Design, Synthesis and Preclinical Characterization of the Selective Androgen Receptor Modulator (SARM) RAD-140

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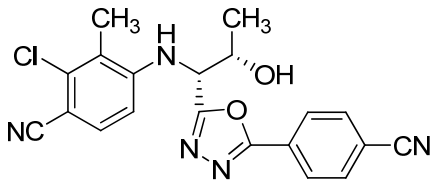
Supplementary Materials

Design, Synthesis and Preclinical Characterization of the Selective Androgen Receptor Modulator (SARM) RAD-140

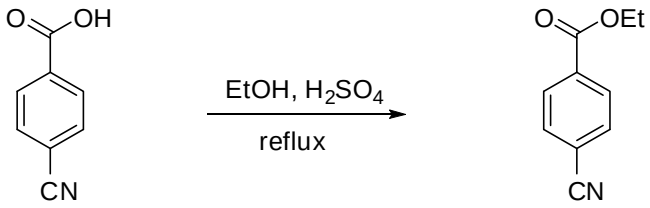
Preparation of compound **7** (**RAD140**)

2-chloro-4-((1*R*,2*S*)-1-(5-(4-cyanophenyl)-1,3,4-oxadiazol-2-yl)-2-hydroxypropylamino)-3-methylbenzonitrile



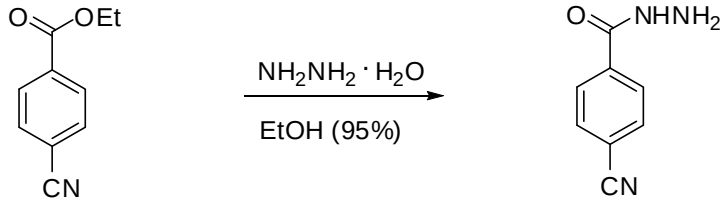
General Methods. All solvents were commercially available and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel plates (60 F254; EMD Chemicals) which were visualized using ultraviolet light, iodine vapor, or vanillin stain. Flash chromatography was performed on silica gel (230-400 mesh, Silicycle) using commercially available high purity solvents. ^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$, MeOH-d_6, DMSO-d_6, or acetone-d_6 using either a Varian 300 MHz, Varian Unity 400 MHz spectrometer or a Varian Unity 500 MHz spectrometer. Proton chemical shifts (δ) are relative to the residual solvent peaks for each deuterated solvent and expressed in ppm. Coupling constants (J) are expressed in hertz. Mass spectra were obtained LC-MS on an Agilent instrument. All chemical reagents are commercially available and were used without further purification unless stated otherwise. Yields reported are unoptimized.

Ethyl 4-cyanobenzoate



To a 250 mL round bottom flask charged with 4-cyanobenzoic acid (19.36 g, 0.13 mol) in abs. ethanol (100 mL) was added concentrated sulfuric acid (3 mL). This mixture was heated to reflux for a period of 28 h and then allowed to cool to ambient temperature overnight. The solvent was removed under reduced pressure and the resulting off-white residue was dissolved in diethyl ether (500 mL). The resulting solution was washed with saturated sodium bicarbonate solution (5 x 100 mL) followed by brine (1 x 100 mL), and dried over magnesium sulfate. The mixture was filtered using a buchner funnel and the filtrate was concentrated to afford an off-white solid as a crude product. The crude product was recrystallized from 95% ethanol (50 mL) to get 16.85 g of the pure product as white crystals (yield 73%). ^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm) 8.14 (AA'XX', J = 8.6 Hz, 2H), 7.74 (AA'XX', J = 8.6 Hz, 2H), 4.42 (q, J = 7.1 Hz, 2H), 1.41 (t, J = 7.1 Hz, 3H).

4-Cyanobenzohydrazide

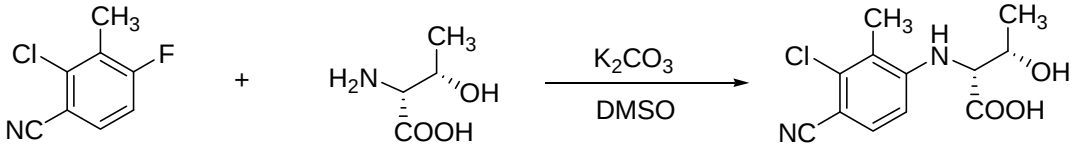


To a 250 mL round bottom flask charged with ethyl 4-cyanobenzoate (16.85 g, 96.2 mmol) in 95% ethanol (75 mL) was added hydrazine mono-hydrate (18.2 mL, 64% solution, 240 mmol). This mixture was heated to reflux for a period of 4.5 h, and then allowed to cool to ambient temperature over a 16 h period. The solvent was removed under reduced pressure to get the crude product as a yellow solid. Ice-cold water (150 mL) was added to the yellow residue and mechanically stirred for ~30 minutes. The light yellow solid was filtered using a Buchner funnel and washed with additional

cold water (50 mL). The solid was then dried under high vacuum to yield the title compound as a light yellow solid (13.98 g, 90%). ^{1}H NMR (400 MHz, DMSO-d_6, δ in ppm) 10.05 (br s, 1H), 7.95 (AA'XX', J = 8.5 Hz, 4H), 4.61 (br s, 2H).

Intermediate **9**

(2*R*,3*S*)-2-(3-chloro-4-cyano-2-methylphenylamino)-3-hydroxybutanoic acid

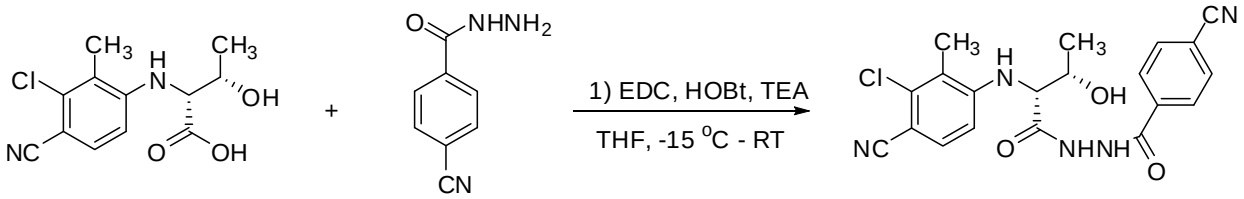


D-Threonine

2-chloro-4-fluoro-3-methylbenzonitrile (CAS 796600-15-2, 45 g, 265.4 mmol) was mixed together with H-D-Thr-OH (37.92 g, 318.4 mmol) in DMSO (250 mL). K_2CO_3 (73.35 g, 530.7 mmol) was added to the reaction mixture and the reaction mixture stirred at 75 ^{o}C for 24 h. The reaction mixture was cooled to room temperature and poured slowly into a 10% citric acid solution and stirred for 10 min at room temperature. The solution was extracted with EtOAc several times to get the crude product. The crude product was chromatographed on silica gel with a gradient of hexanes/EtOAc and then with EtOAc, 100% to get the purified final product (39.0 g, 54%)^{1}H NMR(500 MHz, Acetone-d_6, δ in ppm) 7.49 (d, J = 9 Hz, 1H), 6.70 (d, J = 9 Hz, 1H), 5.38 (d, J = 10 Hz, 1H), 4.47 (d, J = 6 Hz, 1H), 4.25 (m, 1H), 2.34 (s, 3H), 1.33 (d, J = 6 Hz, 3H); MS-API (pos) 269.1 ($M+H^+$).

Intermediate **10**

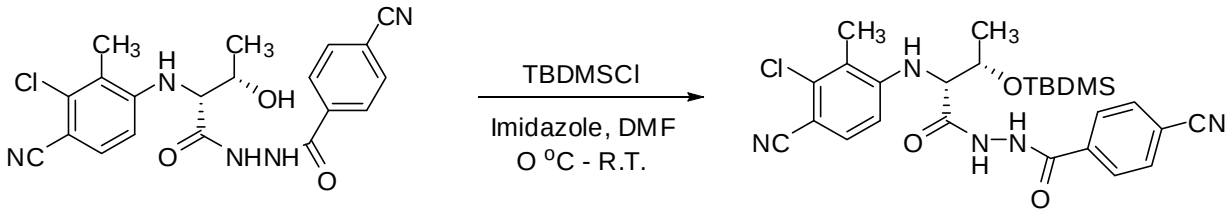
N'-((2*R*,3*S*)-2-(3-chloro-4-cyano-2-methylphenylamino)-3-hydroxybutanoyl)-4-cyanobenzohydrazide



(2*R*,3*S*)-2-(3-chloro-4-cyano-2-methylphenylamino)-3-hydroxybutanoic acid **9** (10.03 g, 37.3 mmol) and 4-cyano-benzohydrazide (6.02 g, 37.3 mmol) were mixed together in approximately 200mL THF and cooled to -15°C under a N_2 atmosphere. To the pre-cooled solution was added hydroxybenzotriazole (HOBT) (5 g, 37 mmol), triethylamine (6 g, 59 mmol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI) (11 g, 56 mmol). The reaction was allowed to stir at approximately 1 hour and then allowed to warm to ambient temperature and stirred overnight. The urea was filtered off and the solution was washed with water, 5% citric acid followed by 5% $NaHCO_3$ to provide the crude hydrazide prouct the crude product was purified by boiling in chloroform (100 mL) followed by cooling to 0 °C and filtration to yield product as a light yellow solid (12.05 g, 78%). ^{1}H NMR (400 MHz, acetone-d_6, δ in ppm) 9.81 (br s, 2H), 8.08 (AA'XX', *J* = 8.6 Hz, 2H), 7.91 (AA'XX', *J* = 8.7 Hz, 2H), 7.51 (d, *J* = 8.7 Hz, 1H), 6.69 (d, *J* = 8.8 Hz, 1H), 5.57 (d, *J* = 7.0 Hz, 1H), 4.40 (m, 1H), 2.34 (dd, *J* = 3.6, 6.9 Hz, 1H), 2.34 (s, 3H), 1.35 (d, *J* = 6.4 Hz, 3H); MS-API (pos) 412.1 ($M+H^+$).

Intermediate **10a**

N'-((2*R*,3*S*)-3-(tert-butyldimethylsilyloxy)-2-(3-chloro-4-cyano-2-methylphenyl-amino)butanoyl)-4-cyanobenzohydrazide

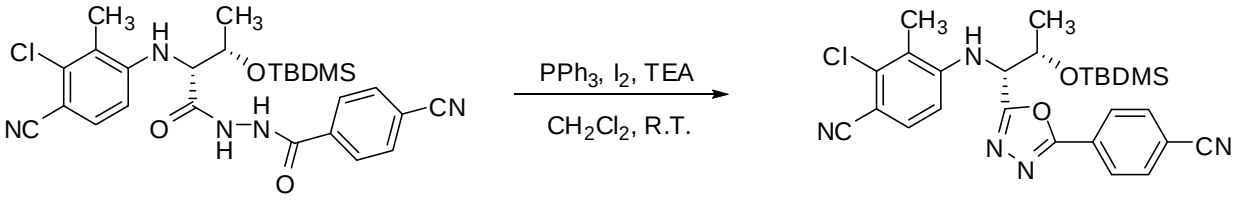


N'-((2*R*,3*S*)-2-(3-chloro-4-cyano-2-methylphenylamino)-3-hydroxybutanoyl)-4-cyano benzohydrazide (intermediate **10**) (12.1 g, 29.3 mmol) in DMF under N_2 was cooled to 0 °C and treated with TBDMSCl (13.2 g, 88.0 mmol) and imidazole (68.1 g, 146.0 mmol) . The reaction was slowly allowed to ambient temperature and allowed to stir overnight. Purification of the compound was accomplished by pouring the reaction mixture into ice-cold water and filtering off the solid precipitate. This was taken up in methylene chloride (400 mL) and sequentially washed with water (2 x 100 mL) and brine (100 mL). This was dried over sodium sulfate, filtered and concentrated down to roughly 100 mL. Hexanes were then added producing a white solid. This was filtered out and washed with hexanes to give the product as a white solid (12.92 g, 84%). ^{1}H NMR (400 MHz, $CDCl_3$, δ in

ppm) 9.32 (m, 2H), 7.89 (AA'XX', *J* = 8.4 Hz, 2H), 7.71 (AA'XX', *J* = 8.6 Hz, 2H), 7.37 (d, *J* = 8.4 Hz, 1H), 6.47 (d, *J* = 8.8 Hz, 1H), 5.37 (d, *J* = 6.2 Hz, 1H), 4.49 (m, 1H), 3.99 (m, 1H), 2.33 (s, 3H), 1.25 (d, *J* = 6.2 Hz, 3H), 0.92 (s, 9H), 0.14 (s, 3H), 0.09 (s, 3H); MS-API (pos) 526.2 ($M+H^+$).

Intermediate **10b**

4-((1*R*,2*S*)-2-(tert-butyldimethylsilyloxy)-1-(5-(4-cyanophenyl)-1,3,4-oxadiazol-2-yl)propylamino)-2-chloro-3-methylbenzonitrile

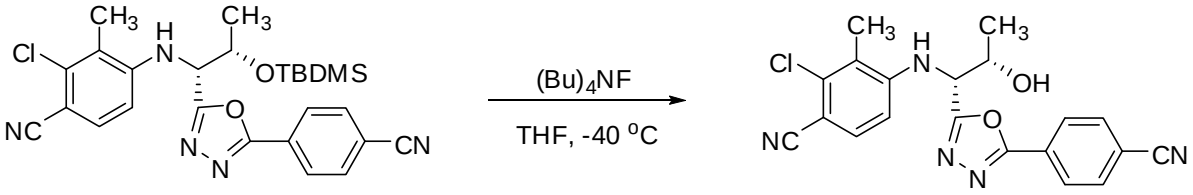


To a 3-neck 1 L round bottom flask equipped with a mechanical stirrer, nitrogen inlet and septum was added triphenylphosphine (12.3 g, 47.0 mmol) under an atmosphere of nitrogen. To the flask was added methylene chloride (500 mL), iodine crystals (11.91 g, 47.0 mmol) and the stirring was started. This reaction mixture was allowed to stir at ambient temperature for a period of 10 minutes and then cooled to 0 °C. Triethylamine (13.1 mL, 94 mmol) was then slowly added to this reaction (**CAUTION**: Exothermic). This mixture was allowed to stir at ambient temperature for an additional 10 minutes. At this point, the reaction mixture became a thick slurry. N'-((2*R*,3*S*)-3-(tert-butyldimethylsilyloxy)-2-(3-chloro-4-cyano-2-methylphenyl-amino) butanoyl)-4-cyanobenzohydrazide (12.35 g, 23.5 mmol) was dissolved in methylene chloride (200 mL) in a separate flask and slowly added to the stirred reaction mixture. After 10 minutes of stirring, the progress of the reaction was monitored by TLC (Solvent system, 1:1 EtOAc:hexanes). The reaction was then quenched by slow addition of 10% sodiumthiosulfate/water (500 mL). The organic layer was then isolated and washed with additional 10% sodium thiosulfate/water (1 x 200 mL), brine (1 x 200 mL) and then dried with sodium sulfate. The solution was then filtered and concentrated under reduced pressure to get a brown oil/solid. The crude product was purified via silica gel chromatography eluting with 25% ethylacetate in hexanes to provide the title compound as a white solid (9.98 g, 84%):. ^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm) (AA'XX', *J* = 8.8 Hz, 2H), 7.79 (AA'XX', *J* = 8.8 Hz, 2H), 7.33 (d, *J* = 8.7 Hz, 1H), 6.44 (d, *J* = 8.7 Hz, 1H), 5.35 (d, *J* = 8.8 Hz, 1H),

4.82 (dd, *J* = 2.0, 8.7 Hz, 1H), 3.54 (m, 1H), 2.37 (s, 3H), 1.42 (d, *J* = 6.2 Hz, 3H), 0.85 (s, 9H), 0.07 (s, 3H), -0.22 (s, 3H); MS-API (pos) 508.2 ($M+H^+$).

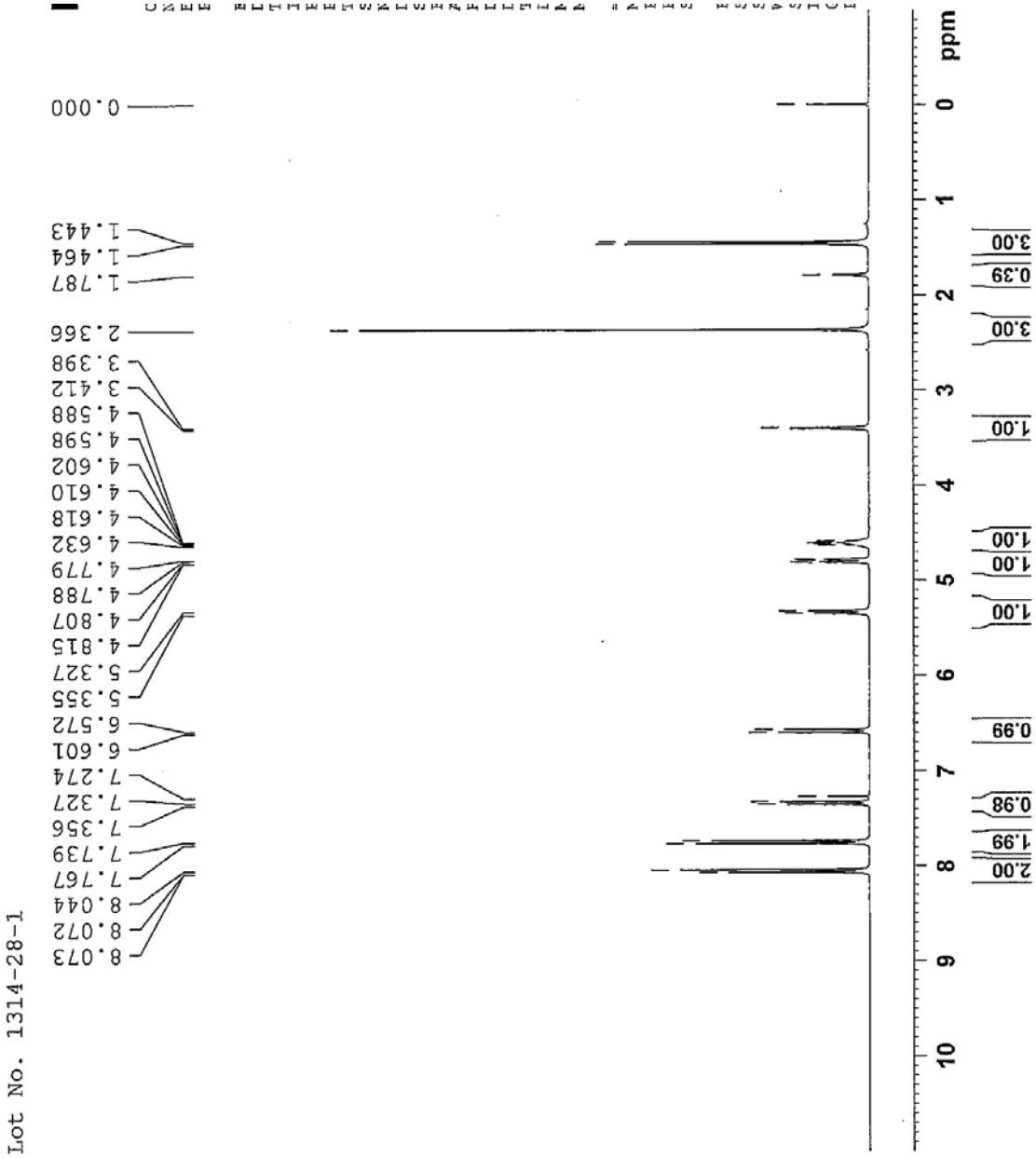
Compound **7** (**RAD140**)

2-chloro-4-((1*R*,2*S*)-1-(5-(4-cyanophenyl)-1,3,4-oxadiazol-2-yl)-2-hydroxypropylamino)-3-methylbenzonitrile

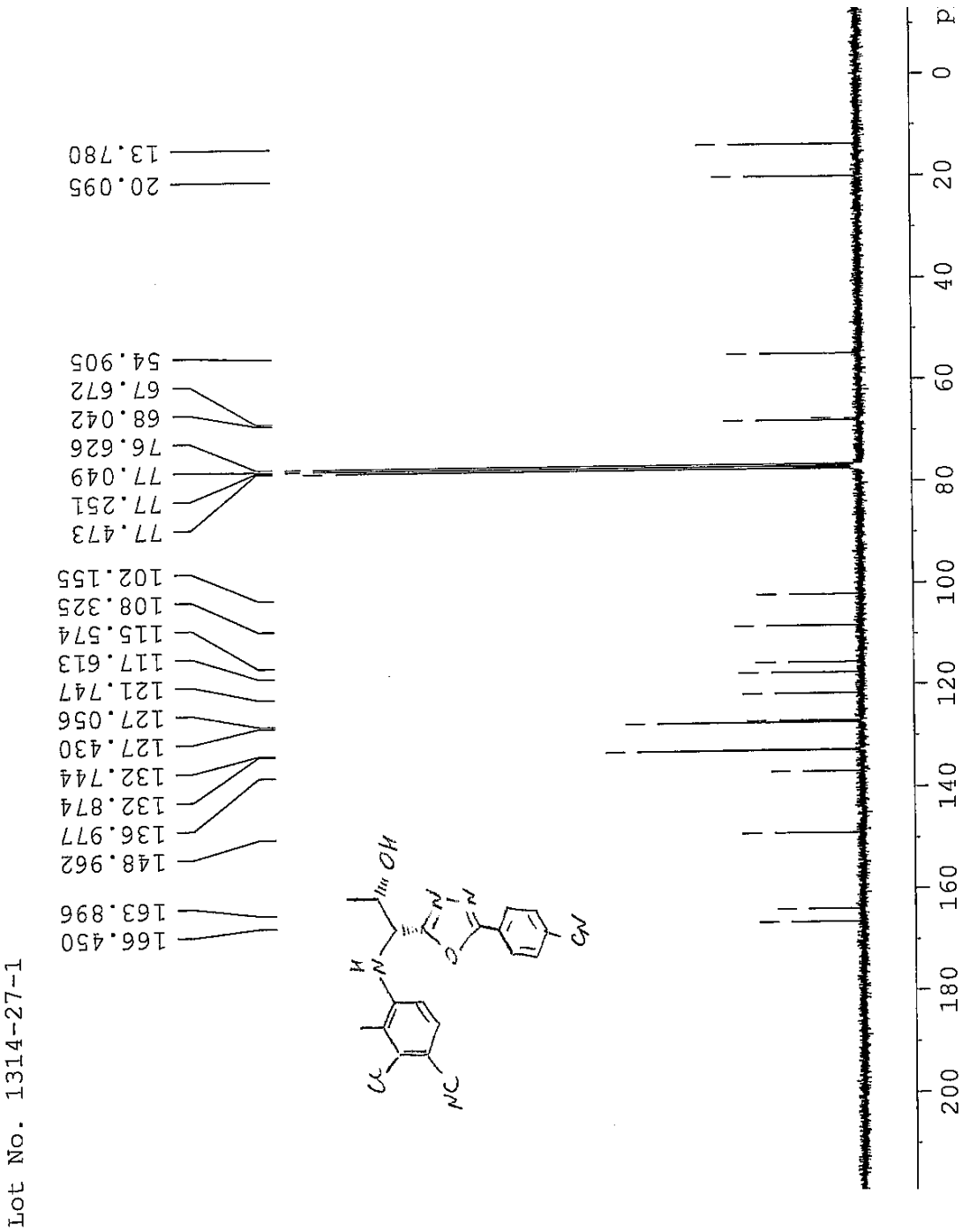


To a 500 mL round bottom flask equipped with a magnetic stirrer and a septum was added 4-((1*R*,2*S*)-2-(tert-butyldimethylsilyloxy)-1-(5-(4-cyanophenyl)-1,3,4-oxadiazol-2-yl)propylamino)-2-chloro-3-methylbenzonitrile (9.98 g, 19.6 mmol), followed by anhydrous THF (245 mL) under an atmosphere of nitrogen. The reaction mixture was cooled to -40 °C using dry ice/acetone bath. To this was added tetrabutylammonium fluoride as a 1M solution in THF (22.54 mL, 22.54 mmol) producing an instant color change to yellow. The reaction mixture was allowed to warm to ambient temperature and stirred at that temperature for a period of 4 h. The solvent was then removed under reduced pressure to yield a black residue. This residue was dissolved in ethyl acetate (200 mL) and washed with water (2 x 250 mL), brine (1 x 200 mL) and dried over sodium sulfate. The solution was then filtered and concentrated under reduced pressure to afford a yellow solid. The crude solid was purified via silica gel chromatography eluting with hexanes-ethylacetate (20:80) to give **RAD140** as a white solid (5.23 g, 68%). ^{1}H NMR (500 MHz, $CDCl_3$, δ in ppm) 8.10 (d, *J* = 8.4 Hz, 2H), 7.80 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 1H), 6.65 (d, *J* = 8.4 Hz, 1H), 5.27 (d, *J* = 8.0 Hz, 1H), 4.80 (dd, *J* = 2.8, 8.4 Hz, 1H), 4.62-4.65 (m, 1H), 2.91 (br s, 1H), 2.37 (s, 3H), 1.46 (d, *J* = 6.4 Hz, 3H); MS-API (pos) 394.1 (M+H+); CHN calc'd for $C_{20}H_{16}ClN_5O_2$: 61% C; 4.09% H; 17.78% N; 9% Cl. Found: 61.17% C; 4.02%; 17.88% N; 9.21% Cl.

300 MHz ^{1}H NMR of RAD140 in $CDCl_3$



75 MHz ^{13}C NMR of RAD140 in $CDCl_3$



Note on synthetic procedures: The synthetic procedures for intermediates **10a** and **10b** were translated from the general procedure(s) provided in US2010/0041721. The mass spectra of all intermediates, the images of the $^{1}H/^{13}C$ NMR for the final product and the elemental analysis for the final product were taken from one of the GMP runs for the synthesis of this compound. We were able to consistently prepare the desired intermediates and final compound by the methods provided in this paper, but since the physical characterization was more complete for the GMP synthesis, we augmented the analytical detail from the laboratory experimental with the GMP-derived analytical data. The GMP synthesis utilized the same general route but was done under GMP conditions on GMP scale.

Biological Assays

COMPOUND BINDING ASSAYS

The AR-binding assay was performed as specified by the manufacturer (Invitrogen, Madison, WI). Briefly, 1 μL of 10 mM compound was added to 500 μL of AR screening buffer in a 1.5mL eppendorf tube to make a $2x10^{-5}$ M stock. 10-fold serial dilutions of the test compounds were prepared ranging in concentration from 10^{-5} M to 10^{-12} M. Each dilution was added in triplicate to a black 384-microtiter plate. The test compounds will be diluted 2-fold in the final reaction. 2x AR-Fluormone™ complex (Fluormone is fluorescent-tagged R1881 AR-ligand) was prepared with 2 nM Flourmone AL Green™ and 30 nM AR. 25 μL of 2x complex was aliquoted to each reaction well, such that the final reaction volume was 50 μL per well. Plate was sealed with a foil cover and incubated in the dark at room temperature for 4 h. Polarization values for each well were measured. The polarization values were plotted against the concentration of the test compound. The concentration of the test compound that results in half-maximum shift equals the IC_{50} of the test compound. As a control, a competition curve for R1881 (methyltrienolone) was performed for each assay. Curve Fitting was performed using GraphPad Prism® software from GraphPad™ Software Inc. Conversion from IC_{50} to Ki was made by application of the Cheng-Prusoff equation:

$Ki=((IC_{50}/(1+ [S]/Km))$, where Km = Kd

wherein [S]=1 nM and Kd for the AR kit ligand (Fluormone) = 25 nM.

C2C12 cells are seeded on 96-well plates at a density of 5,000 cells per 100 µL DMEM with 10% non-HI FBS per well and incubated overnight. The next day, cells are transfected with 0.2 µg pRSV-AR using TransIT-LT1 Transfection Reagent (Mirus, MIR2305). (The transfection reagent is made up in 25 µL serum-free MEM at 1:3 ratio (0.6 µL per cell), and added directly to cells.) Cells were incubated overnight with DNA, and next day, the media was removed, and 200 µL of 10^{-6} to 10^{-10} M compound were added to the cells, and incubation continued for a period of 5 days. Add 1.26 mL of medium to wells 2-5 on assay block; dilute 1 uL of 2 mM compound in 2 mL of medium with continuous vortexing while adding, then transfer to well A; make dilution series by diluting 140 uL of 1 into 2 and so on; pre-wet tips by pipetting up and down 2 times, before taking the 140 uL; mix with pipet for 20 times. After 5 days, cells are washed with cold PBS, and frozen in 50 uL of water. After 2 freeze- thaw cycles, 50 uL of reaction mix containing 8.4 mg/mL para-nitrophenylphosphate, 0.2% Triton X-100, 33.3 mM $MgCl_2$ in 0.1 M glycine, pH 10.2 is added to the well. The plate is incubated at 37°C for 30 minutes, and the reaction stopped by adding 100 uL 0.2M NaOH. Absorbance is measured at 405 nM. Blanks (no cell) is subtracted from the raw values to obtain net values.

IN VIVO RAT MODEL OF ANDROGEN AND ANABOLIC ACTIVITY-RAT HERSCHBERGER ASSAY

Castrated Male Rat 11day Model

Sexually immature rats were castrated or SHAM-castrated at Charles River Laboratories (CRL) 21 days after birth and were acclimated for 4-5 days prior to study initiation. At approximately five weeks of age, animals were randomized by body weight into treatment groups (n=5), and dosing began and continued for 11 days. Treatment groups included vehicle, testosterone propionate (TP; 1 mg/kg), and RAD140 (10, 3, 1, 0.3, 0.1 mg/kg). TP (Sigma) was dissolved in 5% DMSO/95% corn oil and was administered by subcutaneous (SQ) injection. RAD140 doses were administered by oral gavage (PO) and prepared in 0.5% carboxyl methylcellulose (CMC; Sigma). Vehicle animals were dosed with 0.5% CMC by oral gavage. Twenty-four hours after the last dose all rats were euthanized by carbon dioxide inhalation. Body weight, prostate, seminal vesicle and muscle (levitor ani-bulbacavernous; LABC) weights were recorded for each rat.

Castrated Male Rat 4day Model

Sexually immature rats were castrated or SHAM-castrated at Charles River Laboratories (CRL) 21 days after birth and were acclimated for 4-5 days prior to study initiation. At approximately five weeks of age, animals were randomized by body weight into treatment groups (n=5), and dosing began and continued for 4 days. Treatment groups included vehicle, testosterone proprionate (TP; 1 mg/kg), and RAD30047 (compound **5**) or RAD30117 (compound **6**) as indicated. TP (Sigma) was dissolved in 5% DMSO/95% corn oil and was administered by subcutaneous (SQ) injection. RAD140 doses were administered by oral gavage (PO) and prepared in 0.5% carboxyl methylcellulose (CMC; Sigma). Vehicle animals were dosed with 0.5% CMC by oral gavage. Twenty-four hours after the last dose, all rats were euthanized by carbon dioxide inhalation. Body weight, prostate, seminal vesicle and muscle (levitor ani-bulbacavernous; LABC) weights were recorded for each rat.

Intact Male Rat 11day Model

Male rats at approximately five weeks of age were randomized by body weight into treatment groups (n=8) and dosing began and continued for 11 days. Treatment groups included vehicle, testosterone propionate (TP; 0.5 mg/kg), and RAD140 (30, 10, 3, 1, 0.3, 0.1 mg/kg). TP (Sigma) was dissolved in 5% DMSO/95% corn oil and was administered by subcutaneous (SQ) injection. RAD140 doses were administered by oral gavage (PO) and prepared in 0.5% carboxyl methylcellulose (CMC; Sigma). Vehicle animals were dosed with 0.5% CMC by oral gavage. Twenty-four hours after the last dose, all rats were euthanized by carbon dioxide inhalation. Body weight, prostate, seminal vesicle and muscle (levitor ani-bulbacavernous; LABC) weights were recorded for each rat.

Intact Male Monkey Model

Nine non-naïve male Cynomolgus macaques aged between 3-4 years old were selected and randomized to one of 3 groups with 3 animals each. After undergoing the 7-day acclimation period and 14-day baseline period, the monkeys were dosed daily with 5 mL/kg of the RAD140 at dose levels of 0.01, 0.1 and 1.0 mg/kg/day for 28 days. RAD140 doses were administered by oral gavage (PO) and prepared in 0.5% carboxyl methylcellulose. A washout period of three weeks subsequently followed the dosing period.

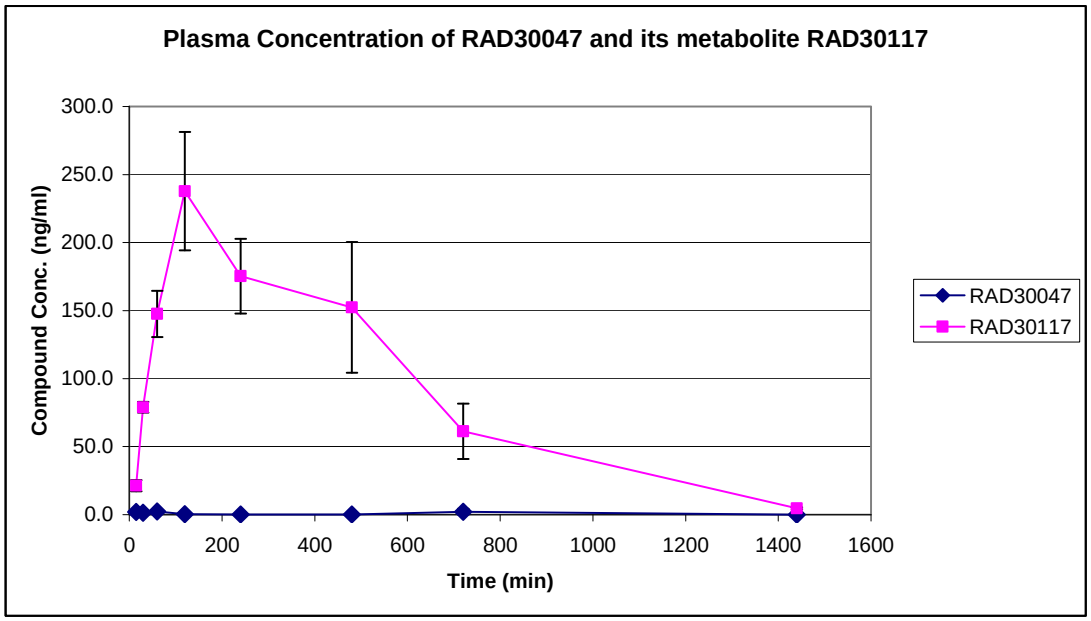
The general health, clinical condition and food intake of the animals were monitored daily. Weekly body weight measurements were done until the end of the washout period. Blood samples for serum biomarker analysis were obtained on Days 1 (pre-dose) and 28 (4 h post-dose).

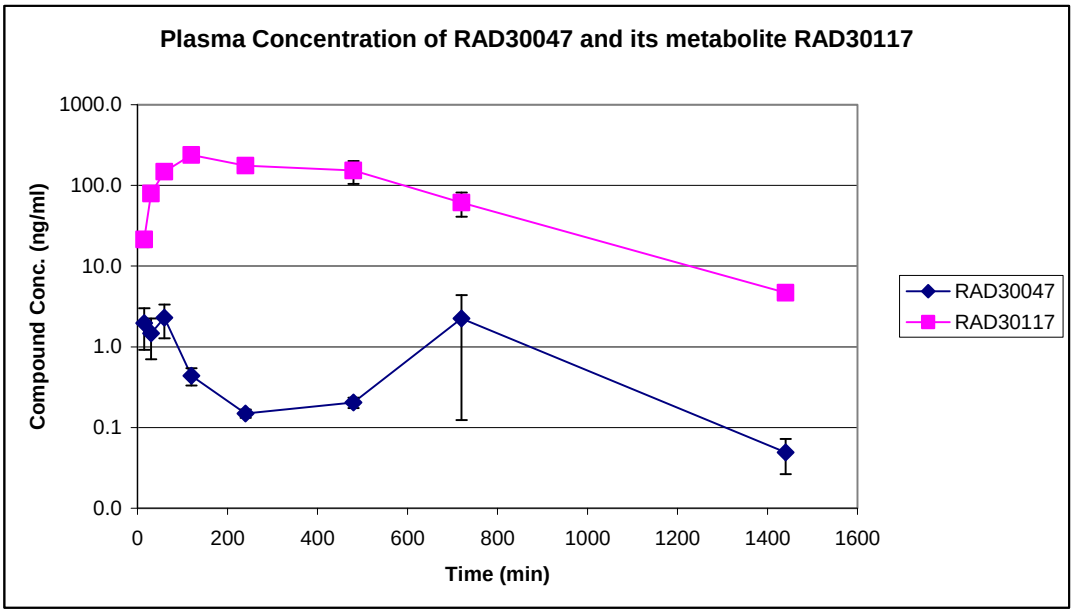
Statistical Analysis

All data are presented as the mean and the standard deviation (SD). The difference was evaluated by Student's *t* test when comparing two groups where $p<0.05$ was considered to be statistically significant.

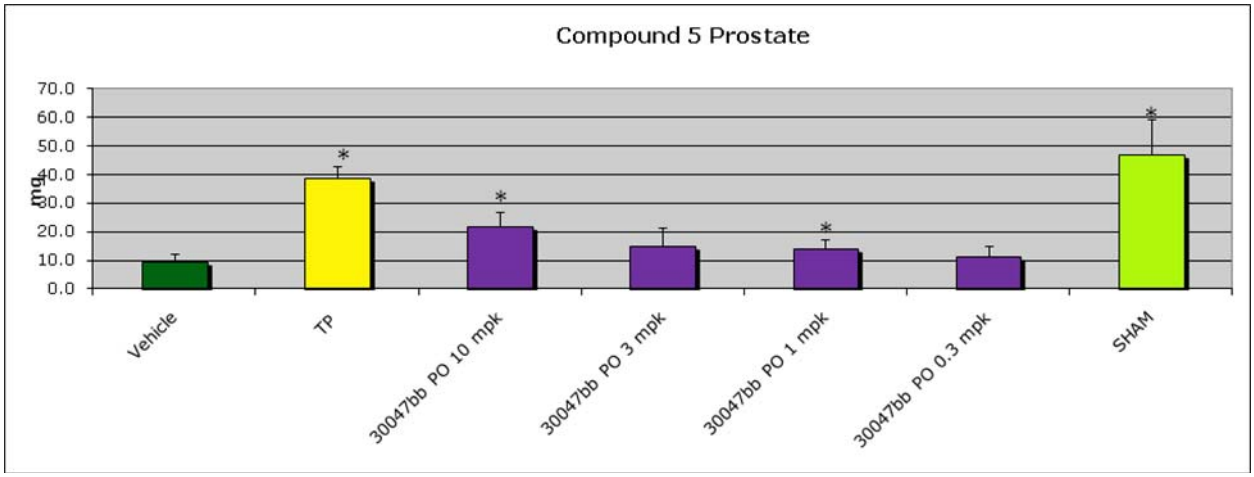
Biological Data on Compounds **5** and **6**

Footnote 10 refers to the metabolism of compound **5** (RAD30047) to compound **6** (RAD30117), as explained in the text. The figures shown immediately below demonstrate the very low levels of compound **5** after oral dosing and the rapid appearance of compound **6**. The first graph is the normal exposure, and the second graph is plotted on a log scale.

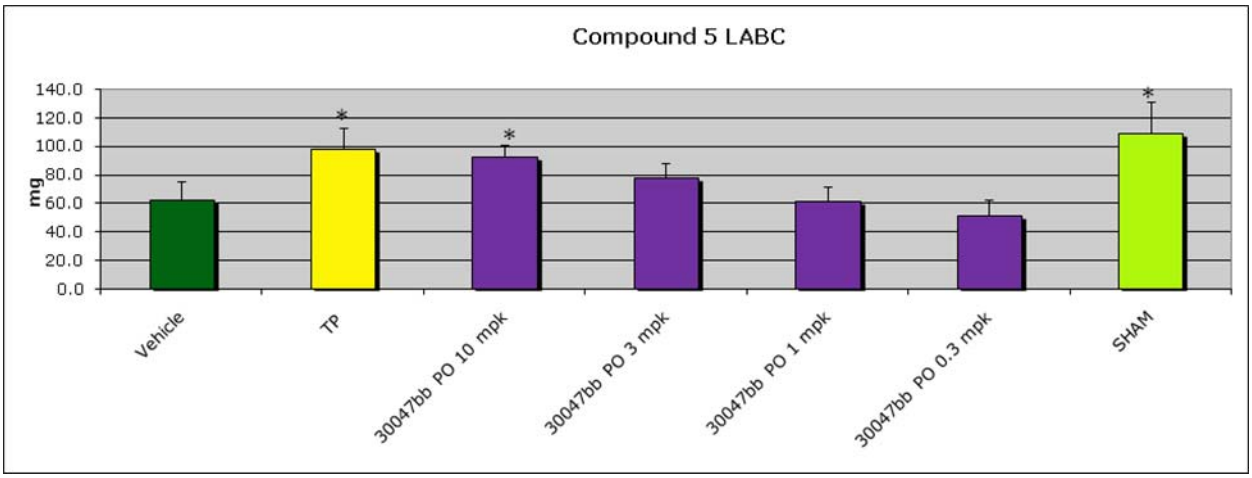



Plasma Concentration of RAD30047 and its metabolite RAD30117
Compound Conc. (ng/ml)
1000.0
100.0
10.0
1.0
0.1
0.0
0
200
400
600
800
1000
1200
1400
1600
Time (min)
RAD30047
RAD30117

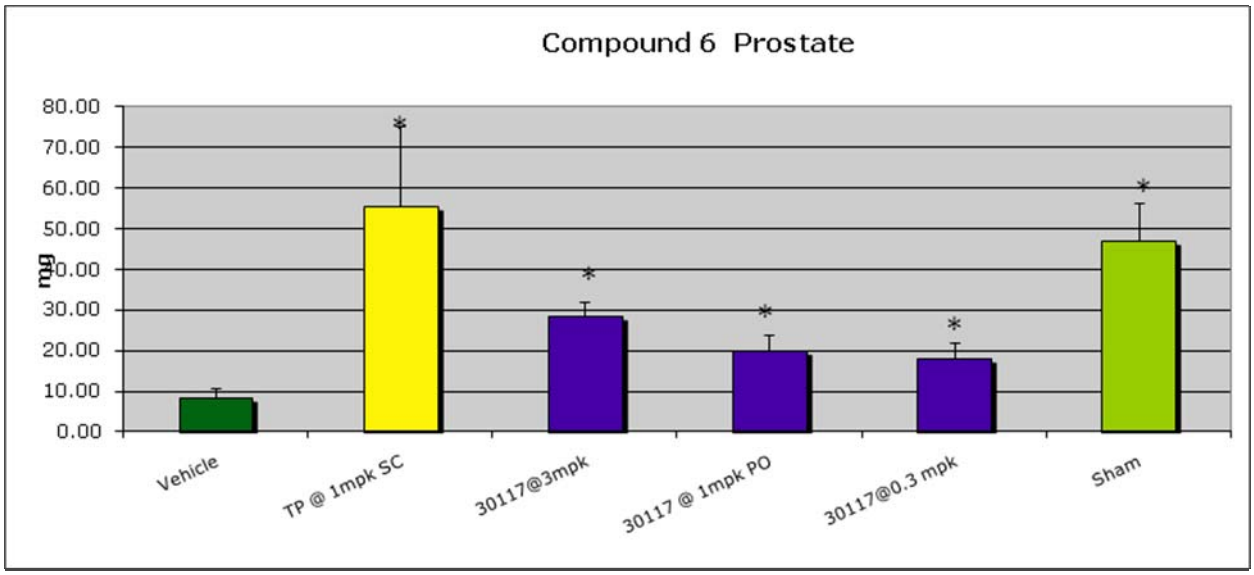
In vivo activity of compounds **5** and **6** in the 4-day rat Herschberger assay



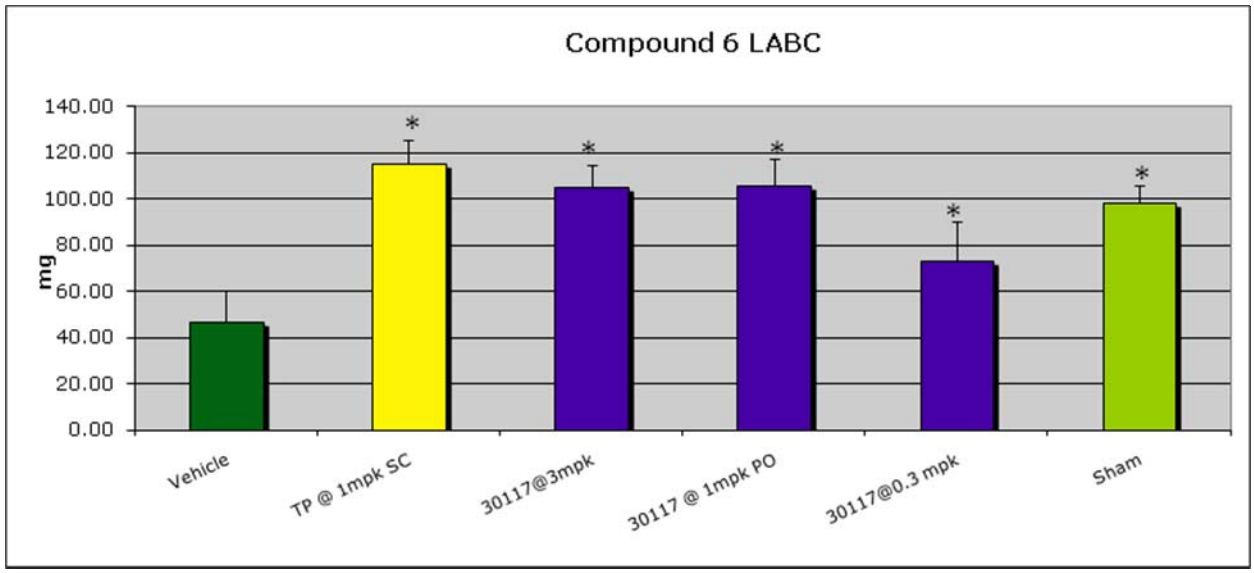
*Asterisks denote statistically significant effect relative to vehicle ($p < 0.05$)



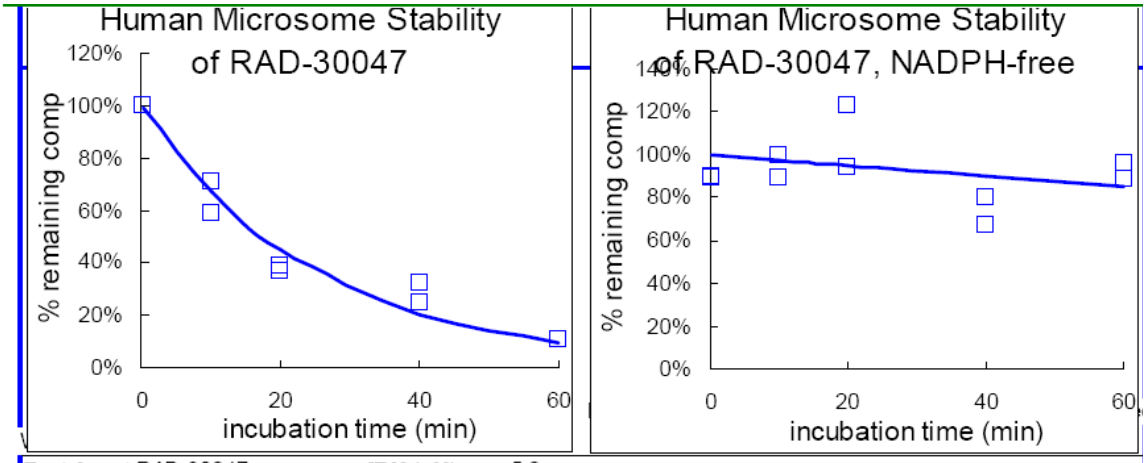
*Asterisks denote statistically significant effect relative to vehicle ($p < 0.05$)



*Asterisks denote statistically significant effect relative to vehicle.($p < 0.05$)



Stability of compound **5** (RAD30047) in human microsomes

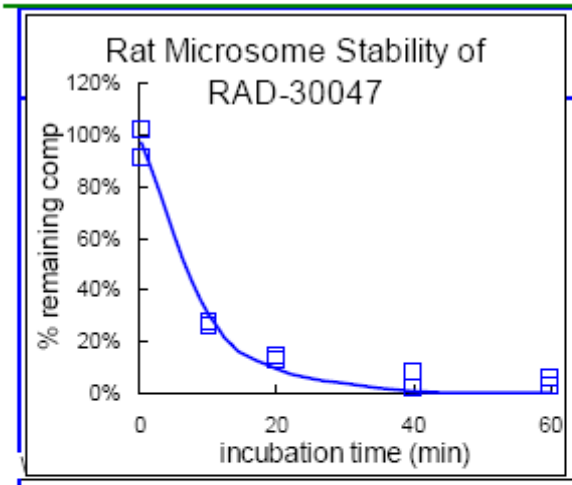


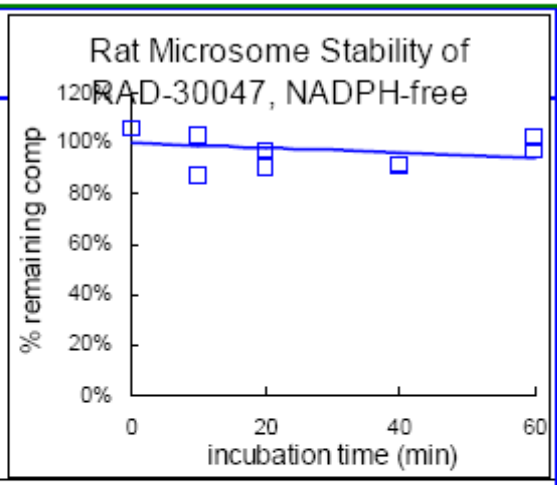
Test Agent RAD-30047 **[TA] (uM):** 5.0

Species: Human

	W NADPH				W/O NADPH			
	CLint	133	**T1/2**	17	**CLint**	9	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	100%	100%	100%	99%	89%	90%	89%	100%
10	59%	71%	65%	67%	99%	89%	94%	97%
20	37%	39%	38%	45%	123%	94%	109%	95%
40	25%	32%	29%	20%	80%	67%	74%	90%
60		11%	11%	9%	95%	89%	92%	85%

Stability of compound **5** (RAD30047) in rat microsomes





Test Agent RAD-30047 **[TA] (uM):** 5.0

Species: Rat

	W NADPH				W/O NADPH			
	CLint	400	**T1/2**	6	**CLint**	3	**T1/2**	>180
time	%RE1	%RE2	mean	calc	%RE1	%RE2	mean	calc
0.25	92%	102%	97%	97%	106%	106%	106%	100%
10	26%	28%	27%	30%	87%	103%	95%	99%
20	14%	13%	13%	9%	90%	97%	94%	98%
40	2%	8%	5%	1%	91%	91%	91%	96%
60	3%	5%	4%	0%	102%	97%	100%	94%