The Read-Across Hypothesis and Environmental Risk Assessment of Pharmaceuticals

Mariann Rand-Weaver,† Luigi Margiotta-Casaluci,‡ Alpa Patel,†,‡ Grace H. Panter,§ Stewart F. Owen,§ and John P. Sumpter‡

†Biosciences, School of Health Sciences and Social Care, Brunel University, Uxbridge, Middlesex, UB8 3PH, United Kingdom

‡Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, United Kingdom

§AstraZeneca, Brixham Environmental Laboratory, Freshwater Quarry, Brixham, Devon TQ5 8BA, United Kingdom

Supporting Information

Three tables, 16 pages.

Table S1 details the accession numbers of sequences used to construct the phylogenetic tree for 5-alpha reductase. Table S2 collates published laboratory studies to 10 pharmaceuticals that report both measured water and plasma concentrations. Table S3 shows the predicted drug plasma concentration for studies[28,33,36-42,44,45] which observed mode of action related effects, but where drug plasma concentrations were not measured.

Table S1. Accession numbers of the 5-alpha reductase protein sequences used for the construction of the phylogenetic tree.

Common name	Latin name	5α-Reductase Type	Accession No.	Database
Human	*Homo sapiens*	1	NP_001038.1	NCBI
Human	*Homo sapiens*	2	NP_000339	NCBI
Dog	*Canis lupus familiaris*	1	XP_535799	NCBI
Dog	*Canis lupus familiaris*	2	XP_532922	NCBI
Mouse	*Mus musculus*	1	EDL37018	NCBI
Mouse	*Mus musculus*	2	NP_444418	NCBI
Rat	*Rattus norvegicus*	1	NP_073202	NCBI
Rat	*Rattus norvegicus*	2	NP_058766	NCBI
African clawed frog	*Xenopus laevis*	1	NP_001092166	NCBI
Western clawed frog	*Xenopus tropicalis*	1	NP_001006841	NCBI
Western clawed frog	*Xenopus tropicalis*	2	NP_001017113	NCBI
Japanese wrinkled frog	*Glandirana rugosa*	1	BAH16707	NCBI
Zebrafish	*Danio rerio*	1	AAI64429	NCBI
Zebrafish	*Danio rerio*	2	NP_001017703	NCBI
Medaka	*Oryzias latipes*	1	ABQ09262	NCBI
Medaka	*Oryzias latipes*	2	ENSORLP00000015262	Ensembl
Salmon	*Salmo salar*	2	ACI68485	NCBI
Fugu	*Takifugu rubripes*	1	ENSTRUP00000030532	Ensembl
Stickleback	*Gasterosteus aculeatus*	1	ENSGACP00000011845	Ensembl

Amphioxus	*Branchiostoma belcheri*	Unknown	BAI44851	NCBI
Purple sea urchin	*Strongylocentrotus porpuratus*	Unknown	SPU_010958	SpBase
Great pond snail	*Limnea stagnalis*	Unknown	Translated from ES571494	NCBI (ESTs)
Owl limpet	*Lottia gigantea*	Unknown	194080	Lottia gigantea Database
Nematode	*Caenorhabditis elegans*	Unknown	F42F12.3	WormBase
Nematode	*Caenorhabditis remanei*	Unknown	XP_003101195	NCBI
Nematode	*Caenorhabditis brenneri*	Unknown	EGT46677	NCBI
Thale cress	*Arabidopsis thaliana*	Unknown	AT2G38050	TAIR
Upland cotton	*Gossypium mexicanum*	Unknown	Q2QDF6	UniPortKb
Grape	*Vitis vinifera*	Unknown	D7THB8	UniPortKb
Tomato	*Solanum lycopersicum*	Unknown	Q5K2N1	UniPortKb
Maize	*Zea mays*	Unknown	B6TIF9	UniPortKb
Rice	*Oryza sativa*	Unknown	Q53NE6	UniPortKb

Table S2. Published laboratory studies of fish exposed to 10 pharmaceuticals that report measured water and plasma concentrations.

Pharmaceutical (CAS no.)	**Solvent**	**Exposure duration**	**Nominal water concentration (µg/L)**	**Measured water concentration (µg/L)**	**Measured plasma concentration (ng/mL)**	**Species / weight / temperature/ pH**	**Reference**	**Human therapeutic range (ng/mL)**
Atenolol free base (29122-68-7)	None	21 days	100; 320; 1000; 3200; 10,000	Reported as 77.10 to 95.67% of nominal	Males – 4.2; 10.8; 48.6; 52.6; 164.3 ; females >LOQ; 30.1; >LOQ; 108.9; 291.0	Fathead minnows /Male 3.28 to 4.34 g; female 1.66 to 2.13 g / 24.93 to 25.06 ˚C / pH 7.64 to 7.76	Winter et al., 2008. http://www.sciencedirect.com/science/article/pii/S0166445X07004237	A= 100 – 2000
Carbamazepine (CAS 298-46-4)	Dimethyl-formamide (0.01 %) CAS 68-12-2	7 days (sampled on days 1; 3; 6; 7)	100	83	Day 1 = 11,500; day 3 = 2,500; day 6 = 1,000; day 7 = zero ? *? = read from graph, appears that continuous exposure leads to clearance*	Channel catfish / NA / 19 ± 2˚C	Garcia et al., 2012. http://www.sciencedirect.com/science/article/pii/S0147651312002369	A = 2,000-12,000 B = 4,000 – 12,000
Clofibric acid (882-09-7)	NaOH: final concentration not reported	28 days	100; 1000; 10,000	122; 625; 11,000	26 ± 6; 90 ± 31; 2,876 ± 867	Female Rainbow trout / 26.90 ± 3.04; 26.81 ± 5.74; 18.21 ± 5.12 g / 15 ± 0.03 ˚C / pH 7.49	Owen et al., 2010. http://onlinelibrary.wiley.com/doi/10.1002/etc.351/abstract	A= 50,000 - 250,000
Diclofenac sodium	None	14 days	1; 10; 100	1.6 ± 0.93; 11.5 ± 3.98; 81.5 ± 14.09	8?; 60?; 369.5	Rainbow trout / 40 g / 13.0 ±0.1 ˚C	Cuklev et al. 2011. http://onlinelibrary.wiley.com/doi/1	A = 500 – 3,000 B = 500 –

(Not supplied)					*? = read from graph*		0.1002/etc.599/abstract	3,000
Gemfibrozil (Not supplied)	Dimethyl-sulfoxide 0.0003% final	96 hours and 14 days	1500 or 10,000 for 96h; 1.5 or 1,500 over 14 days	Not reported for 96h; 0.34 and 851.9 after 14 days	>75,000 in all fish after 96h at nominal 1500 and 10,000 µg/L water; 170 ± 20 and 78,000 ± 5000 in 1.5 and 1500 over 14 days respectively, with the control unexposed having 16 ± 5	Male goldfish, *Carassius auratus* / 25.6 ± 0.8 g 96h and 26.4 ± 0.7 g for 14 days / 18 ± 1˚C/ pH Not reported	Mimeault et al., 2005. http://www.sciencedirect.com/science/article/pii/S0166445X05000731	B= approx 25,000
Ibuprofen: (15687-27-1)	DMF; final concentration not reported	1 and 7 days	250	223.9 ± 6.2	227.19 ± 35.09 after 1 day; 314.85 ± 55.19 after 7 days	channel catfish (Ictalurus punctatus)/ ~50g/20˚C/ pH not reported	Nallani et al., 2011. http://www.sciencedirect.com/science/article/pii/S0045653511005339	A=10,000 – 30,000 with 50,000 in the normal range
Ketoprofen (Not supplied)	None	14 days	1; 10; 100	1.2 ± 0.21; 7.2 ± 2.59; 90.5 ± 32.98	0.05 ± 0.02; 0.19 ± 0.08; 0.62 ± 0.49	Rainbow trout / 39.4 ± 6.6 g /12.9˚C	Cuklev et al. 2012. http://www.sciencedirect.com/science/article/pii/S0304389412005808	A = 1000 – 5000 B = 1000 – 6000 but up to 20,000 is normal

Norethindrone (68-22-4)	Dimethyl-formamide 0.003% in exposure tanks	7 days	100	82.5 ± 8.5	1000? *? = read from graph*	channel catfish (Ictalurus punctatus)/ 49.9 ± 9.7 g / 21.3 ± 0.6˚C/ pH 7.4 ± 0.3	Nallani et al., 2012. http://rd.springer.com/article/10.1007%2Fs00244-011-9691-x#page-1	
Propranolol Hydrochloride (318-98-9)	None	6; 25; 98 hours	1	mean1120 ± 160 n=5 range 990 – 1330	138.2 ± 42.7; 254.4 ± 18.5; 385.1± 127.7 for 6; 25; 98h exposure respectively	Female Rainbow trout / NA /15.1˚C/ 7.4	Bartram et al. 2012. http://onlinelibrary.wiley.com/doi/10.1002/tox.20684/abstract;jsessionid=274D4E884205EB183F0E38B6D58E6C91.d03t04	A = 20 – 300 B = 20 – 300
Propranolol Hydrochloride (318-98-9)	None	21 days	0.001 to 10	1.3; 8; 78; 970	Males: 555; 6,050; 340,000; 15,000,000 Females: 690; 8,550; 210,000; 5,750,000	Fathead minnows /2.4-5.7g males; 0.8 – 2.5g female / 25 ± 1 ˚C	Giltrow et al., 2009. http://www.sciencedirect.com/science/article/pii/S0166445X0900304X	Cites 146 ng/mL in manuscript
Propranolol hydrochloride (318-98-9)	None	28 days	1; 10; 100; 1000; 10,000	0.92; 10.98; 83.77; 1007.81; 8,696.71	0.94; 3.3 ± 0.4; 16 ± 7; 280 ± 116; 5200 ± 1333	Female Rainbow trout / 23.66±3.67; 23.96±3.64; 23.69±3.60; 22.33±4.38; 16.52±3.87g/ 15 ± 0.03 ˚C / pH 7.4	Owen et al., 2009. http://www.sciencedirect.com/science/article/pii/S0166445X09001635	

Sertraline hydrochloride (Not supplied)	None	28 days	3; 10; 30	2.8; 9.4; 28.1	305; 610; 1927	Male Fathead minnows/ not reported - adult / 25 ± 1 ˚C / pH 8.5	Valenti et al., 2012. http://pubs.acs.org/doi/abs/10.1021/es204164b	A= 30 – 200 B= 50 -250

Human Therapeutic concentrations from A= Rogenthal R, Krueger M, Koeppel C, Preiss R (1999) Drug levels: Therapeutic and toxic serum / plasma concentrations of common drugs. J Clin Monit 15, 529-554; B= Schulz M, Iwersen-Bergmann S, Andresen H, Schmoldt A (2012) Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. Critical Care 16:R136

NA = not reported in study

? = data read from figures and are therefore estimates

Table S3. Fish steady state plasma concentrations ($F_{SS}PC$) of different pharmaceuticals calculated by using the Fish Plasma Model[1] and the exposure concentrations used in the cited studies. A range of H_TPC is provided (source reference is in brackets), and two predicted partitioning factors were used for the calculation - Log P (A) and Log $D_{7.4}$ (B), unless the two values were identical. The predicted $F_{SS}PC$ are compared to the Human Therapeutic Concentration (H_TPC), and the potential conflict with the read across hypothesis is indicated as "Yes, No, or unknown (?)".

Drug name	Reference in manuscript	H_TPC (ng/mL)	Partitioning factor	Exposure concentration (µg/L)	Predicted $F_{SS}PC$ (ng/mL)	Ratio $F_{SS}PC/H_TPC$	Conflict with the Read-Across Hypothesis?
Ethinylestradiol (CAS 57-63-6)	36	0.011-0.082 (RxList)	4.1 ACD/Log P ACD/Log $D_{7.4}$	0.0002	0.03	0.4-2.7	NO
				0.001	0.13	1.6-11.8	
				0.004*	0.52	6.3-47.3	
				0.016*	2.1	25.6-191	
				0.064*	8.3	101.2-754.5	
Beclomethasone dipropionate (CAS 5534-09-8)	37	0.78-1.344 (RxList)	3.63 ACD/Log P ACD/Log $D_{7.4}$	0.1*	5.0	3.7-6.4	NO
				1*	50	37.2-64.1	
				10*	498	370.5-638.7	
Levonorgestrel (CAS 797–63–7)	33	1-14 (RxList)	3.72 (A) ACD/Log P 3.37 (B) ACD/Log $D_{7.4}$	0.0008*	A: 0.06 B: 0.03	A: 0.004-0.06 B: 0.003-0.03	*Underestimated uptake ? (see Fick et al., 2010)*
				0.0033*	A: 0.23 B: 0.13	A: 0.02-0.20 B: 0.01-0.13	
				0.0296*	A: 2.03 B: 1.13	A: 0.15-2.0 B: 0.08-1.1	

Drospirenone (CAS 67392–87–4)	33	38-70 (RxList)	3.63 (A) ACD/Log P 3.39 (B) ACD/Log $D_{7.4}$	0.00033	A: 13 B: 19	A: 0.2-0.3 B: 0.3-0.5	NO
				0.0065*	A: 383 B: 256	A: 5.5-10.1 B: 3.7-6.7	
				0.070*	A: 4121 B: 2753	A: 58.9-108.4 B: 39.3-72.5	
Gestodene (CAS 60282-87-3)	38	2.3-13.4 (Dibbelt et al., 1992)	3.65 ACD/Log P ACD/Log $D_{7.4}$	0.001*	0.06	0.005-0.03	YES ? *Lack of effects at the medium conc.* →
				0.01	0.6	0.05-0.26	
				0.1*	6	0.45-2.6	*LOEC require replication.*
Desogestrel (CAS 54024-22-5)	38	2.2-4.1 (RxList)	5.35 ACD/Log P ACD/Log $D_{7.4}$	0.1	106	25.9-48.2	NO
				1	1060	258.5-481.8	
				10*	10605	2586.6-4820.5	
Tamoxifen citrate (CAS 54965-24-1)	39,40	40-183 (DailyMed)	7.882 (A) ACD/LogP 6.58 (B) ACD/Log $D_{7.4}$	0.18	A: 13463 B: 1509	A: 73.6-336.6 B: 8.3-37.7	? *Possible overestimated uptake*
				0.56	A: 41884 B: 4694	A: 228.9-1047.1 B: 25.6-117.4	
				1.8	A: 134627 B: 15089	A: 735.8-3365.7 B: 82.5-377.2	
				5.6*	A: 418840 B: 46945	A: 2288.7-10471.0 B: 256.5-1173.6	

				18*	A: 1346271 B: 150894	A:7356.7-33656.8 B:824.6-3772.4	
Fadrozole (CAS 102676-31-3)‡	41	2-10 (Estimated from Kochak et al., 1993)	1.484 (A) ACD/LogP 1.29 (B) ACD/Log $D_{7.4}$	2*	A: 3.2 B: 2.3	A: 0.3-1.6 B: 0.2-1.2	NO
				10*	A: 16 B: 12	A: 1.6-8.0 B: 1.2-6.0	
				50*	A: 80 B: 58	A: 8.0-40.0 B: 5.8-29.0	
Letrozole (CAS 112809-51-5)	42	13-35.75 (MHRA, Letrozole Public Assessment Report; Fick et al., 2010)	1.92 ACD/LogP ACD/Log $D_{7.4}$	1	3.3	0.1-0.3	NO
				5	17	0.5-1.3	
				25*	83	2.3-6.4	
				125*	415	11.6-32.0	
				625*	2077	58.1-160.0	
Fluoxetine (CAS 56296-78-7) ‡	44	97-302 (Amsterdam et al., 1997)	4.09 (A) ACD/Log P 1.56 (B) ACD/Log $D_{7.4}$	23.2*	A: 2959 B: 42	A: 9.8-30.5 B: 0.14-0.4	NO
				51.4*	A: 6569 B: 94	A: 21.8-67.7 B: 0.3-1.0	
				101.9*	A: 12998 B: 185	A: 43.0-134.0 B: 0.6-1.9	
Citalopram	45	25-88	3.48 (A)	1	A: 46	A: 0.5-1.8	NO

(CAS 59729-33-8) ‡		(de Mendoca Lima et al., 2005)	ACD/Log P 1.35 (B) ACD/Log $D_{7.4}$		B: 1.3	B: 0.015-0.03	
				10	A: 458 B: 13	A: 5.2-18.3 B: 0.15-0.5	
				100	A: 4575 B: 127	A: 52.0-183.0 B: 1.4-5.1	
Oxazepam (CAS 604-75-1) ‡	28	263-510 (Smink et al., 2008)	2.01 (A) ACD/Log P 2.21 (B) ACD/Log $D_{7.4}$	1.8*	A: 7 B: 10	A: 0.015-0.03 B: 0.02-0.04	YES?
				910*	A: 3518 B: 5008	A: 6.9-13.4 B: 9.8-19.0	

‡ CAS not provided in the paper. * Effect concentrations for the most relevant endpoint.

Comments

We used the Fish Plasma Model[1] (FPM) to predict if the exposure concentrations used in the studies cited would produce a $F_{SS}PC$ below or above the H_TPC; furthermore, we then related the predictions with the concentrations at which effects on the most relevant endpoints were observed.

The accuracy of the FPM has not yet been fully experimentally validated; therefore, a degree of uncertainty exists with respect to its range of applicability. Since the Log K_{OW} is the driving factor of the model, the accumulation of some compounds (e.g. highly

lipophilic drugs) may be overestimated. At the same time, the FPM does not consider the role of other factors, such as the presence of steroid hormone binding globulins in the gills, that may increase the uptake rate of pharmaceuticals form the surrounding water (i.e. steroids). The Log $D_{7.4}$ itself may be a source of uncertainty, since an experimental value is unavailable for the vast majority of compounds; hence, a computationally estimated value is used, carrying an intrinsic computational method error. Further sources of uncertainty may also be represented by: small differences of pH in the exposure system, which may in some cases affect the actual Log K_{OW}; differences in the Area Under the Curve (AUC) for fish and humans with equal C_{max}; differences between the duration of fish exposure and human treatment; lack of replicated experiments that reproduce the effects at the lowest concentrations.

Using the FPM and considering the uncertainties described above, the predictions for 8 out of 12 pharmaceuticals are not in conflict with the read-across hypothesis. These compounds had an effect at concentrations that are predicted to produce $F_{SS}PC$ equal or higher to the relative H_TPC. The reliability of the predictions for 2 out of 12 compounds were identified as "unknown", since the FPM is believed to overestimate (i.e. tamoxifen citrate) or underestimate (i.e. levonorgestrel) the uptake of the compound from the water via the gills. In the case of tamoxifen citrate the predictions do not disprove the hypothesis (effects occurring only when $F_{SS}PC > 250$-fold H_TPC), however the very high Log $D_{7.4}$ of this compound (6.58) may generate a significant overestimation of the predicted plasma accumulation. On the other hand, the predictions for levonorgestrel apparently disprove the hypothesis (the LOEC produces a $F_{SS}PC << H_TPC$); however, a strong discrepancy has been demonstrated between the predicted plasma bioconcentration factor (36-68) and the experimental one (8500-12000) (Fick et al., 2010).[13] This discrepancy indicates that the model may significantly underestimate the uptake of levonorgestrel from the surrounding water.

Only the predictions for gestodene and oxazepam apparently disprove the read-across hypothesis. In both cases the LOEC would produce $F_{SS}PC << H_TPC$. In the first case, Runnals et al. (2013)[38] observed reproductive effects only at the lowest and highest concentrations, but not at the intermediate one. This indicates that the effects observed at the lowest concentration will need to be replicated in order to properly interpret the results. If the high effect concentration is used as the LOEC, then the predictions fully support the read-across hypothesis. A similar situation occurs with oxazepam, where fish were exposed at two concentrations of oxazepam (1.8 and 910 μg/L), and effects were observed at both concentrations. The effects observed at the highest concentrations would fit with the read-across hypothesis (producing a $F_{SS}PC$ equal to 7-19-fold the H_TPC), whereas effects observed at the lowest concentration would, if replicated, disprove it. In this case, the experimental design of the study prevents a proper interpretation of the results: only two concentrations were used, and there is a 505-fold difference between them (a factor of 3.2 up to 100-fold difference between concentrations is generally used to establish a dose response). For both gestodene and oxazepam, only the replication of the effects observed at the lowest concentrations will clarify if these compounds represent exceptions of the hypothesis that human pharmaceuticals elicit mode of action-related effects in fish only if the $F_{SS}PC$ is equal or above the H_TPC.

In conclusion, the predictions obtained by the FPM indicate that the results obtained for the 12 pharmaceuticals discussed above do not disprove the read-across hypothesis, with the possible exceptions of gestodene and oxazepam; however, the LOECs reported for these two compounds have not yet been replicated, and only additional experiments will clarify the observed discrepancies.

The FPM is considered to be a useful tool that can be used to guide the experimental design, the selection of the exposure concentrations and the definition of the environmental risk. However, the FPM output should be carefully evaluated to avoid significant misinterpretations of the predictions.

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Superscripted references are those contained in the manuscript.

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