Supplementary Data

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TAS05567, a Novel Potent and Selective Spleen Tyrosine Kinase Inhibitor, Abrogates Immunoglobulin-Mediated Autoimmune and Allergic Responses in Rodent Models

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Results

Table S1. Selectivity profile of TAS05567 and R406 for kinase from the Profiler Pro Kit.

Enzyme	TAS05567 % inh at 50 nM	R406 % inh at 500 nM	Enzyme	TAS05567 % inh at 50 nM	R406 % inh at 500 nM	Enzyme	TAS05567 % inh at 50 nM	R406 % inh at 500 nM
ABL	1	20	EPHB3	2	25	NuaK1	31	87
Abl(H396P)	-11	-3	EPHB4	2	21	p38a	2	27
Abl(Q252H)	-17	-7	Erk1	-1	14	p38alpha/SAPK2a (T106M)	-11	-4
Abl(T315I)	-55	-36	Erk2	3	30	p38-beta2	-2	13
ABL1(E255K)	-15	-6	Fer	55	96	p38-delta	4	36
ABL1(G250E)	-16	-7	FES	2	27	p38-gamma	-1	14
ABL1(Y253F)	-12	-5	FGFR1	-3	11	p70S6K	0	17
AKT1	13	68	FGFR1 (V561M)	-19	-16	PAK2	1	20
AKT2	0	17	FGFR2	2	24	PAK3	9	56
AKT3	-1	14	FGFR2(N549H)	-18	-8	PAK4	77	100
ALK	62	99	FGFR3	-3	9	PAK5 (PAK7)	80	100
AMPK	3	29	FGFR3 [K650E]	-18	-14	PASK	-5	2
AMPK-alpha2/ beta1/gamma1	0	17	FGFR4	-5	3	PDGFR beta	-1	15
Arg	-3	9	FGR	-7	1	PDGFR_alpha	4	32
AurA	10	63	FLT1	-5	4	PDGFRA (D842V)	-9	0
AurB	2	23	FLT3	17	73	PDGFR-alpha(V561D)	-10	-2
AurC	-5	4	Flt3(D835Y)	-8	1	PhKg1	-2	12
AXL	1	20	FLT4	4	33	PhKg2	-3	11
BLK	27	86	FMS	-2	13	PIM1	-4	6
BMX	11	65	FRK	23	83	PIM2	56	97
BRSK1	-4	7	FYN	34	90	PIM3	0	19
BRSK2	-1	14	GCK	52	93	PKA	10	60

BTK	25	85	GSK3-alpha	8	53	PKC-alpha	27	86
CaMK1a	-5	4	GSK3b	9	58	PKCb2	-5	3
CamK1d	-2	12	Hck	5	46	PKC-beta1	18	73
CAMK2	0	18	HER4	4	31	PKC-delta	-3	9
CaMK2a	3	29	HGK	6	49	PKC-epsilon	7	52
CAMK4	-2	12	HIPK1	3	29	PKC-eta	8	55
CaMKII_beta	-6	2	HIPK2	-1	14	PKC-gamma	19	76
CaMKII_gamma	17	73	IGF1R	33	89	PKC-theta	23	83
Casein kinase 1g2	-1	16	IKBKE (IKK epsilon)	23	85	PKCz	-2	12
CDK1/Cycline B1	-5	3	IKK-beta	60	98	PKD1	-1	16
CDK2	5	43	INSR	32	87	PKD2	12	68
CDK3	-5	3	IRAK4	21	83	PKD3	-4	5
CDK5/p25	-4	4	ITK	5	45	PKG1-beta	0	18
CHK1	12	66	JAK2	79	100	PKGa	2	21
CHK2	17	71	KDR	4	32	PRAK	10	63
CK1d	0	18	KIT	-7	1	PRKCI (PKC-iota)	1	20
CK1-epsilon	-1	16	KIT[T670I]	-74	-45	PRKX	1	20
CK1g3 (CSNK1G3)	-3	8	LCK	18	76	PYK2	29	86
CK1-gamma1	-1	16	LOK	59	97	RET	49	92
CLK2	1	20	LTK	18	76	Ret (V804L)	-12	-5
c-Raf	-1	15	LYN	40	91	RET Y791F	-9	-2
CSNK1A1	-2	11	LYNB	50	92	ROCK1	15	69
c-TAK1	-7	1	MAPKAPK2	9	59	ROCK2	25	86
DAPK1	4	36	MAPKAPK3	7	52	ROS (ROS1)	54	94
DCAMKL1	-4	6	MARK1	4	35	RSK1	3	28
DCAMKL2	-6	2	MARK2	-3	9	RSK2	3	31
DDR2	-6	2	MARK4	8	55	RSK3	9	56
DYRK1a	-5	3	MELK	-2	13	RSK4	4	34
DYRK1B	5	40	Mer	32	87	SGK1	8	55
DYRK3	0	20	MET	5	43	SGK2	6	47

DYRK4	2	27	MET M1250T	-10	-2	SGK3	11	64
EGFR	-3	9	MINK	16	70	SRC	39	90
EGFR (ErbB1) T790M L858R	-8	0	MNK1 (MKNK1)	16	70	SRM (SRMS)	-4	6
EGFR(T790M)	-33	-34	MSK1	0	18	SYK	99	93
EPHA1	11	66	MSK2	-5	3	TEC	-5	2
EPHA2	5	39	MST1	64	99	TRKC (NTRK3)	13	69
EPHA3	7	49	MST1R	2	21	TSSK1	58	97
EPHA4	2	22	MST2	20	78	TSSK2	20	81
EPHA5	5	38	MST3 (STK24)	3	30	TXK	-4	6
EPHA8	5	42	NEK1	-4	5	TYRO3	7	53
EPHB1	4	32	NEK2	-4	7	Yes	32	87
EPHB2	5	43	NTRK2 (TRKB)	13	69	ZIPK (DAPK3)	72	99

Table S2. Off-target receptor binding assays.

Receptor	TAS05567 % inh at 10 µM	Receptor	TAS05567 % inh at 10 µM	Receptor	TAS05567 % inh at 10 µM
Adenosine A1	75	Epidermal Growth Factor	-19	Neuropeptide Y Y2	4
Adenosine A2A	2	Estrogen ERalpha	1	Nicotinic Acetylcholine	-10
Adenosine A3	5	Transporter, GABA	15	Nicotinic Acetylcholine Alpha1, Bungarotoxin	0
Adrenergic alpha1A	12	GABAA, Muscimol, Central	-4	Opiate delta1 (OP1, DOP)	0
Adrenergic alpha1B	7	GABAA, Flunitrazepam, Central	24	Opiate kappa (OP2, KOP)	11
Adrenergic alpha1D	10	GABAB1A	-3	Opiate mu (OP3, MOP)	9
Adrenergic alpha2A	-7	Glucocorticoid	9	Phorbol Ester	-9
Adrenergic beta1	-5	Glutamate, Kainate	8	Platelet Activating Factor (PAF)	-10
Adrenergic beta2	15	Glutamate, NMDA, Agonism	3	Potassium Channel [KATP]	-3
Transporter, Norepinephrine	12	Glutamate, NMDA, Glycine	4	Potassium Channel hERG	26
Bradykinin B1	15	Glutamate, NMDA, Phencyclidine	-3	Prostanoid EP4	-2
Bradykinin B2	-3	Histamine H1	-7	Purinergic P2X	5
Calcium Channel L-Type, Benzothiazepine	15	Histamine H2	4	Purinergic P2Y	0
Calcium Channel L-Type, Dihydropyridine	28	Histamine H3	8	Rolipram	34
Calcium Channel N-Type	5	Imidazoline I2, Central	12	Serotonin (5-Hydroxytryptamine) 5-HT1A	-8

Cannabinoid CB1	6	Interleukin IL-1	-10	Serotonin (5-Hydroxytryptamine) 5-HT2B	-9
Dopamine D1	-1	Leukotriene, Cysteinyl CysLT1	11	Serotonin (5-Hydroxytryptamine) 5-HT3	-13
Dopamine D2S	-5	Melatonin MT1	4	Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	-4
Dopamine D3	3	Muscarinic M1	0	Sigma1	-2
Dopamine D4.2	-1	Muscarinic M2	-1	Sodium Channel, Site 2	30
Transporter, Dopamine	24	Muscarinic M3	-7	Androgen (Testosterone) AR	-8
Endothelin ETA	11	Tachykinin NK1	6	Thyroid Hormone	3
Endothelin ETB	0	Neuropeptide Y Y1	-1		

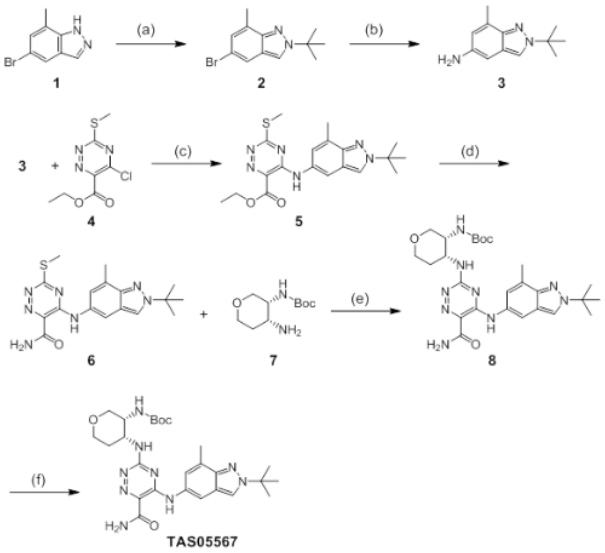
Off-target effects of 10 μM TAS05567 were assessed using the LeadPrifilingScreen commercial assay. Results that show an inhibition or stimulation >50% are considered to represent significant effects on the test compound.

Table S3. Plasma protein binding of TAS05567.

Species	Unbound fraction (%)
	TAS05567
Mouse	15.4 ± 0.5
Rat	18.3 ± 1.1
Human	21.9 ± 0.1

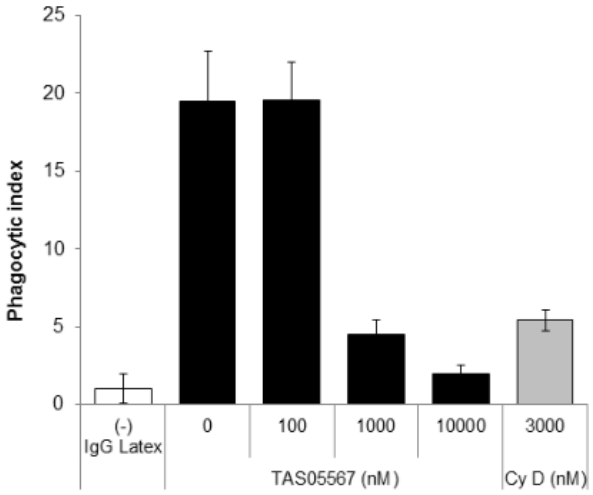
TAS05567 was tested at a final concentration of 1 μM in mouse, rat, and human plasma. An aliquot of plasma containing TAS05567 was in the well of equilibrium dialysis device. The plate containing plasma and buffer was equilibrated at 37°C for 6 h, with constant shaking at 80 rpm on an orbital shaker. Samples were collected and precipitated using organic solvents. All samples were centrifuged at 850*g* for 5 min at 4°C, and then were analyzed by liquid chromatography–tandem mass spectrometry. Data are presented as mean ± SD (n = 3).

Additional file 1: Figure S1



Supplemental Figure 1. Scheme of the design and synthesis of TAS05567.

Additional file 1: Figure S2



Supplemental Figure 2. TAS05567 inhibits FcγR-mediated phagocytosis in macrophages. RAW264.7 cells were pretreated with TAS05567 or cytochalasin D for 1 h, followed by incubation with latex beads coated with FITC-labeled IgG (1:500) for 4 h. The cells were collected and analyzed by flow cytometry. Data were obtained from triplicate analyses and are presented as mean ± SD. Cy D indicates cytochalasin D.

Supplementation Experimental Procedure

Synthetic procedures and characterization data for the synthesis of TAS05567

Reagents and Conditions:

(a) AcOtBu, $MeSO_3H$, toluene, 80 °C, 28 h; (b) 28% NH_3 aq., Cu_2O, NMP, 95 °C, 14 h; (c) (i) chlorine, chloroform, 0 °C (ii) iPr_2NEt, THF, 0 °C; (d) DMA, iPr_2NEt, room temperature, 6 h; (e) 8M NH_3-MeOH, room temperature, 6 days; (f) TFA, room temperature, 1.5 h

(a) 5-bromo-2-(tert-butyl)-7-methyl-2H-indazole (compound 2)

tert-butyl acetate (405 ml) and methanesulfonic acid (19.5 ml, 300 mmol) were added to a solution of 5-bromo-7-methyl-1H-indazole (compound 1, 63.3 g) in toluene (190 ml) at room temperature. The mixture was stirred at 60 °C for 1 day and at 70 °C for 2 days. Water was added, followed by extraction with toluene. The organic layer was washed with water, and dried over anhydrous sodium sulfate. The solvent was distilled off under reduced pressure. The resulting residue was purified by silica gel column chromatography (hexane/ethyl acetate) to obtain compound 2 as pale red oil (69.7 g, 87%).

(b) 2-(tert-butyl)-7-methyl-2H-indazol-5-amine (compound 3)

A mixture of compound 2 (23.77g, 88.97 mmol) and copper (I) oxide (1.27g, 8.88 mmol) in 1-methyl-2-pyrrolidone (120 mL) and concentrated ammonia solution (120 ml) in stainless steel sealed tube was heated at 95 °C (internal temperature) for 14 h.

The mixture was allowed to cool to room temperature and then partitioned between ethyl acetate and water. The organic layer was washed with water and brine, dried over Na_2SO_4 and evaporated in vacuo. To the residue was added diisopropyl ether and the precipitate was collected by filtration and dried over at 50 °C. Compound 3 (7.98 g, 44%) was obtained as white solid. The filtrate was purified by flash column chromatography on silicagel to afford the 2nd crop of compound 3 (5.53 g, 31%)

(c) ethyl 5-((2-(tert-butyl)-7-methyl-2H-indazol-5-yl)amino)-3-chloro-1,2,4-triazine-6-carboxylate (compound 5)

To the mixture of ethyl 5-chloro-3-methylsulfanyl-1,2,4-triazine-6-carboxylate (5.95 g, 25.5 mmol) in chloroform (60 ml) was added chlorine gas at 0 °C. After the starting material was disappeared in LCMS, the mixture was concentrated in vacuo. The residue was diluted by THF (100 mL) and cooled at 0 °C.

To the mixture was added N,N-diisopropylethylamine, followed by a solution of compound 3 (5.69 g, 28 mmol) in THF (50 ml). The mixture was quenched by addition

of 10% H_3PO_4 aqueous solution, and the mixture was extracted by ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4 and evaporated in vacuo. To the residue was added diisopropyl ether and the precipitate was collected by filtration and dried over at 50 °C. Compound 5 (6.78 g, 69%) was obtained as pale yellow solid. The filtrate was purified by flash column chromatography on silicagel to afford the 2nd crop of compound 5 (0.97 g, 10%)

(d) ethyl 3-(((3R,4R)-3-((tert-butoxycarbonyl)amino)tetrahydro-2H-pyran-4-yl)amino)-5-((2-(tert-butyl)-7-methyl-2H-indazol-5-yl)amino)-1,2,4-triazine-6-carboxylate (compound 7).

To a yellow suspension of compound 5 (35.0 g , 90 mmol) in dimethylacetamide (100 ml), a solution of compound 6 (21.4 g, 98.9 mmol) in dimethylacetamide (80 ml) and diisopropylethylamine (15.1 g, 117 mmol) were added at room temperature. The dark red reaction mixture was stirred for 6 h. After water (300 ml) was added, reaction mixture was stirred for 3 h. Precipitate was collected by filtration and washed with water, and dried over at 50 °C. A crude material was added to ethanol (350 ml). The mixture was heated, and then water (350 ml) was added to the mixture. The mixture was stirred for 3 h at room temperature, then precipitate was collected by filtration and washed with 50% aqueous ethanol, and dried over at 50 °C to afford compound 7 (47.7

g, 93.2%) as a yellow solid.

(e) tert-butyl ((3R,4R)-4-((5-((2-(tert-butyl)-7-methyl-2H-indazol-5-yl)amino)-6-carbamoyl-1,2,4-triazin-3-yl)amino)tetrahydro-2H-pyran-3-yl)carbamate (compound 8)

8 M ammonia in methanol solution (500 ml, 4.00 mol) was added to Compound 7 (47.2 g, 83.0 mmol), and the mixture was stirred for 6 days at room temperature. Methanol was removed under reduced pressure, and 30% methanol was added to the residue, and the mixture was stirred for 2 h. Precipitate was collected by filtration and washed with 30% methanol aqueous solution, and dried over at 50 °C to afford a compound 8 (44.1 g, 98.5 %) as a pale yellow solid.

(f) 3-(((3R,4R)-3-aminotetrahydro-2H-pyran-4-yl)amino)-5-((2-(tert-butyl)-7-methyl-2H-indazol-5-yl)amino)-1,2,4-triazine-6-carboxamide (TAS05567)

Compound 8 (40.4 g, 74.9 mmol) was added to trifluoroacetic acid (170 ml) and the mixture was stirred for 1.5 h at room temperature. After removal of solvent under reduced pressure, water was added to the residue, and then the mixture was basified (approximately pH 10) with conc. ammonia solution at 0 °C. Precipitate was collected by filtration, washed with water, and dried in vacuo at 50 °C to give TAS05567 as

yellow solid (32.4 g, 98.5 %).

1H-NMR ($CDCl_3$/CD_3OD=1/1) δ: 8.20-7.84 (1H, m), 8.11 (1H, s), 7.23-7.03 (1H, m), 4.30-4.07 (1H, m), 4.01 (1H, d, J = 11.0 Hz), 3.87 (1H, d, J = 12.1 Hz), 3.64 (1H, q, J = 7.0 Hz), 3.58-3.47 (1H, m), 3.16 (1H, s), 2.63 (3H, s), 1.98-1.88 (1H, m), 1.87-1.81 (1H, m), 1.77 (9H, s).

ESI-MS m/z 440 [M+H＋].

Off-target receptor binding assays

TAS05567 was tested at 10 μM for orthosteric radioligand displacement against a panel of 68 primary molecular targets, such as G-protein-coupled receptors, ion channels, and transporters, to characterize its specificity using the LeadPrifilingScreen commercial assay (Eurofins Panlabs Taiwan, Taipei, Taiwan). Results that show an inhibition or stimulation >50% were considered to represent significant effects on the test compound.

Plasma protein binding

TAS05567 (in 50:50 acetonitrile/water) was tested at a final concentration of 1 μM in mouse, rat (Charles River Laboratories Japan, Yokohama, Japan), and human plasma (Biopredic, Rennes, France). An aliquot of 150 μl plasma containing TAS05567 was in

donor side of the well of 96-well micro-equilibrium dialysis device (HTD 96b, HTDialysis, CT, USA). An aliquot of 150 µl PBS was added in reservoir side of the same device. The plate containing plasma and buffer was equilibrated at 37°C for 6 h, with constant shaking at 80 rpm on an orbital shaker. Samples were collected from respective sides after 6 h. All samples were centrifuged at 850*g* for 5 min at 4°C, and then were analyzed by liquid chromatography–tandem mass spectrometry. Data are presented as mean ± SD (n = 3).

FcγRI-mediated Phagocytosis Assay

Phagocytic ability was evaluated by measuring the amount of uptake of latex beads coated with FITC-labeled IgG into cells by a phagocytosis assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the instruction manual. Briefly, RAW264.7 cells (ATCC TIB-71) were seeded in 6-well plates (1×10^6 cells/well) in DMEM containing 5% FBS overnight at 37°C to allow adherence to the plate. On the next day, the latex beads were added directly to the culture medium at a dilution of 1:500 and incubated at 37°C for 4 h. The cells were collected and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).