict of interest statement

The authors have no conflict of interest to disclose.

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Appendix A. Supplementary data

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