Title
Drug Interactions with the Human Organic Anion Transporter 3, OAT3

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Drug Interactions with the Human Organic Anion Transporter 3, OAT3

by

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THESIS

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MASTER OF SCIENCE

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Acknowledgments and Dedication:

I dedicate this work to my wife and children who have supported me in all my efforts and endeavors.

I would like to acknowledge Kathy Giacomini for serving as my PI, mentor, and champion, without whom I would not have been successful in this project.

I would also like to thank Pui-Yan Kwok and Deanna Kroetz who provided my initial training in graduate school and served on my thesis committee.

Lastly, I thank the students and post-doctoral scholars I was lucky enough to learn from and work with, namely, Jim Shima, Swati More, Leslie Chinn, and Stephanie Hesselson.

Abstract:

As an active transporter of anionic molecules in the kidney, OAT3 plays a role in the renal elimination of many drugs and is also thought to be involved in drug-drug interactions in the kidney. A number of drugs have been identified as substrates or inhibitors of OAT3 including several non-steroidal anti-inflammatory drugs (NSAIDs) and various antibiotics. To date, most studies of drug interactions with OAT3 have focused on a few compounds or at most, a small panel of structural analogs. The goal of the current study was to identify FDA approved drugs that interact with OAT3 using a high-throughput screening approach. A library of 937 FDA approved drugs was screened using a cell-based fluorescence assay. A total of 55 drugs were identified as hits including NSAIDS, anti-diabetic agents, and macrolide antibiotics not previously known to interact with OAT3. Kinetics profiles were generated for a selection of drugs as validation for the screen.

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Drug Interactions with the Human Organic Anion Transporter 3, OAT3

Background:

Organic Anion Transporter 3 (OAT3) is the protein product of the gene SLC22A8 and a member of the solute carrier family of membrane transporters. OAT3 shares hallmarks of the major facilitator superfamily (MFS) of proteins with its 12 transmembrane alpha helices, cystolic N- C- termini, intracellular loop connecting two 6-helix halves, and the RXXXR signature conserved sequence between loop 2 and 3[1]. OAT3 is expressed primarily in the kidney and found on the basolateral membrane of the proximal tubules[2]. As an active transporter of anionic molecules in the kidney, OAT3 plays a role in the renal elimination of many drugs and is also thought to be involved in drug-drug interactions in the kidney. A number of drugs have been identified as substrates or inhibitors of OAT3 including several non-steroidal anti-inflammatory drugs (NSAIDs) and various antibiotics[3,4]. Because many OAT3 inhibitors have inhibition constants (K_i's) well above their therapeutic unbound plasma concentrations, their relevance as inhibitors of active tubular secretion in vivo is questionable.

Study Objective:

To date, most studies of drug interactions with OAT3 have focused on a few compounds or at most, a small panel of structural analogs. Thus comprehensive information about potential drugs that interact with OAT3 or about the structural features of inhibitors of OAT3 has not been available. The goal of the current study was to identify FDA approved drugs that interact with OAT3 using a high-throughput screening approach. With this approach, we hoped to comprehensively identify inhibitors that may be candidates for clinical drug-drug interactions, i.e., drugs that inhibit OAT3-mediated

transport at clinically relevant concentrations. A secondary objective was to generate data that could be used in detailed follow-up analyses to elucidate important structural features of molecules that inhibit transport mediated by OAT3.

Methods:

Cell Based High-Throughput Screening

Human Embryonic Kidney cells (HEK293) stably expressing human OAT3 were cultured in Dulbecco's modified Eagle medium and supplemented with 10 % Fetal Bovine Serum (FBS), 5% penicillin G/streptomycin, and 100 μg/mL hygromycin B. Cells were plated into Greiner BioOne CellCoat D-Lysine coated, 96-well clear-bottom plates at $2.5x10^6$ cells per well and grown for 48 hours until approximately 90% confluent. Experiments were carried out using the Biomek FX^P liquid handling robot. The liquid handling procedure proceeded as follows: wash 3 times with 100 μL calcium free PBS, add 100 μL of 50 μM compound/10 μM 6-carboxyfluorescein in 5% DMSO calcium free PBS solution and incubate for 3 minutes, remove solution and wash 3 times with 100 μM probenecid in calcium free PBS stop solution, invert and centrifuge to 650 RPM. All plates were read on the Analyst HT (Applied Biosystems) fluorescent reader. Read settings were as follows: 485 nm excitation, 530 nm emission, 6 reads per well, $1x10^6$ μsec integration time, bottom read at 0 mm from bottom. The screen was subsequently repeated in full and data points were averaged to produce average inhibition values.

ICONIX Library

Screening compounds came from the 910-compound ICONIX library of FDA approved drugs provided by the UCSF Small Molecule Discovery Center (SMDC) and 27 additional antibiotic compounds for enriched coverage. The ICONIX library covers

every major class of prescription drug and is a representative sample of the prescription drug chemical space. The compound library is dissolved at 1 mM in 100% DMSO, thus limiting the drugs that can be dissolved and the effective concentration usable in a cell-based assay.

Inhibition Curves

To determine the K_i's of selected inhibitors, we performed competitive uptake experiments with 10 μM 6-CF and compounds selected from a spectrum of inhibition percentages produced by the high throughput screen. All experiments were done using the same OAT3 overexpressing HEK-293 cell line as used for screening. Concentration-dependent inhibition was measured using the same uptake conditions described in the screening experiments except for the DMSO concentration was lowered to 1%. All compound concentrations varied from 136 nM to 75 μM.

Data Analysis

Screening data were normalized and analyzed using the Pipeline Pilot software. Hits were classified as <2 standard deviations above the mean. Inhibition dose response curves were analyzed using the nonlinear fit One Site Competition algorithm Prism 4 software.

Results

Initial studies demonstrate that 6-carboxyfluorescein (6-CF) is taken up by OAT3.

6-CF was found to be a substrate of OAT3 stably expressing HEK293 cells, which took up 38 times more of the fluorescent probe than empty vector (EV) cells. A Michaelis Menton transport rate curve was generated in order to characterize the kinetics of the interaction (Figure 1). A Km of 70 (±5.7) μM and a Vmax of 3.8mM/min were

determined from the data set using the Single-Site Binding nonlinear fit algorithm in the Prism 4 software.

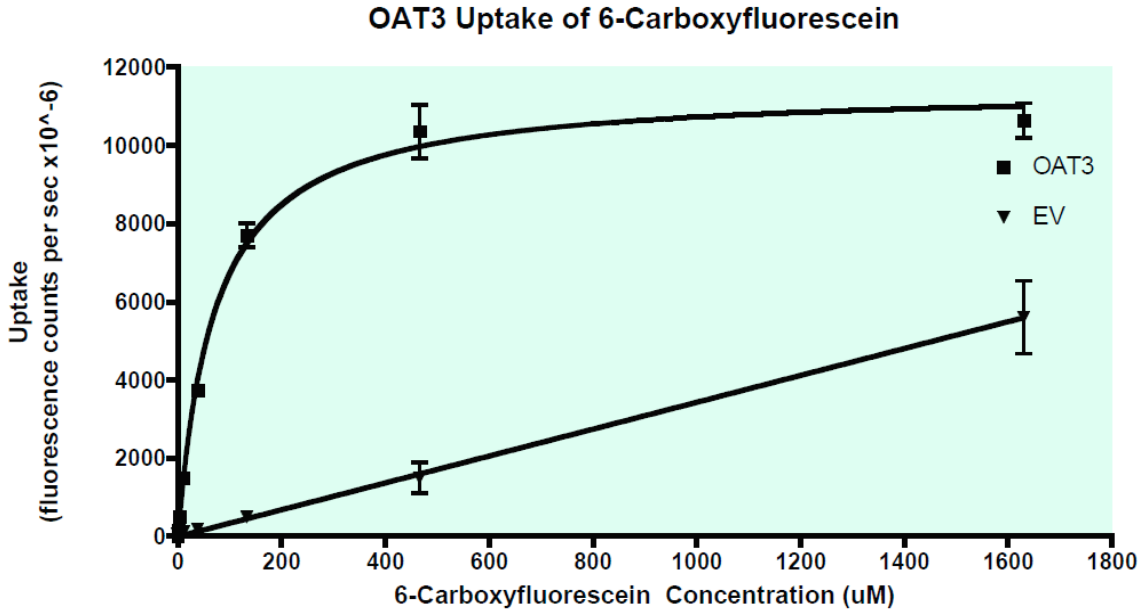


Figure 3. Dose-response curve for OAT3 and 6-carboxyfluorescein. 3 minute uptake at 2% DMSO. OAT3 values have the EV fluorescence subtracted.

DMSO affects 6-CF uptake by OAT3 in a concentration dependent fashion.

A 3-minute uptake of 6-CF (15 μM) with DMSO concentrations ranging from 0.19% to 50% showed a concentration dependent effect of DMSO on 6-CF uptake by OAT3 (Figure 2). DMSO appeared to have an optimum effect on OAT3 facilitated 6-CF transport at concentrations of about 1.5%. At high concentrations (>3%), DMSO nonspecifically inhibited 6-CF uptake. However, z-values were above the 0.5 critical threshold for up to 5% DMSO. The ICONIX library compounds being dissolved at 1 mM in DMSO limited the concentration at which the assay could be run. To screen drugs at sufficient concentrations and still maintain assay quality, we limited DMSO concentrations to 5% in the current study.

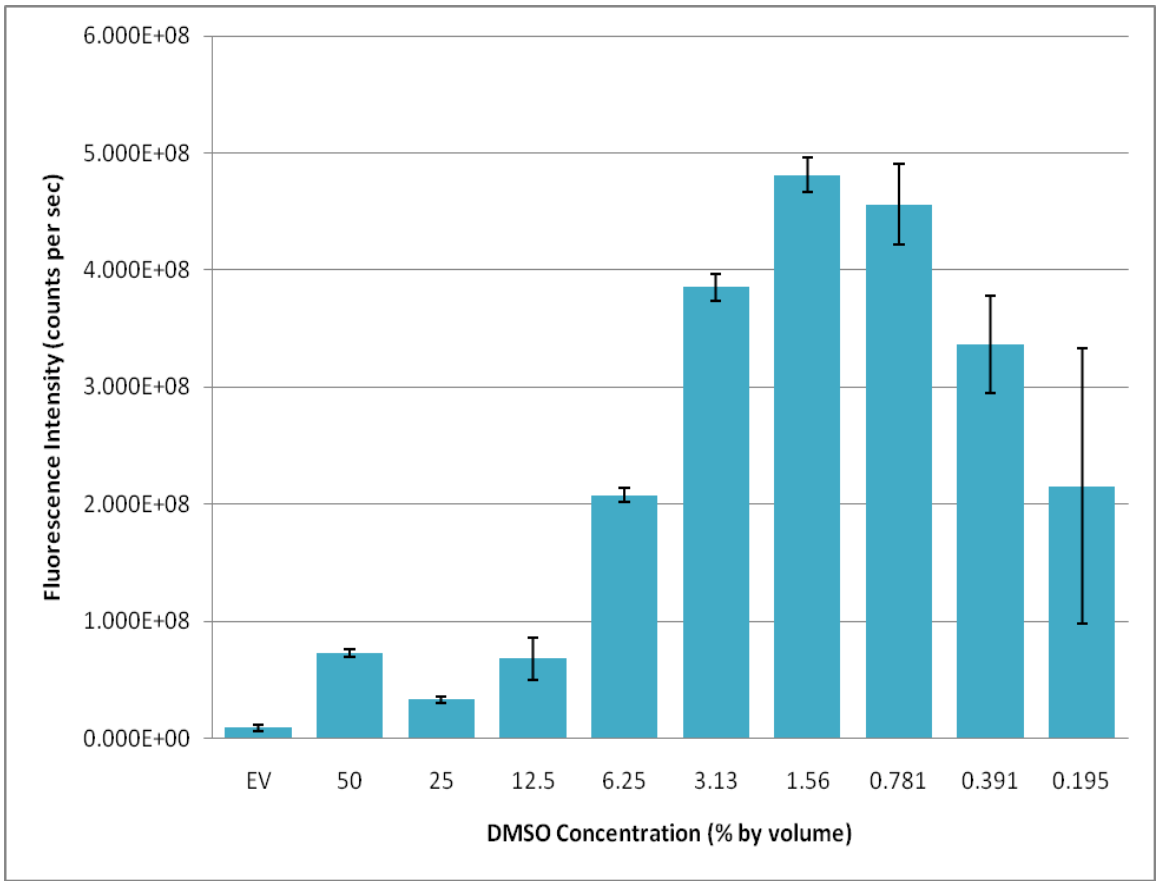


Figure 4. DMSO Effect on 6-CF Uptake. Uptake of 6-carboxyfluorescein (15 µM) at 3 minutes was determined in OAT3 expressing cells exposed to varying concentrations of DMSO. Error bars indicate standard error.

Drug Name	Average % Inhibition
IRBESARTAN	119
OXYMETHOLONE	114
MEFENAMIC ACID	113
Sulindac sulfide	108
TANNIC ACID	106
PIOGLITAZONE	106
ZAFIRLUKAST	105
OXAPROZIN	105
DIFLUNISAL	104
3,3',4',5-tetrachlorosalicylanilide	104
NIMODIPINE	103
NS-398	103
ACEMETACIN	103
NAFENOPIN	101
TOLFENAMIC ACID	100
GLYBURIDE	100
PROBENECID	100
ERYTHROMYCIN PROPIONATE	100
ANTIMYCIN A	99
BROMFENAC	99
CAPSAICIN	99
ZAPRINAST	98
SULFINPYRAZONE	98
NATEGLINIDE	97
TICRYNAFEN	95
IDEBENONE	95
NIFLUMIC ACID	94
TELMISARTAN	94
CANDESARTAN	93
DEXIBUPROFEN	93
TENIDAP	93
Sulindac sulfone	93
BITHIONOL	93
INDOMETHACIN	93
GW-1929	93
MYCOPHENOLATE MOFETIL	92
NIMESULIDE	92
Closantel	92
NANDROLONE	91
NIFEDIPINE	91
LOSARTAN	90
FLUNOXAPROFEN	90
MEDROXYPROGESTERONE	90
GLIMEPIRIDE	90
MECLOFENAMIC ACID	89
1-(2-methylbenzoyl)-4-(phenylmethyl)-piperidine	89
FLUFENAMIC ACID	89
MELOXICAM	89
KETOROLAC	88
WARFARIN	87
SULFASALAZINE	87
DEXKETOPROFEN	87
NORETHINDRONE ACETATE	87
GLIPIZIDE	87
Oxacillin	86
TRANILAST	86
CARMOFUR	85
PICLAMILAST	85
RWJ-68354	85
ZOMEPIRAC	85
DEXIBUPROFEN, 3-(-)Ibuprofen	84
DICLOFENAC	84
BENOXAPROFEN	84
FUROSEMIDE	84
GEMFIBROZIL	83
ACECLOFENAC	83
KETOPROFEN	83
FENBUFEN	83
IBUPROFEN	83
NIFURSOL	82
FENOPROFEN	82
Fusidic acid	82
MEGESTROL ACETATE	82
VALSARTAN	82
2-	82

ACETYLAMINOFLUORENE	
MOSAPRIDE	81
FLURBIPROFEN	81
DROPERIDOL	80
EXEMESTANE	80
SALSALATE	80
1-(2-Chlorobenzoyl)-4-(phenylmethyl)-piperidine	80
DIPYRIDAMOLE	79
PIROXICAM	79
CYPROTERONE ACETATE	79
TRAZODONE	79
ETHACRYNIC ACID	79
R(-)-IBUPROFEN	79
VALDECOXIB	79
TRETINOIN	78
TENOXICAM	78
BENDAZAC	78
alpha-ERGOCRYPTINE	78
VX-745	78
LANSOPRAZOLE	78
DIAZOXIDE	77
NORETHINDRONE	77
PHENYLBUTAZONE	76
MEDROXYPROGESTERONE ACETATE	76
PANTOPRAZOLE	76
LEFLUNOMIDE	76
BALSALAZIDE	76
CILOSTAZOL	75
CHLOROTHIAZIDE	75
RUBITECAN	75
SULINDAC	74
HEXACHLOROPHENE	74
CAFFEIC ACID PHENETHYL ESTER	73
NEFAZODONE	73
EPALRESTAT	73
17-METHYLTESTOSTERONE	73
4-(Phenylmethyl)-1-[2-(trifluoromethyl)benzoyl]-piperidine	73
ETHINYLESTRADIOL	73
DIGITONIN	73
ERGOCORNINE	73
CICLOPIROX	72
CORTISONE	72
4-NITROBENZOIC ACID	72
OXATOMIDE	71
MESTANOLONE	71
CHLORMADINONE ACETATE	71
CLONAZEPAM	70
REBAMIPIDE	70
NISOLDIPINE	69
1-(2-Fluorobenzoyl)-4-(phenylmethyl)-piperidine	68
URSODEOXYCHOLIC ACID	68
BETAMIPRON	67
BENDROFLUMETHIAZIDE	67
ANDROSTERONE	67
FELBINAC	67
RABEPRAZOLE	67
BUTYLPARABEN	66
DONEPEZIL	65
NITRAZEPAM	65
CHLOROXYLENOL	65
ACLARUBICIN	65
PYROGALLOL	65
RIFAMYCIN B	64
Penicillin V	64
ROFLUMILAST	62
6-METHOXY-2-NAPHTHYLACETIC ACID	62
GENISTEIN	62
THIOCTIC ACID	62
BEXAROTENE	61
BEZAFIBRATE	61
ETODOLAC	61
MILRINONE	60
6(5H)-PHENANTHRIDINONE	60
ANISINDIONE	59
PRAZIQUANTEL	59
NITRENDIPINE	59
RILUZOLE	59

(R)-BICALUTAMIDE	58
NIFEKALANT	58
FLUDROCORTISONE ACETATE	58
ESTRIOL	58
CHLORQUINALDOL	58
ZOPICLONE	58
SILDENAFIL	57
CERIVASTATIN	57
PEROSPIRONE	57
NOCODAZOLE	56
BUTAMBEN	56
SEMUSTINE	56
Cefaclor	55
MONTELUKAST	55
FLUMAZENIL	54
NILUTAMIDE	54

BISPHENOL A	53
IBUDILAST	53
NABUMETONE	53
ADRENOSTERONE	53
beta-ESTRADIOL	53
NALIDIXIC ACID	53
NICARDIPINE	53
BAY 11-7085	52
alpha-NAPHTHOFLAVONE	52
NITROFURANTOIN	52
FINASTERIDE	52
OMEPRAZOLE	52
DAPIPRAZOLE	51
NORGESTREL	51
RITONAVIR	51
GENTIAN VIOLET	50

Table 1. Drugs presenting >50% average inhibition of 6-CF (15 µM) transport by OAT3 with two independent experiments. Concentrations of drugs used in this screen were 50 µM.

Screening 937 compounds in the ICONIX Library reveals a hit rate of 5.9%.

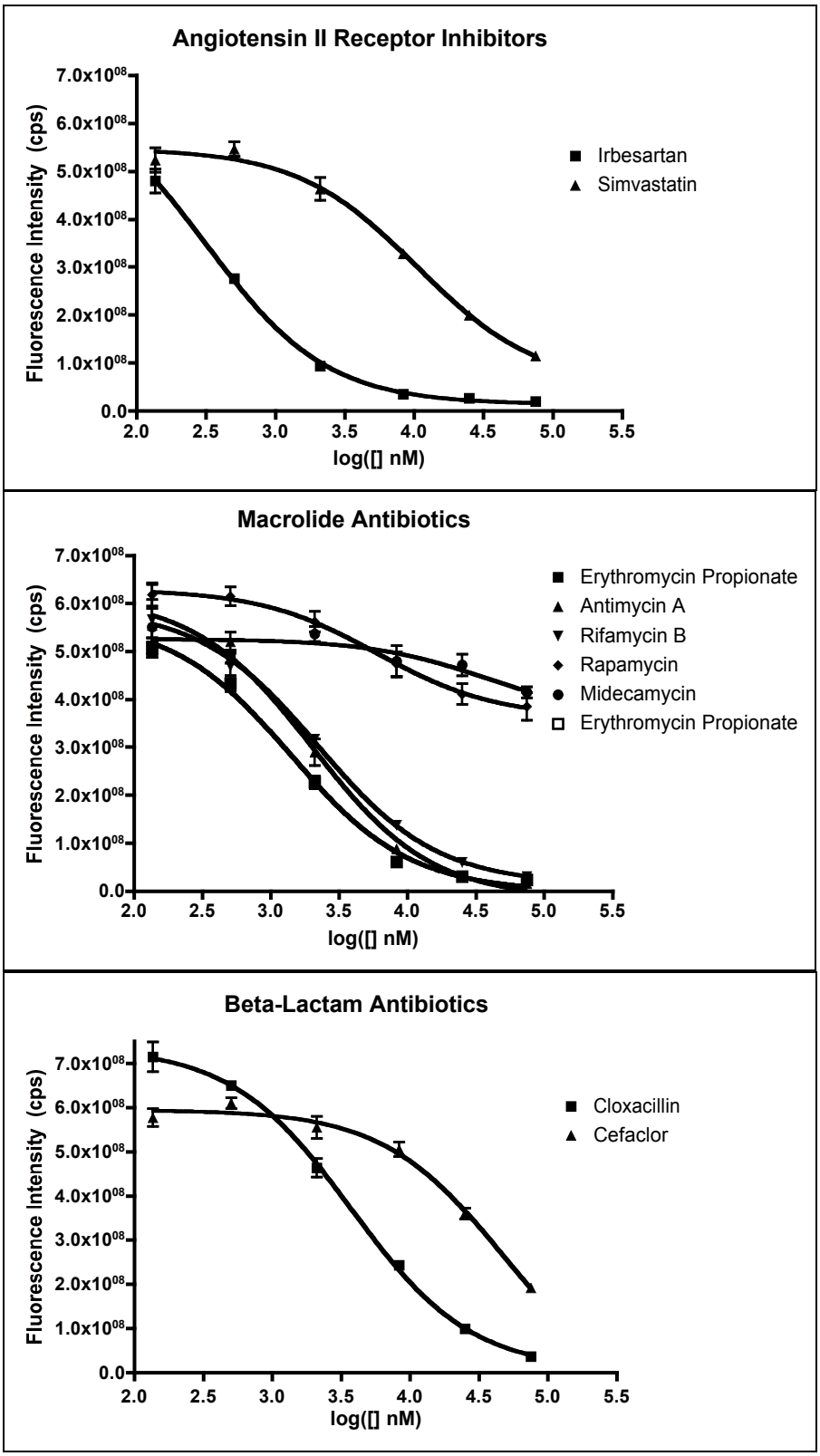
There was a 5.9% (55/937) hit rate for hits > 2 standard deviations (86% inhibition) above the mean and 0.1% (1/937) hit rate for > 3 standard deviations (118% inhibition) above the mean. Compounds listed in Table 1 are those above 50% average inhibition in two independent screening experiments. Screen results reveal enrichment of angiotensin II receptor antagonists, antidiabetic agents, statins, and especially NSAIDs. For example, the relatively small class of angiotensin II receptor agonists was represented by irbesartan as the top hit, followed by telmisartan, candesartan, and losartan. NSAIDs included mefenamic acid, sulindac sulfide, acemetacin, bromfenac, and many ibuprofen derivatives. Antidiabetics such as pioglitazone, glimepride, and nateglinide also were potent inhibitors.

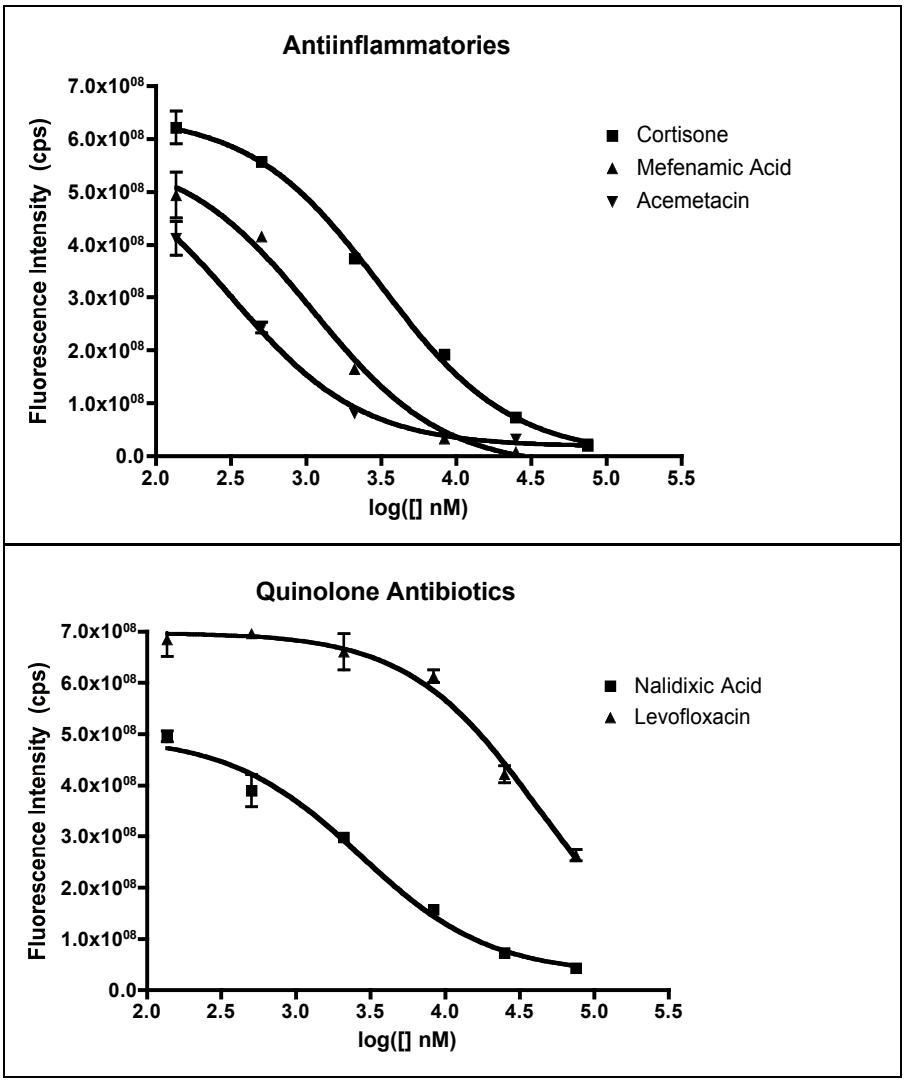
Drug Name	% inhibition from screen	K_i (nM)	Max unbound conc. (nM)	R squared
Acemetacin	103	276.8	14.9*	0.98
Irbesartan	119	282.6	30.3	0.99
Estrone	48.9	594.3	N/A	0.97
Mefenamic Acid	113	947.6	4,144	0.98
Erythromycin propionate	99.5	1223	41.1*	0.99
Fusidic Acid	81.9	1708	N/A	0.99
Antimycin A	99.3	1727	N/A	0.98
Rifamycin B	64.4	2030	N/A	0.95
Idebenone	94.9	2145	0.0025	0.98
Nalidixic Acid	52.6	2341	823	0.98
Cortisone	72.0	2730	N/A	0.99
Cloxacillin	46.2	3238	N/A	0.99
Bithionol	93.3	4217	190*	0.98
Rapamycin	29.7	5322	N/A	0.88
Simvastatin	40.7	9092	0.802	0.97
Midecamycin	35.4	32933	374	0.41
Levofloxacin	2.5	35545	N/A	0.96
Cefaclor	55.5	39517	3,405	0.97
Erythromycin	18.4	Does Not Converge	823	
Folic Acid	6.44	Does Not Converge	N/A	

Table 2. Inhibition constants (Ki values) of various compounds obtained in inhibition studies of 6-CF transport by OAT3. Uptake of 6-CF uptake in 1% DMSO was determined in the presence of various concentrations of inhibitor drugs (listed in column 1). Max concentrations from literature[5] or desk references[6,7]. N/A= not available. *assumed 5% fraction unbound.

Inhibition studies validate eight drugs as inhibitors of OAT3

Inhibition curves for 20 drugs selected from several drug classes and varying inhibition levels resulted in K_is ranging from 277 nM to 39.5 μM (see Table 2). Two drugs, folic acid and erythromycin, did not produce curves consistent with Michaelis-Menten kinetics and are not considered to interact with OAT3 as predicted from screening (Figure 3).


Angiotensin II Receptor Inhibitors
Fluorescence Intensity (cps)
7.0x10^08
6.0x10^08
5.0x10^08
4.0x10^08
3.0x10^08
2.0x10^08
1.0x10^08
0.0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
log([] nM)
Irbesartan
Simvastatin
Macrolide Antibiotics
Erythromycin Propionate
Antimycin A
Rifamycin B
Rapamycin
Midecamycin
Erythromycin Propionate
Beta-Lactam Antibiotics
Cloxacillin
Cefaclor


Antiinflammatories
Fluorescence Intensity (cps)
7.0x10^08
6.0x10^08
5.0x10^08
4.0x10^08
3.0x10^08
2.0x10^08
1.0x10^08
0.0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
log([] nM)
Cortisone
Mefenamic Acid
Acemetacin
Quinolone Antibiotics
Fluorescence Intensity (cps)
7.0x10^08
6.0x10^08
5.0x10^08
4.0x10^08
3.0x10^08
2.0x10^08
1.0x10^08
0.0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
log([] nM)
Nalidixic Acid
Levofloxacin

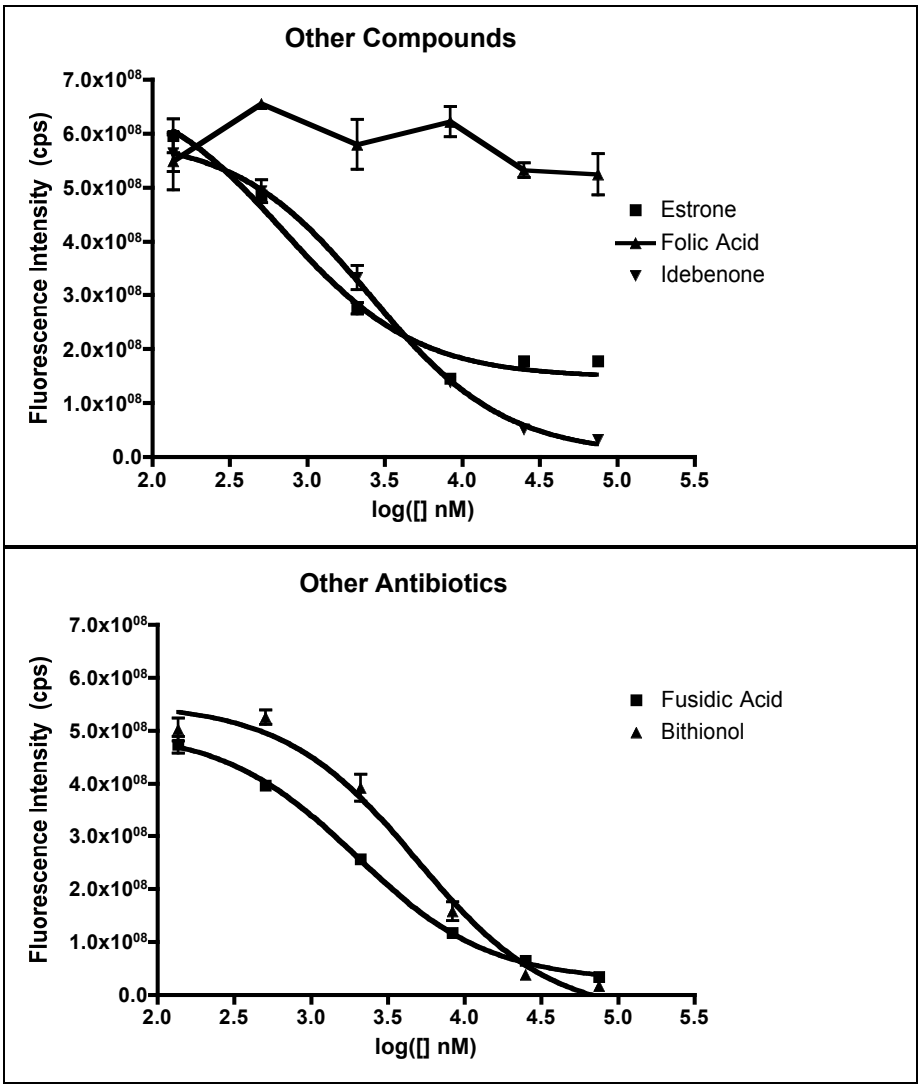


Figure 3. Inhibition curves for selected compounds inhibiting 6-CF transport by OAT3. A 3 minute uptake in OAT3 expressing HEK-293 with 6-CF in 1%DMSO was performed in the presence of various concentrations of test compounds. Error bars indicate standard error (n=3).

The overall ranking of compounds by K_i trends with the inhibition value from the screen with the exception of estrone and bithionol, which may have been compromised by the high DMSO concentration in the HTS assay.

Discussion/Conclusions

Initial studies demonstrate that 6-carboxyfluorescein (6-CF) is taken up by OAT3.

6-CF has been previously reported to interact with organic anion transporters such as OAT4[8]. The kinetics of 6-CF with OAT3 specifically has been characterized in mice

and rabbits, but no previous kinetics have been reported for the interaction with human OAT3 (hOAT3).

In this study, we determined the interaction of 6-CF with hOAT3 and found the K_m of 6-CF with OAT3 to be 70 μM, on par with previous studies with mouse and rabbit OAT3, which showed K_ms near 10 μM [9,10]. In four trial runs at 5% DMSO, the average Z-value was 0.7. This Z-value qualifies as an excellent assay as per Zhang et al[11].

Screening 937 compounds in the ICONIX Library revealed a hit rate of 5.9%.

Previous studies have identified inhibitors of OAT3, but most of these studies involved small-scale screens or isolated inhibition studies. In this study, we screened 937 FDA approved drugs and observed 55 hits (5.9%) above the two standard deviation threshold (86% inhibition). Consistent with previous small scale studies, we found that NSAIDS and angiotensin II receptor agonists were potent inhibitors[12,13]. We also found model OAT3 inhibitors, probenecid and indomethacin, to be potent inhibitors.

In the current study, we identified novel drug inhibitors of OAT3. For example, we determined that several anti-diabetic drugs were potent inhibitors of OAT3, thus implicating this therapeutic class of drugs as potentially important inhibitors and substrates of OAT3. Further, though various NSAIDs have been well-established as inhibitors of OAT3, in this study we identified additional NSAIDs as potent OAT3 inhibitors, e.g., acemetacin, oxaprozin, and diflunisal. Macrolide antibiotics, erythromycin propionate and antimycin A were also not known previously to interact with OAT3.

Inhibition studies validate ten drugs as inhibitors of OAT3

We performed further studies on eight of our 55 hits and 12 compounds that did not qualify as "hits" based on our criteria. The determined K_is ranged from 276.8 nM to 39.5 μM. Consistent with previous studies in the literature, we observed K_i values of 948 nM for mefenamic acid and 39.5 μM for cefaclor. Wang et al showed K_is of 780 nM and 120 μM respectively. Of the compounds for which we obtained kinetics data, the most likely to be clinically significant is mefenamic acid. It has a K_i above the unbound concentration found clinically. Of the compounds with kinetic profiles, folic acid had the weakest interaction, consistent with the screening results and no mention of a direct interaction in the literature. Other compounds that may be potentially important inhibitors from a clinical standpoint are compounds that have Ki values within 10 times the therapeutic unbound concentration range.

It is possible that many of the inhibitors of OAT3 identified in this study may also be substrates of OAT3. The studies reported here are focused on identification of inhibitors. Studies directly testing the uptake of the compounds using specific assays for individual compounds are needed to identify potential substrates of OAT3. Such studies should focus on drugs that are renally cleared by active tubular secretion.

Conclusion

In conclusion, 937 compounds were screened revealing a host of novel inhibitors of OAT3. At least one drug, mefenamic acid, is likely to be a clinically significant inhibitor of OAT3 and should be studied in situ or in vivo to elucidate its importance. Several of the drugs shown here to have K_i lower than the plasma unbound concentration may also be important inhibitors of renal clearance of various OAT3 substrates under

clinical conditions and should be considered in future clinical drug-drug interaction studies[14]. Further study is needed to determine the mechanisms of inhibition for all potent inhibitors. This data set can also be used to perform structure-activity relationships (SAR) to elucidate structural features important for OAT3 interactions, which are not currently well understood.

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