Award Number: W81XWH-10-1-0735

TITLE: Development of F-18 Labeled Radiotracers for PET Imaging of Brain Alpha-1 Noradrenergic Receptors: Potential PTSD Vulnerability and Diagnostic Biomarkers

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REPORT DATE: September 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
1 September 2012	Annual	1 Sep 2011 – 31 Aug 2012

4. TITLE AND SUBTITLE
Development of F-18 Labeled Radiotracers for PET Imaging of Brain Alpha-1 Noradrenergic Receptors: Potential PTSD Vulnerability and Diagnostic Biomarkers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0735

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Washington
Seattle, WA 98195-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research
and Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT: Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Increased central nervous system (CNS) alpha-1 noradrenergic receptor (α1NAR) responsivity may be a vulnerability and/or diagnostic biomarker for posttraumatic stress disorder (PTSD). Development of α1NAR PET radiotracers would allow *in vivo* interrogation of CNS α1NAR responsivity in combat-exposed active duty and Veteran warriors with PTSD. During the initial period of this award, we synthesized a 6-[18-F]-Fluoro-5'-Iodo analog of the α1NAR antagonist, 1-(2H)-naphthalenone-3,4-dihydro-2-(((2-p-hydroxyphenyl)-ethylamino)-methyl)-HCl (HEAT). PET imaging studies of [18F]-fluoro-5'-iodo-HEAT binding to CNS α1NAR in 2 Macaques demostrated rapid uptake but negligible efflux of radioactivity from the brain over 120 minutes, consistent with high levels of non-specific binding. Analysis of venous plasma samples indicated that 6-[18-F]-Fluoro-5'-iodo-HEAT decomposed or was metabolised to a lipophilic product as early as four minutes after administration – rendering it unsuitable for PET imaging of brain α1NAR. Subsequently, we synthesized another 8 HEAT analogs and their binding affinities for cloned human α1NARs and other neurotransmitter receptors was performed by the NIMH Psychoactive Drug Screening Pro-gram (PDSP). The HEAT analog exhibing the best profile of α1NAR vs. off-target neurotransmitter receptor binding was 6-Fluoro-5'-Iodo-HEAT, the compound we had previously found to be rapidly metabolised *in vivo*. Based upon these findings, we have chosen to abandon the HEAT structue as a platform for developing α1NAR radiotracers. An extensive review of structure-activity relationships among α1NAR antagonists has identified a small series of molecular scaffolds based on pendent nicotinyl- and uracil-amines, which exhibit sub-nanomolar α1NAR affinity in addition to varying degrees of resistance to metabolism *in vivo*, favorable lipophilicity, and/or tractable routes to radiofluorination (Lopez FJ. Bioorganic Med Chem Lett. 2003; 13:1873–1878; Elworthy TR. J Med Chem 1997; 40:2674-2687; Chern J-W. J Med Chem 1998; 41:3128-3141). These syntheses are in progress. The compounds will be submitted to the NIMH PDSP for assessment of α1NAR vs. off-target receptor binding, with the goal of identifying a promising lead compound for radiolabeling and pilot PET imaging.

15. SUBJECT TERMS: Positron Emission Tomography; PET; Posttraumatic stress disorder; PTSD; norepinephrine; alpha-1 noradrenergic receptor; radiotracer; HEAT; BE-2254

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
U					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	16	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

No vulnerability or diagnostic biomarkers of Combat Stress Symptoms (CSS) or Posttraumatic Stress Disorder (PTSD) with potential for translation to military or Veterans Affairs (VA) clinical settings have yet been identified. However, findings from neurobiological and clinical studies suggest that increased responsivity of central nervous system (CNS) noradrenergic stress-response networks at or downstream of (predominantly post-synaptic) alpha-1 noradrenaline (NA) receptors [α1NAR] may play a crucial role in the development of CSS and PTSD. (1-12) That increased CNS α1NAR responsivity in CSS and PTSD is clinically significant is supported by studies demonstrating that prazosin [a non-sedating drug that blocks excessive stimulation of CNS α1NARs (13)] robustly reduces combat-related nightmares and sleep disturbance and improves overall CSS in active duty and veteran Operation Iraqi Freedom/Operation Enduring Freedom (OIF/OEF) warriors (14) and Vietnam War combat veterans with chronic PTSD. (15-17) These findings suggest that increased CNS α1NAR responsivity may be a vulnerability and/or diagnostic biomarker for CSS and PTSD. At present, interrogation of CNS NA and/or α1NAR responsivity in human subjects is hampered by a lack of minimally invasive assessment methods acceptable for use in clinical (particularly active duty military) populations. The development of radiotracer compounds for PET imaging of CNS α1NAR would open up a completely new avenue of research into biomarkers of CNS NA and/or α1NAR responsivity implicated in the pathogenesis of CSS and PTSD in combat-exposed active duty and veteran warriors. The development and evaluation (in non-human primates) of [18-F]-labeled analogs of 1-(2H)-naphthalenone-3,4-dihydro-2-(((2-p-hydroxyphenyl)-ethylamino)-methyl)-HCl (HEAT), a well characterized α1NAR antagonist compound, as potential radiotracer compounds for PET imaging of α1NARs *in vivo* is the goal of our proposed studies.

BODY

1. Statement of Work Task I. Establish and troubleshoot F-HEAT analog synthesis methods;
2. Statement of Work Task II. Establish and troubleshoot [18F]-HEAT analog radiolabeling methods; &
3. Statement of Work Task IV. [18F]-HEAT analog production for primate PET imaging studies.

In prior work, our laboratory had developed a scheme for the radiosynthesis of 2-[18-F]-1-(1H)-naphthalenone-3,4-dihydro-2-(((2-p-hydroxyphenyl)-ethylamino)-methyl)-HCl (2-[18-F]-HEAT), a prospective alpha-1 noradrenergic receptor (α1NAR) PET radiotracer which exhibited good blood brain barrier (BBB) penetration and regionally selective uptake into macaque brain by PET imaging. However, the synthesis scheme for 2-[18-F]-HEAT required two separations using preparative high performance liquid chromatography (HPLC), resulting in low specific activity at the time of tracer injection, as well as delivery of a significant radiation dose to the chemist's hands. Therefore, in the present work, we have initiated investigations of other HEAT analogs as prospective α1NAR PET radiotracers, including a series of 5′-halogenated analogs for which α1NAR binding affinity is known to be approximately ten-fold greater than HEAT itself.(18) A current listing of prepared compounds appears in **Table 1**. Compound [***#8***] has been radiolabeled with fluorine-18 and preliminary PET imaging studies have been accomplished with non-human primates. All compounds have been characterized by a panel of spectroscopic (i.e., ^{1}H-nuclear magnetic resonance [NMR] and mass spectrometry) and chromatographic (HPLC, radio-HPLC) methods to confirm their identity and specific activity.

Table 1: Current list of test compounds for α1NAR PET tracer development.

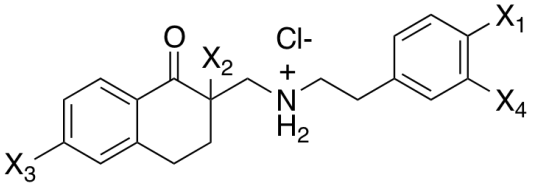

Figure 1a. Basic molecular structure of HEAT Substituents X_1 - X_4 shown in columns 4-7 (below).

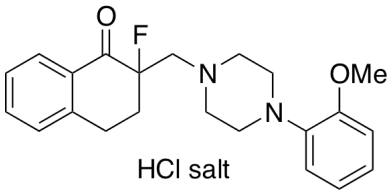

Figure 1b. Compound [#10]: 2-F-2-MMP-HEAT HCl

Compound Lab ID #	**Compound PDSP #**	**Compound Short Name**	**Compound Structural Substituents**			
			X_1	**X_2**	**X_3**	**X_4**
[***#1***]	unassigned	HEAT HCl	OH	H	H	H
[***#2***]	**14485**	2-Fluoro-HEAT HCl	OH	F	H	H
[***#3***]	**22373**	6-Fluoro-HEAT HCl	OH	H	F	H
[***#4***]	**22374**	6-[Me_2]N-HEAT HCl	OH	H	N(Me_2)	H
[***#5***]	**22375**	Desoxy-HEAT HCl	H	H	H	H
[***#6***]	**22376**	5'-Iodo-HEAT HCl	OH	H	H	I
[***#7***]	**22377**	6-Iodo-HEAT HCl	OH	H	I	H
[***#8***]	**22378**	6-Fluoro-5'Iodo-HEAT HCl	OH	H	F	I
[***#9***]	**22379**	5'-Bromo-4'-OMe-HEAT HCl	OMe	H	H	Br
[***#10***]	**22380**	2-Fluoro-2-MMP-HEAT HCl	See Figure 1b (above)			

H: Hydrogen, **C**: Carbon, **Cl**: Chlorine, **OH**: Hydroxy, **F**: Fluorine, **I**: Iodine, **Br**: Bromine, **Me**: Methyl [CH_3] **OMe**: O-methyl [O-CH_3], **N(Me2)**: N-dimethyl [N-$(CH_3)_2$], **MMP**: [(2-methoxyphenyl-1-piperazinyl)-methyl]

Chromatographic (HPLC) analysis of a sub-series of these compounds was explored to provide a rank-index of their inherent lipophilicities, as illustrated in **Figure 2**. Compounds with longer retention times have a stronger lipophilic character. The logD (determined by means of octanol:water partition studies) for 6-Fluoro-5′-Iodo-HEAT [***#8***] was determined to be 3.5, which is high enough for it to exhibit passive diffusion through the BBB. This method will be used, in part, to screen compounds in structure-activity relationships, in order to select an optimized labeled compound with a logD in the range of 1.6-2.3, which is considered ideal for rapid brain uptake and minimal non-specific binding. Our synthesis of [***#2***], [***#3***], [***#8***], [***#9***], and [***#10***]

is particularly significant, since [I-125]-5′-Iodo-HEAT has been the "gold standard" radioligand for performing *in vitro* autoradiographic studies of the neuroanatomical distribution, density, and affinity of α1NARs in the brains of multiple species, including man. The reported K_d value for [I-125]-5′-Iodo-HEAT binding to α1NARs is 0.2 nM and this value will serve as a benchmark against which we will measure the performance of our HEAT analogs. In general, targeted brain radiopharmaceuticals with K_d values in this range have been more successfully developed for PET imaging applications.

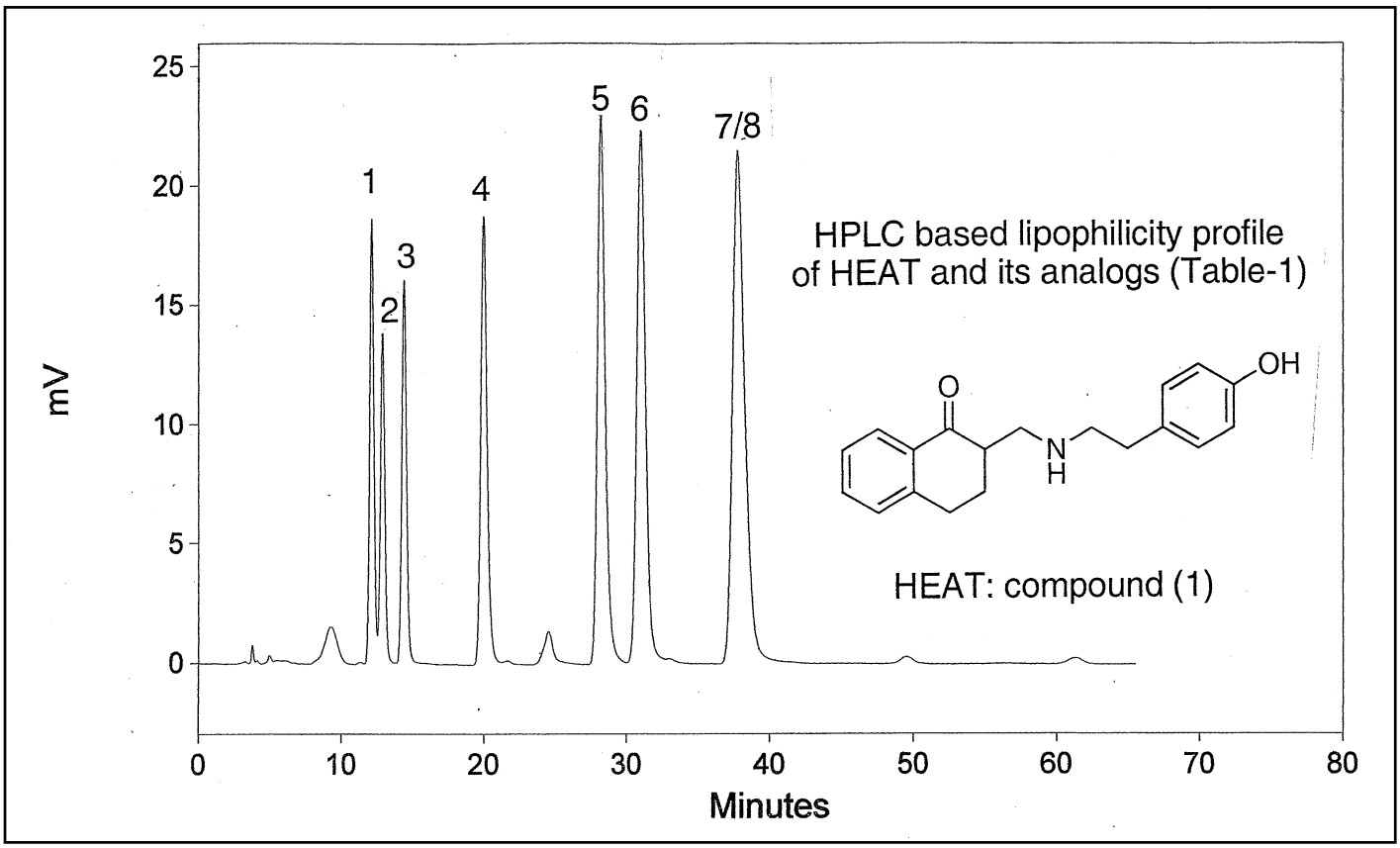

Figure 2: HPLC comparisons of HEAT and selected HEAT-analogs (*Compounds numbered as given in Table 1*). Separation of the calibration mixture components was achieved by differential "mix-mode partitioning" (lipophilic-ion exchange) at pH 4.5, using a C-18 column (Phenomenex Gemini, 5 mm particle, 250 x 2 mm) and a mobile phase of 27% acetonitrile/73% aqueous ion-pairing solution (7 mM $HexSO_3Na$/10 mM NaOAc/10 mM HOAc, pH 4.5) at 0.2 ml/min and 40^oC with UV detection (250 nm).

The α1NAR is known to exhibit enantioselectivity with regard to HEAT-like structural elements. We plan to leverage that fact in compound optimization. All of our HEAT analogs have an asymmetric carbon and it is one of our interests to characterize the preferential stereochemistry of HEAT analogs for α1NARs. As a preliminary approach to this question, we developed a chiral-HPLC method for the separation of the optical isomers of 2-Fluoro-HEAT [**#2**], as illustrated in **Figure 3**. In this example, the absolute stereochemistry is not assigned and the separation of isomers was not optimized. However, we anticipate that this method can be generalized to assay optical purity during preparative chemical resolutions or be adapted for in-line purification of our labeled compounds.

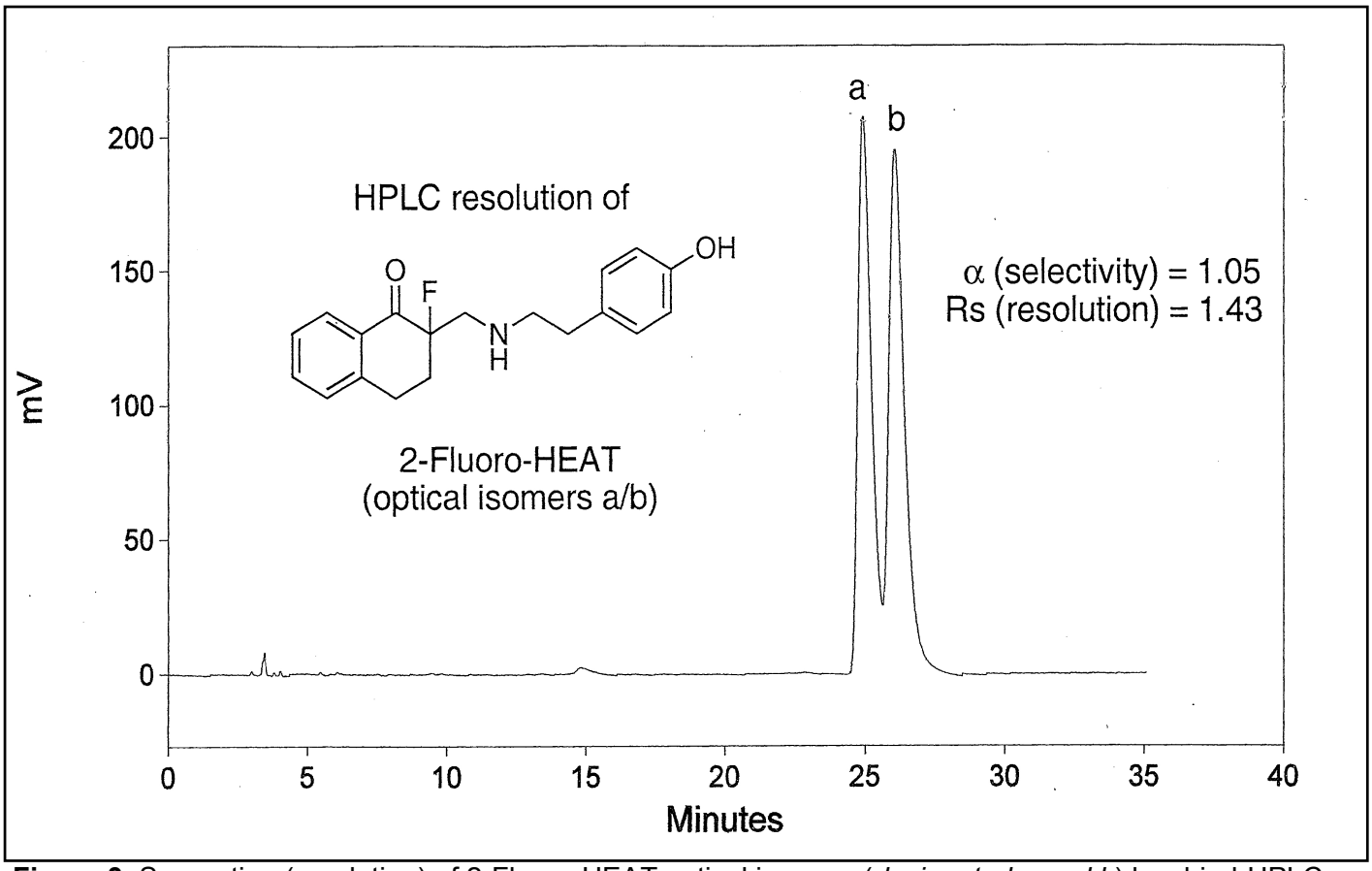

Figure 3: Separation (resolution) of 2-Fluoro-HEAT optical isomers (*designated a and b*) by chiral-HPLC using a silica-immobilized cellulose column (Phenomenex Lux-1, 3 mm particle, 250 x 4.6 mm); and a mobile phase of 10% *i*-PrOH/90% hexane/0.1% diethylamine at 1 ml/min and 40°C with UV detection (250 nm). *Capacity factors*: $k'_{(a)}$ 7.40. $k'_{(b)}$ 7.77.

Progress in radiolabeling HEAT analogs with fluorine-18 is illustrated in **Figures 4** and **5**. We have investigated radiofluorinating the teralone portion of the HEAT molecule at two different positions. Adopting either strategy permits the synthesis of analogs with structural variations within the side-chain tyramine moiety (i.e., the portion of the HEAT molecule with the X_1 and X_4 substituents, as shown in **Table 1**). These radiolabeling methods were not optimized during the first year of funding, as we wished to first determine if the compounds were able to cross the BBB in non-human primates.

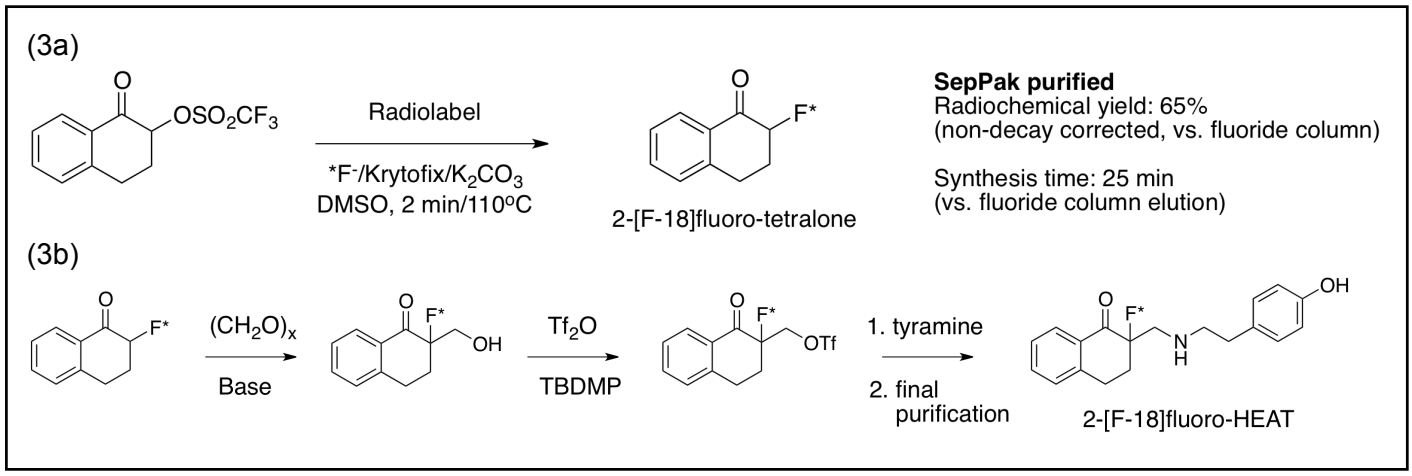

Figure 4: Radiosynthesis of 2-[F-18]Fluoro-teralone, showing: (3a) initial labeling; and (3b) subsequent synthetic conversions.

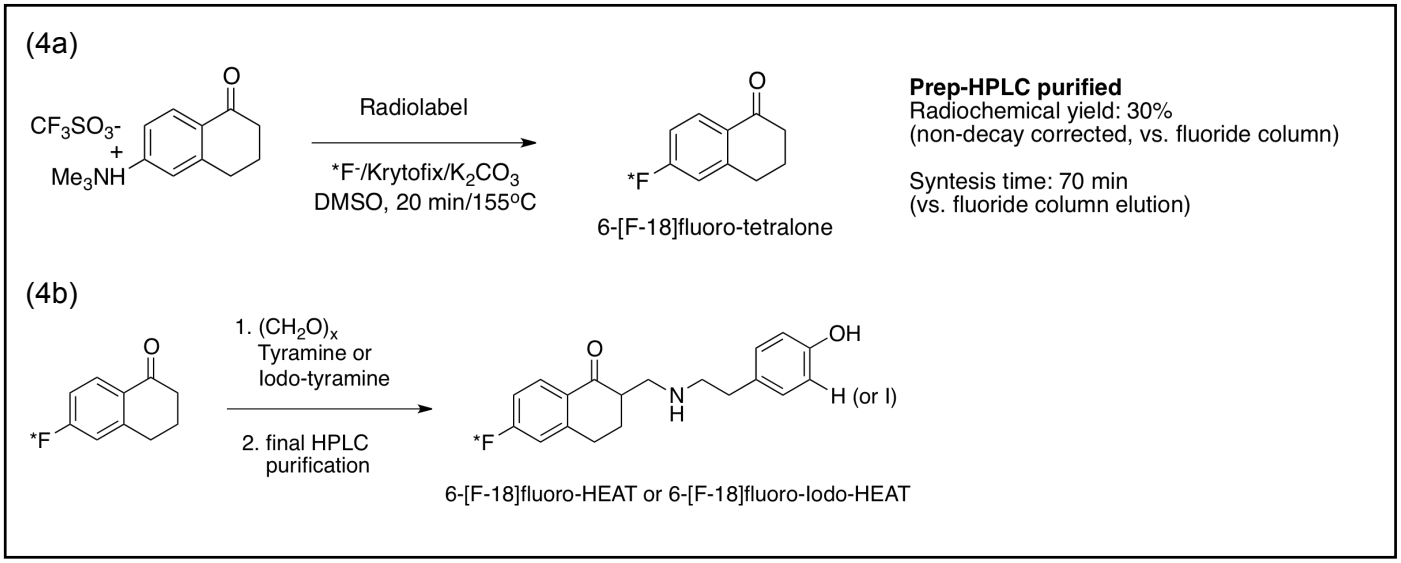

Figure 5: Radiosyntheses of 6-[F-18]fluoro-tetralone and its conversion to 6-[F-18]fluoro-HEAT **[#3]** and 6-[F-18]fluoro-5'-Iodo-HEAT **[#8]**, showing: (4a) initial labeling; and (4b) subsequent synthetic conversion.

4. Statement of Work Task III. F-HEAT analog *in vitro* receptor binding studies performed by National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (PDSP).

Unlabeled forms of compounds **[#2]** through **[#10] (Table 1)** were synthesized and submitted to the PDSP for *in vitro* studies of binding affinities at the following transmitter receptors:

Norepinephrine: α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3
Norepinephrine uptake transporter (NET)
Serotonin: (5-HT)$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_3$, 5-HT$_{5A}$, 5-HT$_6$, and 5-HT$_7$
Serotonin uptake transporter (SERT)
Dopamine: D1, D2, D3, D4, and D5
Dopamine uptake transporter (DAT)
Histamine: H1, H2, and H3
Acetylcholine: M1, M2, M3, M4, and M5
Endogenous opioid: delta, kappa, and mu
Sigma 1 and Sigma 2
Gamma Amino Butyric Acid (GABA) type A
Rat brain benzodiazepine receptor (BZP)

The PDSP *in vitro* binding screen Is a two-stage process (see Roth BL. University of North Carolina at Chapel Hill, National Institute of Mental Health, Psychoactive Drug Screening Program, Assay Protocol Book, at https://pdspdb.unc.edu/html/tutorials/UNC-CH%20Protocol%20Book.pdf, accessed 27 September, 2012). All receptor binding studies are carried out using cloned human receptors transiently or stably transfected in one of several cell lines (e.g., HEK293, COS, CHO, NIH3T3).

Primary binding assays are performed to obtain qualitative estimates of receptor binding affinities. Quadruplicate samples of sample compounds at 10 micromolar (uM; 10^{-6} M) concentration are incubated with crude membrane fractions from cell cultures expressing the cloned human receptor, a radiolabeled "gold standard" compound known to bind to the receptor with high affinity, and a 10 uM concentration of a non-labeled reference compound (also known to bind to the receptor with high affinity) which blocks the receptor and allows calculation of non-specific binding (e.g., to non-receptor proteins, membrane lipids, and plastic surfaces of assay tubes, etc.). The specific radioligands and reference compounds for each of the PDSP receptor assays relevent to the present studies are shown in **Table 2** on page 9. Compounds that inhibit binding of the radiolabeled "gold standard" compound by 50% or more are then subjected to a secondary binding screen.

In the secondary binding screen, the test compound and the reference compound for each receptor are added to the radioligand binding assay in several concentrations (typically 10-12 concentrations between 10 picomolar [pM; 10^{-9} M] and 10 uM) and binding affinities (expressed as the negative log of the compound concentration that inhibits radioligand binding by 50% [IC50]) are calculated using non-linear regression and the Chen-Prusoff equation (19).

Table 2. PDSP *in vitro* Receptor Binding Assays: Neurotransmitters, Receptors, Receptor Subtypes, Radioligands, and Reference Compounds

Transmitter	Receptor	Radioligand	Reference Compound
NOREPINEPHRINE	α1A	[^{3}H]Prazosin	Urapidil
	α1B	[^{3}H]Prazosin	Corynanthine
	α2A	[^{3}H]Clonidine	Oxymetazoline
	α2B	[^{3}H]Clonidine	Prazosin
	α2C	[^{3}H]Clonidine	Prazosin
	β1	[125I]Iodopindolol	Atenolol
	β2	[125I]Iodopindolol	ICI118551
	β3	[125I]Iodopindolol	ICI118551
	NET	[^{3}H]Nisoxetine	Desipramine
SEROTONIN	5-HT$_{1A}$	[^{3}H]8-OH-DPAT	Methysergide
	5-HT$_{1B}$	[^{3}H]GR127543	Ergotamine
	5-HT$_{1D}$	[^{3}H]GR127543	Ergotamine
	5-HT$_{1E}$	[^{3}H]5-HT	5-HT
	5-HT$_{2A}$	[^{3}H]Ketanserin	Chlorpromazine
	5-HT$_{2B}$	[^{3}H]LSD	Methysergide
	5-HT$_{2C}$	[^{3}H]Mesulergine	Chlorpromazine
	5-HT$_3$	[^{3}H]LY278584	LY278584
	5-HT$_{5a}$	[^{3}H]LSD	Ergotamine
	5-HT$_6$	[^{3}H]LSD	Chlorpromazine
	5-HT$_7$	[^{3}H]LSD	Chlorpromazine
	SERT	[^{3}H]Citalopram	Amitriptyline
DOPAMINE	D1	[^{3}H]SCH233930	SKF38393
	D2	[^{3}H]N-methylspiperone	Haloperidol
	D3	[^{3}H]N-methylspiperone	Chlorpromazine
	D4	[^{3}H]N-methylspiperone	Chlorpromazine
	D5	[^{3}H]SCH233930	SKF38393
	DAT	[^{3}H]WIN35428	GBR12909
HISTAMINE	H1	[^{3}H]Pyrilamine	Chlorpheniramine
	H2	[^{3}H]Tiotidine	Cimetidine
	H3	[^{3}H]alpha-methylhistamine	Histamine
	H4	[^{3}H]Histamine	Clozapine
ACETYLCHOLINE	M1	[^{3}H]QNB	Atropine
	M2	[^{3}H]QNB	Atropine
	M3	[^{3}H]QNB	Atropine
	M4	[^{3}H]QNB	Atropine
	M5	[^{3}H]QNB	Atropine
OPIOIDS	Delta Opioid	[^{3}H]DADLE	Naltrindole
	Kappa Opioid	[^{3}H]U69593	Salvinorin A
	Mu Opioid	[^{3}H]DAMGO	DAMGO
UNCLEAR	Sigma 1	[3H]Pentazocine	Haloperidol
	Sigma 2	[3H]DTG	Haloperidol
GABA	GABAA	[^{3}H]Muscimol	GABA
Benzodiazepine	BZP	[^{3}H]Flunitrazepam	Diazepam

Review of the receptor binding data available from the PDSP web site as of 26 September, 2012 (**Table 3**) showed that the "gold standard" α1NAR ligand (5'-Iodo-HEAT) exhibited unexpected high binding affinity (defined as less than 10 nM and/or less than 10 times its Ki for α1NARs) at several off-target receptors, including α_{2A}NAR, α_{2B}NAR, α_{2C}NAR, 5-HT_{1A}, Dopamine D3, and Sigma 2. In addition, of the 10 HEAT analogs submitted for screening, the analog exhibiting the highest α1NAR binding affinities (1.7, 0.7, and 1.0 nM at, respectively, α_{1A}, α_{1B}, and α_{1D}NARs) and the least high-affinity binding to off-target receptors (4.8 nM at α_{2B}NAR, 1.9 nM at α_{2C}NAR, 17.0 nM at 5-HT_{1A}, 7.1 nM at Dopamine D3, and 1.7 nM at Sigma 2 receptors) was 6-F-5'-Iodo-HEAT (Compound **[#8]**. PDSP #22378). Unfortunately, we had previously radiofluorinated compound **[#8]**, investigated its uptake into nonhuman primate brain by means of PET imaging, and found that exhibited extremely slow washout from the brain due to high levels of non-specific bindings that analysis of plasma samples suggested was due to rapid metabolism to radiolabeled metabolites. These findings were reported in this laboratories annual report dated 9 November, 2011, and are included here for reference in Section 6 (Statement of Work Task VI. Primate PET studies of [18F]-HEAT analog brain uptake and α1 receptor binding selectivity [prazosin reversibility]) and Figure 6.

Table 3. ***In vitro*** **receptor binging affinities (Ki values [nM]) for HEAT analog compounds at cloned human receptors transiently or stably expressed in HEK293, COS, CHO, or NIH3T3 cell lines, as performed by NIMH Psychoactive Drug Screening Program (PDSP). Data accessed 26 September, 2012.**

Compound # - Petrie Lab:		6	2	3	4	5	7	8	9	10
Compound # - PDSP:		22376	14485	22373	22374	22375	22377	22378	22379	22380
Compound Name:		5'-I-HEAT	2-F-HEAT	6-F-HEAT	6-(Me2)N-HEAT	Desoxy-HEAT	6-I-HEAT	6-F-5'-I-HEAT	5'-Br-4'-OMe-HEAT	2-F-2-MPP-HEAT
TRANSMITTER	RECEPTOR									
NOREPINEPHRINE	Alpha 1A	**1.9**	19.6	4.5	25.0	124.0	9.8	***1.7***	13.1	2413.0
	Alpha 1B	**1.4**	28.4	2.0	71.5	75.0	15.0	***0.7***	4.3	NB
	Alpha 1D	**1.4**	25.1	2.3	13.0	98.0	3.8	***1.0***	8.7	2840.0
	Alpha 2A	**6.0**	67.2	8.8	8.9	30.0	16.0	11.5	12.5	NB
	Alpha 2B	**1.4**	59.9	4.6	18.0	16.0	6.3	***4.8***	16.0	NB
	Alpha 2C	**2.1**	57.6	6.9	21.0	12.8	3.7	***1.9***	2.6	35.0
	Beta 1		NB							
	Beta 2		NB							NB
	Beta 3		NB					NB		NB
	NET	603.0	NB	1981.0	338.0	NB	556.0	NB	341.0	NB
SEROTONIN	5-HT1A	**5.6**	245.0	22.0	111.0	7.3	31.0	***17.0***	4.2	294.0
	5-HT1B	94.0	329.0	221.0	878.0	RPT	433.0	327.0	251.0	NB
	5-HT1D	RPT	18.6	33.0	351.0	65.0	54.0	80.0	59.0	1378.0
	5-ht1e	NB	NB	NB	NB	NB	NB	NB	NB	NB
	5-HT2A	281.0	NB	NB	2556.0	NB	1499.0	218.0	358.0	NB
	5-HT2B	21.0	216.2	86.0	14.0	66.0	53.0	46.0	19.0	105.0
	5-HT2C	61.0	NB	NB	475.0	NB	>10,000	NB	711.0	NB
	5-HT3	NB	NB	NB	NB	NB	NB	NB	NB	NB
	5-HT5a	45.5	1106.5	193.5	2349.5	116.0	1019.5	132.5	111.5	NB
	5-HT6	554.0	NB	1589.0	99.0	NB	227.0	924.0	NB	>10,000
	5-HT7	**8.1**	662.2	99.0	50.0	84.0	69.0	14.0	11.0	1352.0
	SERT	1967.0	9020.0	893.0	817.0	3664.0	769.0	953.0	1784.0	4176.0
DOPAMINE	D1	NB	NB	NB	124.0	NB	NB	NB	NB	NB
	D2	28.0	NB	>10,000	1600.0	245.0	34.0	41.0	155.0	4365.0
	D3	**4.7**	NT	7.1	6.2	40.0	5.9	***7.1***	17.0	NB
	D4	246.0	NT	1181.0	221.0	784.0	48.0	284.0	276.0	730.0
	D5	NB	NT	NB	NB	NB	2354.0	NB	RPT	NB
	DAT		NB							
HISTAMINE	H1		NT	NB	NB	NB	NB			NB
	H2		NT	NB		NB				NB
	H3	NB	NT	NB		NB	NB	NB	NB	NB
ACETYLCHOLINE	M1	NB	NT	NB	NB	NB	NB	3448.0	NB	NB
	M2	NB	NT	78.0	NB	NB	790.0	RPT	6802.5	7876.5
	M3	NB	NT	NB	NB	NB	NB	NB	NB	NB
	M4	NB	NT	NB	NB	NB	NB	NB	NB	NB
	M5	NB	NT	NB	NB	NB	NB	NB	NB	
OPIOIDS	MOR	NB	NT	NB	NB	NB	552.0	NB	NB	NB
	KOR	NB	NT	NB	NB	NB	NB	NB	NB	NB
	DOR	NB	NT	NB	NB	NB	NB			
SIGMA	SIGMA 1		NT					49.0	26.0	NB
	SIGMA 2	**4.2**	NT	2.9	3.1	13.0	0.9	***1.7***	2.4	154.0
GABA A	GABA A	NB	NT				NB	NB	NB	NB
BENZODIAZEPINE	BZP (rat)	NB	NT	NB	NB	NB	NB	NB	NB	NB

Empty Cell: (Assay requested but not yet performed). **NB:** (no significant binding in primary assay screen). **NT:** (compound not tested at this receptor).
RPT (assay variability > 20% - assay being repeated)

COMMENTS:
Row for 5'- Iodo-HEAT is shaded for emphasis because it represents the "gold standard" radioligand for α1NAR binding.
Bolded values indicate 5'-Iodo-HEAT α1NAR binding affinity <10nM or 5'-Iodo-HEAT off-target binding affinity <10 times α1NAR Ki
Bolded and italicized values indicate 6-F-5'-Iodo-HEAT α1NAR binding affinity <10nM or 6-F-5'-Iodo-HEAT off-target receptor binding affinity <10 times α1NAR Ki

5. Statement of Work Task V. Submit IACUC application for primate PET imaging protocols.

The animal use protocol associated with this contract was first approved by the University of Washington (UW) Institutional Animal Care and Use Committee (IACUC) on 15 October, 2010 and by the U.S. Army Animal Care and Use Review Office (ACURO) on 15 December, 2010. Therefore, first approval to begin the studies described in the contract did not occur until three and one-half months after the official award date of 01 September, 2010. The currently approved IACUC protocol associated with this project expires on 14 October, 2012. The application for annual renewal of the animal use protocol has been submitted to the University of Washington IACUC.

6. Statement of Work Task VI. Primate PET studies of [18F]-HEAT analog brain uptake and α1 receptor binding selectivity (prazosin reversibility).

6-[18-F]-Fluoro-5'-Iodo-HEAT was synthesized in good radiochemical yield and a bolus injection of 5 mCi was administered to an adult (~6 kg) male macaque under sevoflurane anesthesia. PET images were acquired in a Hamamatsu SHR-7700 primate PET scanner over 180 minutes. Time-activity curves were calculated for a volume-of-interest in the cortical region of the brain. Distribution volume images were created with the use of a reference region in a non-cortical brain region, using NEUROSTAT (University of Washington) (***See Figure 6 on Page 12***).

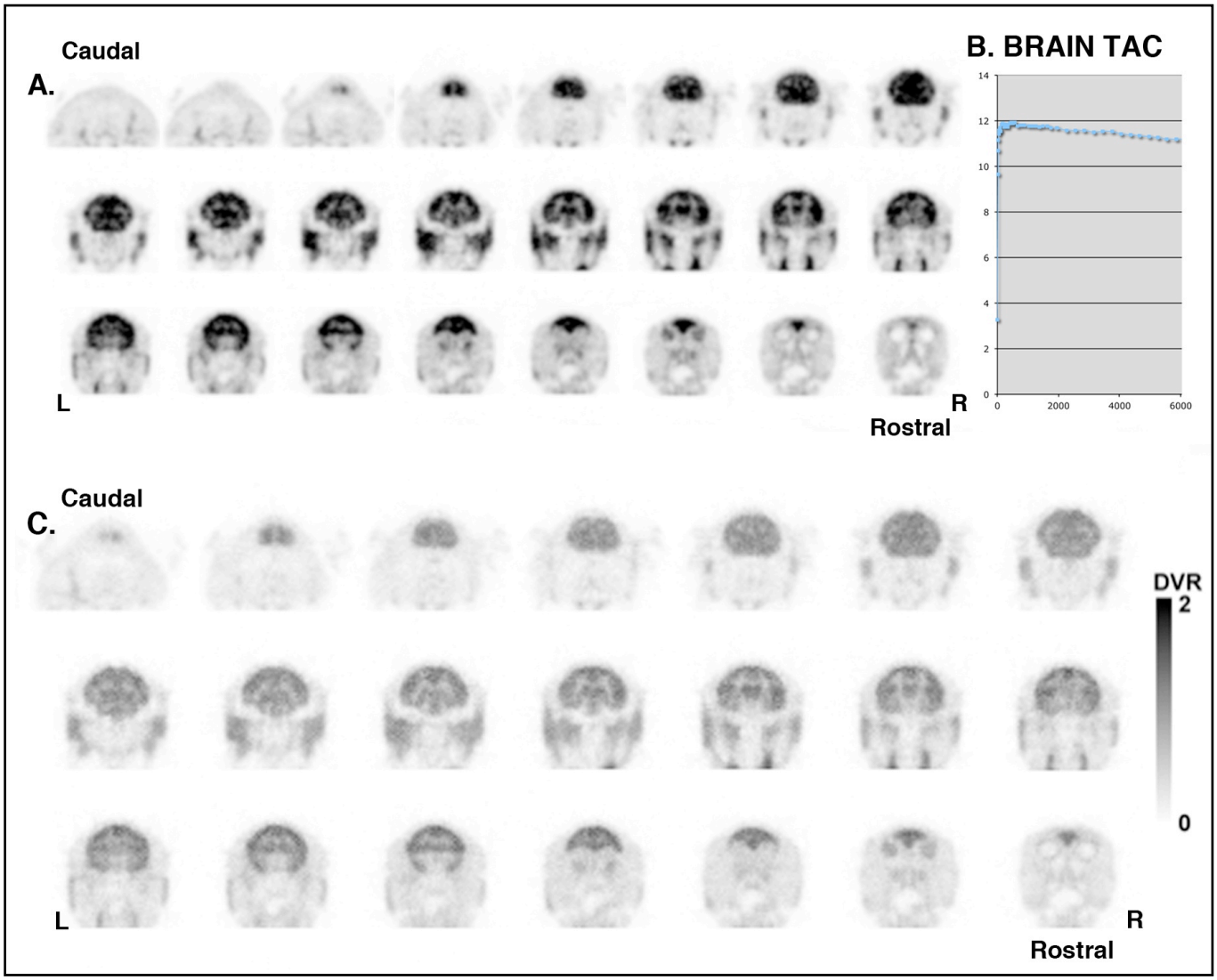

Figure 6. PET images of 6-[18-F]-Fluoro-5′-Iodo-HEAT uptake into the brain of an adult male macaque. (A) Time-averaged images of brain uptake in caudal to rostral image frames at steady state. (B) Time-activity curves of tracer uptake in a cortical brain region. (C) Distribution Volume images generated using non-cortical reference region and Logan Plot equation. Bar shows gray scale vs. Distribution Volume values.

Although Figure 6 shows that 6-[18-F]-Fluoro-5′-Iodo-HEAT and/or one of its metabolites crosses the BBB, the time-activity curve indicates that the efflux of radioactivity from the brain is extremely slow, suggesting the presence of large amounts of non-specific binding. When plasma samples were applied to Oasis MCX (Mixed-mode cation exchange sorbent for bases; Waters Corporation, Milford, MA) separation columns, a significant fraction of radioactivity in samples collected at four minutes after tracer administration could be eluted with 50% acetonitrile, suggesting the presence of a highly lipophilic metabolite present immediately after tracer administration. The remainder of the radioactivity could not be eluted from the column, even with high concentrations of calcium ion or with cold 6-Fluoro-5′-Iodo-HEAT, so confirmation that the non-metabolized radiotracer was adsorbed to the column could not be confirmed.

The logD7.4 of authentic 6-[18-F]-Fluoro-5′-Iodo-HEAT, determined by means of octanol:water partition studies, was greater than 3.5, confirming the highly lipophilic nature of the tracer. These results suggested that: 1) 6-[18-F]-Fluoro-5′-Iodo-HEAT was rapidly metabolized to a lipophilic metabolite soon after administration; and 2) that 6-[18-F]-Fluoro-5′-Iodo-HEAT and/or one of its lipophilic metabolites was able to cross the BBB into the brain, but then bound non-specifically to brain lipids, as evidenced by the extremely slow rate of efflux.

7. Statement of Work X. Data cleaning, double data entry, interim data analysis.

Interim data analyses suggest that [18F]-Fluoro-5'-iodo-HEAT decomposes or is metabolised to a lipophilic product as early as four minutes after administration, rendering the compound unsuitable for PET imaging of CNS α1NAR's.

KEY RESEARCH ACCOMPLISHMENTS

- ▶ Synthesis of:
  - •2-Fluoro-HEAT HCl
  - •6-Fluoro-HEAT HCl
  - •6-[Me2]N-HEAT HCl
  - •Desoxy-HEAT HCl
  - •5'-Iodo-HEAT HCl
  - •6-Iodo-HEAT HCl
  - •6-Fluoro-5'-Iodo-HEAT HCl
  - •5'-Bromo-4'-OMe-HEAT HCl
  - •2-Fluoro-2-MMP-HEAT HCl
- ▶ Leveraging the services of the NIMH PDSP to characterize *in vitro* binding of ten HEAT analogs to membraine preparations of HEK293, COS, CHO, and/or NIH3T3 cell lines transiently or stably expressing the following cloned human neurotransmitter receptors:
  - •Norepinephrine: α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3
  - •Norepinephrine uptake transporter (NET)
  - •Serotonin: (5-HT)$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_{3}$, 5-HT$_{5A}$, 5-HT$_{6}$, and 5-HT$_{7}$
  - •Serotonin uptake transporter (SERT)
  - •Dopamine: D1, D2, D3, D4, and D5
  - •Dopamine uptake transporter (DAT)
  - •Histamine: H1, H2, and H3
  - •Acetylcholine: M1, M2, M3, M4, and M5
  - •Endogenous opioid: delta, kappa, and mu
  - •Sigma 1 and Sigma 2
  - •Gamma Amino Butyric Acid (GABA) type A
- ▶ Radiofluorination of 6-[18-F]-Fluoro-5′-Iodo-HEAT to high specific activity.
- ▶ Demonstration of 6-[18-F]-Fluoro-5′-Iodo-HEAT BBB penetration and uptake into macaque brain by means of PET imaging.
- ▶ Demonstration of rapid 6-[18-F]-Fluoro-5′-Iodo-HEAT metabolism in Macaque venous plasma.

REPORTABLE OUTCOMES

None to date.

CONCLUSION

Because 5'-Iodo-HEAT is the "gold standard" α1NAR radioligand for *in vitro* autoradiography, with a Ki of 0.2 nM, it seemed a promising lead compound for development of a radiotracer for *in vivo* PET imaging of brain α1NARs. Our first HEAT analog was fluorinated at the 2-position on the tetralone ring, in hopes that this would limit metabolism into tyramine and tetralone via a reverse Mannich-type reaction. Although 2-[F-18]-Fluoro-HEAT was able to be radiofluorinated and exhibited favorable brain uptake on PET imaging, the length of the radiosynthesis scheme (requiring two chromatographic purificatioin steps) and the high radiatioin dose delivered to the radiochemist precluded its further development.

Our second HEAT analog, 6-[F-18]-Fluoro-5'Iodo-HEAT, was able to be radiofluorinated within a reasonable time span, resulting in increased specific activity and acceptable radiation delivery to the radiochemist. It also demonstrated good BBB penetration in a Macaque PET imaging study. However, 6-[F-18]-Fluoro-5'Iodo-HEAT proved vulnerable to rapid metabolism in vivo, producing radiolabeled metabolites and unacceptable levels of

non-specific binding.

We then synthesized another eight HEAT analogs (Compounds **[#3]**, [**#4]**, **[#5]**, **[#6]**, **[#7]**, **[#9]**, and **[#10]**) in an effort to identify promising leads that could be rapidly radiofluorinated, exhibit physicochemical properties predictive of good BBB penetration, and would be resistant to metabolic degredation *in vivo*. The results of receptor binding studies of these compounds, carried out by the NIMH PDSP, have become available during the last month (capacity limitations at PDSP had resulted in lower priority being assigned to compounds submitted by studies that were not funded by the National Institutes of Health, but we were able to successfully appeal for higher priority on the basis of being funded by the Department of Defense). Unfortunately, these data indicate that the HEAT analog exhibiting the most promising profile of α1NAR vs. off-target neurotransmitter receptor binding was 6-Fluoro-5'-Iodo-HEAT, the compound we had previously found to be rapidly metabolised *in vivo*.

As a result of our experiences with 2-[F-18]-Fluoro-HEAT and 6-[F-18]-Fluoro-5'-Iodo-HEAT and in light of the receptor binding profiles of our other recently synthesized HEAT analogs (as reviewed earlier in this report), we have chosen to abandon the HEAT structue as a platform for developing α1NAR radiotracer compounds. Based upon an extensive review of structure-activity relationships among α1NAR antagonists, we plan to devote the remaining period of funding to synthesize and screen (via the PDSP) a small series of molecular scaffolds based on pendent nicotinyl- and uracil-amines, which previously published studies have shown to exhibit sub-nanomolar α1NAR affinity (20-22) and which are shown below in **Figure 7**.

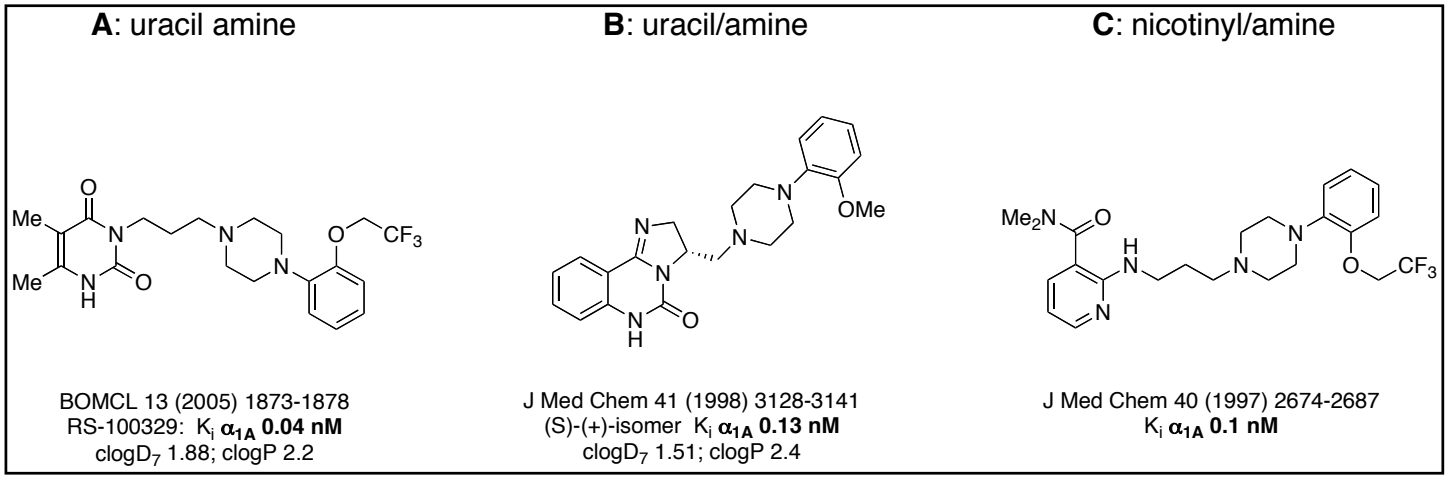

Figure 7. Structures, bibliographic citations, α1NAR binding affinities (Ki values – nM), and other physico-chemical characteristics of molecular scaffolds based on pendent uracil-amine scaffolds (A and B) and a nicotinyl-amine scaffold (C). Structures and data from references 20-22.

The A-type scaffold was selected based upon previous findings suggesting that it would exhibit resistance to metabolism *in vivo*. The B-type scaffold was selected based on its having the most favorable lipophilicity (clogD). Finally, the C-type scaffold was selected based upon previous findings suggesting that it would present the least difficulty with radiolabeling. These syntheses are in progress, with the hope that binding results will identify one or more compounds with sufficiently promising α1NAR vs. off-target binding, resitance to metabolism *in vivo*, lipophilicity, and sufficiently simple and rapid radiolabeling to justify subsequent radiofluorination and *in vivo* Macaque PET imaging studies.

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APPENDIX - PERSONNEL SUPPORTED BY AWARD W81XWH-10-1-0735, Mod P00002

1. Eric C. Petrie, MD, Principal Investigator; 25% effort. (Note: Dr. Petrie's effort on the project is supported by the Department of Veterans Affairs).
2. Satoshi Minoshima, MD, PhD, Co-Investigator, 10% effort.
3. Donna J. Cross, PhD, Co-Investigator, 15% effort.
4. John Grierson, PhD, Research Scientist, 65% effort.
5. Greg Gawin, BA, Research Scientist, 10% effort. (Note: Mr. Gawin replaces Barbara Lewellen, who retired between the date the grant was submitted and the date funds were released).