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Structure-activity relationship study and discovery of indazole 3-carboxamides as calcium-release activated calcium channel blockers

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ABSTRACT. Aberrant activation of mast cells contributes to the development of numerous diseases including cancer, autoimmune disorders, as well as diabetes and its complications. The influx of extracellular calcium via the highly calcium selective calcium-release activated calcium (CRAC) channel controls mast cell functions. Intracellular calcium homeostasis in mast cells can be maintained via the modulation of the CRAC channel, representing a critical point for therapeutic interventions. We describe the structure-activity relationship study (SAR) of indazole-3-carboxamides as potent CRAC channel blockers and their ability to stabilize mast cells. Our SAR results show that the unique regiochemistry of the amide linker is critical for the inhibition of calcium influx, the release of the pro-inflammatory mediators β-hexosaminidase and tumor necrosis factor α by activated mast cells. Thus, the indazole-3-carboxamide **12d** actively inhibits calcium influx and stabilizes mast cells with sub-μM IC_{50}. In contrast, its reverse amide isomer **9c** is inactive in the calcium influx assay even at 100 μM concentration. This requirement of the specific 3-carboxamide regiochemistry in indazoles is unprecedented in known CRAC channel blockers. The new structural scaffolds described in this report expand the structural diversity of the CRAC channel blockers and may lead to the discovery of novel immune modulators for the treatment of human diseases.

Mast cells (MCs) are presented in most tissues including the skin where they form the frontline of defense against invading pathogens. MCs are originated from hematopoietic cells and populate throughout the tissues. When encountered by pathogens, MCs are activated via the ligation of the high affinity immunoglobulin E (IgE) receptor FceRI as well as receptors of growth factors such as the vascular endothelial growth factor receptor (VEGFR). MCs contain cytosolic granules that are composed of preformed inflammatory mediators such as histamine, tryptase, β-hexosaminidase (β-hex), and tumor necrosis factor α (TNFα). Upon activation, MCs are capable of immediately releasing the preformed mediators by a process called MC degranulation, which is followed by *de novo* syntheses of cytokines and growth factors to sustain a long-term effect [1].

Uncontrolled MC activations are implicated in numerous pathological processes including autoimmune diseases and cancer [2-4]. MCs are increasingly recognized as playing a critical role in tissue homeostasis and repairing. Thus, overly activated MCs were found in the skin of diabetic patients and impeded the wound healing process of diabetic foot ulcers [5].

The discovery of MC stabilizers for treating allergy and autoimmune diseases is an active research field [6-9]. MC activation is controlled by the highly calcium selective calcium-release activated calcium (CRAC) channel. The CRAC channel does not share homology or functional similarities with other types of calcium channels (e.g. voltage-gated calcium channel) and operates on a unique mechanism of action [10]. The endoplasmic reticulum (ER) is a major source of intracellular Ca^{2+}. Activations of cell surface receptors on MCs lead to phospholipase C (PLC) mediated hydrolysis of phospholipids to produce diacylglycerol (DAG) and inositol triphosphate (IP_3). Engagement of IP_3 to the IP_3 receptor (IP_3R) on the ER membrane empties the ER Ca^{2+} store, which activates the CRAC channels for influx of extracellular Ca^{2+} to maintain sustained high levels of intracellular Ca^{2+}. Elevated levels of cytosolic calcium ($[Ca^{2+}]_i$) promote the Ca^{2+} dependent degranulation and nuclear translocations of transcription factors [11-16]. The critical role of the CRAC channel in MC effector function is

substantiated by the fact that their genetic ablation in mice severely impaired MC degranulation and the release of pro-inflammatory mediators [17]. Small molecule CRAC channel blockers are shown to potently inhibit MC degranulation [18] and T-cell activation [19]. In addition, CRAC channels are functional in human lung mast cells and their pharmacological inhibition reduces the high-affinity immunoglobulin E receptor (FcεRI) dependent Ca^{2+} influx, and the release of an array of pro-inflammatory cytokines including TNFα[20].

Because of its essential role in mediating MC activation and T cell functions, CRAC channel is widely accepted as a viable drug target for treating autoimmune disorders [21-23]. Many of the known CRAC channel blockers share a common amide bond linker that connects two aryl moieties (**Figure 1**). Our own efforts [24, 25] in the design and synthesis of CRAC channel blockers had previously focused on a series of bi-aryl amides including the widely used pharmacological tool compounds Synta-66 (**1**) and Ro-2959 (**2**) [26-30], which are structurally related to BTP2/YM-58483 (**3**) and Pyr-6 (**4**) [31, 32]. Ring fusion strategies described by Esteve et al. [33] led to the 7-azaindole series as exemplified by compounds **5**. More recently, Derler et al. described the pyrazole amide CRAC inhibitor GSK-7975A (**6**) [29]. Herein we report the structure-activity relationship (SAR) study of novel indazole-3-carboxamides as potent CRAC channel blockers and their effective inhibition of MC activation.

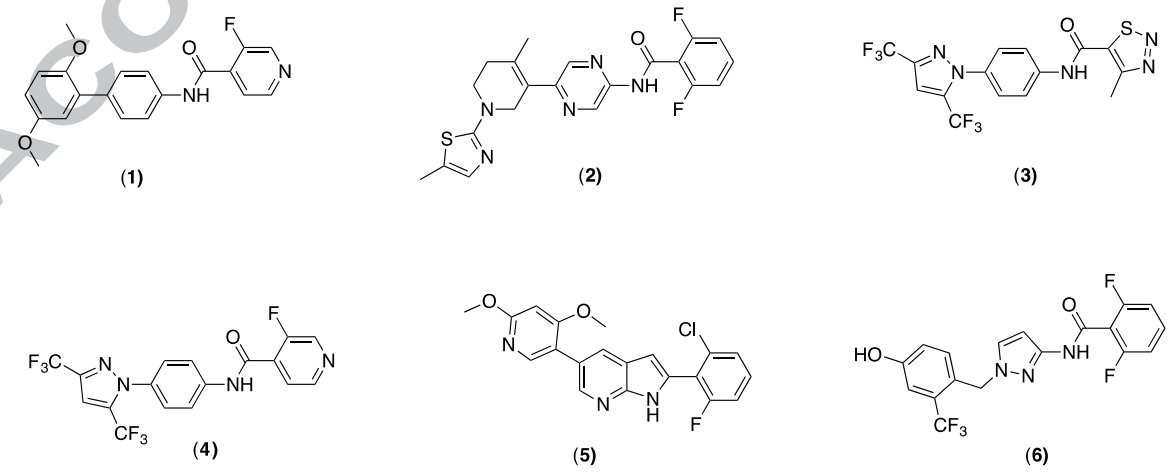
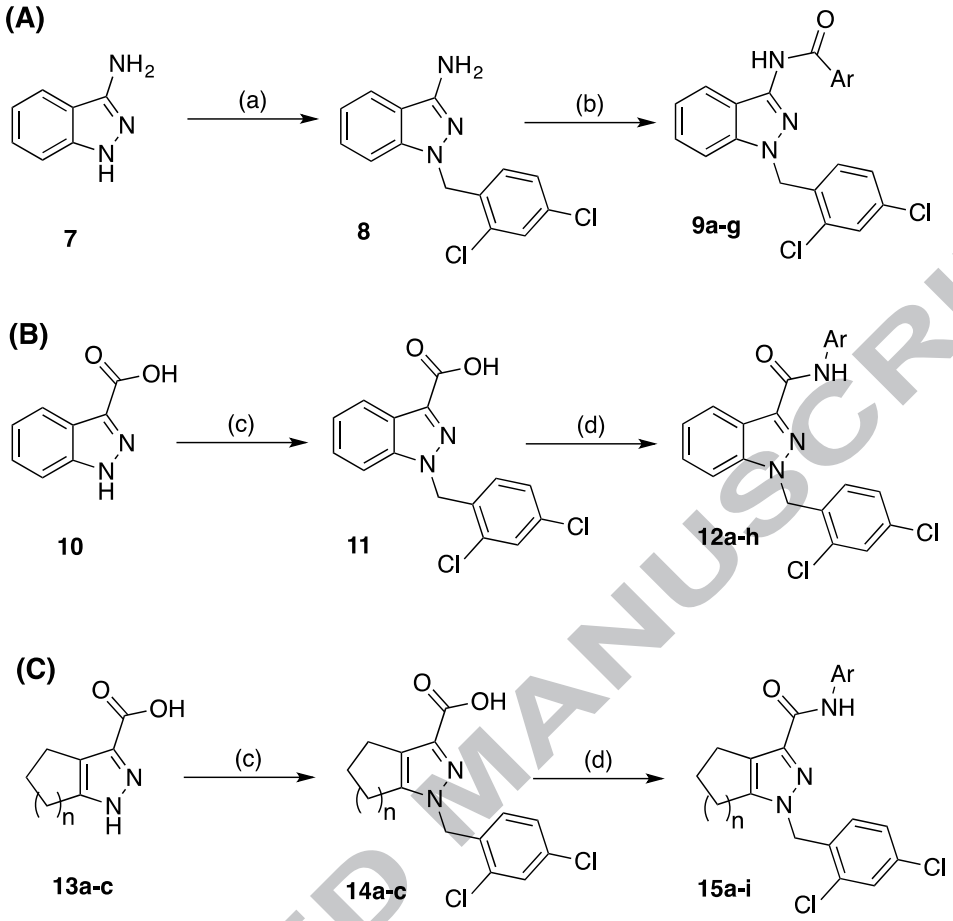


Figure 1. Representative CRAC channel inhibitors described in the literature.

The 3-aminoindazole **7** was used as the starting material for the syntheses of indazole 3-amide derivatives **9a-g** that contained the same amide liker as in the pyrazole amide **6** (**Scheme 1A**). Compound **7** was first treated with KOH, followed by the addition of 2,4-dichlorobenzyl chloride to form regioselectively the 3-amino-1-(2,4-dichlorobenzyl)indazole (**8**). The regioselective alkylation of the NH group of the indazole over the 3-NH_2 group is well documented [34, 35]. In our case the structure assignment of **8** is based on ^{1}H NMR data showing the presence in compound **8** the -NH_2 group at 5.5 ppm (2H, broad singlet), and further supported by the presence of the amide -N**H**-$COAr_2$ proton at 10-11 ppm (1H, singlet) in the subsequent reaction products **9a-g**. Acylation of the 3-amino group with the corresponding acyl chlorides proceeded smoothly to afford the desired 3-amide (-NH-CO-Ar_2) products **9a-g** in good yields (e.g. **9a**: 290mg, 95%) after purification by silica gel chromatography (SGC) or reverse phase high performance liquid chromatography (HPLC).

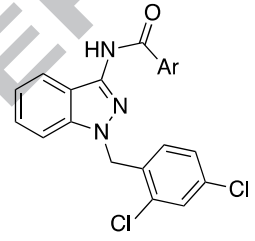
Starting from the indazole-3-carboxylic acid **10**, derivatives **12a-h** containing a 'reversed' 3-carboxamide (-CO-NH-Ar) as compared with **9a-h** were synthesized by three-step reactions (**Scheme 1B**). First, the 3-carboxylic acid was treated with 2,4-dichlorobenzyl chloride in the presence of NaH. The isolated 1-substituted indazole intermediate **11** was treated with oxalyl chloride to form the corresponding acyl chlorides, which were then reacted with aryl amines ($ArNH_2$) to afford the desired 3-carboxamide products **12a-h** in good yields (e.g. **12d**: 120mg, 97%) after purifications by SGC or HPLC. Starting from the pyrazole-3-carboxylic acids fused to a 5-, 6-, or 7-membered saturated carbon ring **13a-c**, the pyrazole 3-carboxamide derivatives **15a-i** were prepared similarly (**Scheme 1C**).



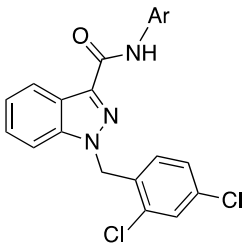
Scheme 1. The syntheses of indazole-3-carboxamides and derivatives. Conditions (a). i) KOH (2.5 eq.), DMSO, rt, overnight; ii) 2,4-dichlorobenzyl chloride (1.25 eq.), rt, 0.5h; iii) H_2O. (b). i) arylcarboxylic acid (1.0 eq.), CO_2Cl_2 (1.5-2.0 eq.), DCM, DMF (cat.), rt, 0.5h; ii) 8 (1.0 eq.), TEA (1.5 eq.), DCM, rt, 0.5h. (c). i) NaH 60% in mineral oil (2.2 eq.), 0 ^{o}C to rt, 0.5-1h; ii) 2,4-dichlorobenzyl chloride (1.2 eq.), 0 ^{o}C to rt, overnight; iii) 2N HCl, rt. (d). i) 11 or 14a-c (1.0 eq.), CO_2Cl_2 (1.5-2.0 eq.), DCM, DMF (cat.), rt, 0.5h; ii) arylamine (1.0 eq.), TEA (1.5 eq.), DCM, rt, overnight. cat: catalytic amount; DCM: dichloromethane; DMSO: dimethyl sulfoxide; DMF: dimethyl formamide; eq: equivalent; h: hour; rt: room temperature; TEA: triethylamine.

To determine the inhibitory activity of calcium influx by the newly synthesized indazole derivatives, we used the RBL-2H3 rodent MC cell line as the primary *in vitro* assay. RBL-2H3 cells are known to express functional CRAC channel. It is a widely used *in vitro* model system to investigate the functions of CRAC channel [36]. Thapsigargin (Tg) is a sarco/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) inhibitor that selectively activates the CRAC channels by depleting Ca^{2+} in the ER store ($[Ca^{2+}]_{ER}$) [37]. Fluo-4NW was used as the molecular sensor to detect the concentration of intracellular calcium ($[Ca^{2+}]_i$). Under our assay conditions, approximately 3.5-fold higher $[Ca^{2+}]_i$ was consistently observed in RBL cells treated with Tg (1 μM) than that in untreated resting MCs. The indazole derivatives **9a-g** and **12a-h** were screened first at 100 and 10 μM concentrations. Those that showed significant inhibition of Ca^{2+} influx at both dose levels were selected for dose-dependent studies to determine their IC_{50} (**Tables 1**). None of the new compounds induced noticeable cytotoxicity or morphological changes in resting RBL cells at concentration as high as 100 μM, as determined by CCK8 cell viability assay.

Table 1. SAR results of indazole derivatives



(**9**)



(**12**)

Compound	**Ar**	**IC_{50} (μM)** [c]
9a	2,6-Difluorophenyl	-[a]
9b	2-Fluorophenyl	29.0
9c	3-Fluoro-4-pyridyl	-[a]
9d	3,5-Difluoro-4-pyridyl	-[a]

9e	4-Methyl-1,2,3-thiadiazol-5-yl	>30 [b]
9f	2,4-Difluorophenyl	- [a]
9g	3-Methyl-4-pyridyl	>30 [b]
12a	2,6-Difluorophenyl	1.51
12b	2-Chloro-6-fluorophenyl	3.23
12c	2-Fluorophenyl	>30 [b]
12d	3-Fluoro-4-pyridyl	0.67
12e	2,4-Difluorophenyl	2.33
12f	3-Methyl-4-pyridyl	- [a]
12g	3,5-Difluoro-4-pyridyl	>30 [b]
12h	1,2,3-Thiadiazol-5-yl	- [a]

Note: [a]: Less than 50% inhibition at 100 μM; [b]: 44-48% inhibition at 30 μM; [c]: standard deviation (SD): <35%.

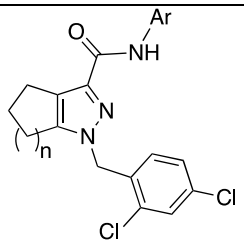
One unexpected finding of the SAR study is that the indazole-3-amide derivatives **9a-g**, which maintain the same amide linker regiochemistry (-NH-CO-Ar) as that of the pyrazole derivative **6** and common to most reported amide type CRAC channel inhibitors (**Figure 1**), are only weakly active in blocking Ca^{2+} influx in Tg-activated RBL cells (**Table 1**). The IC_{50} of the best compound in this series is 29 μM (**9b**), while compounds **9e** and **9g** showed close to 50% inhibition at 30 μM. The others only showed signs of activity at 100 μM.

In contrast, the indazole derivatives **12a-h**, containing the isomeric 'reversed' amide linker (-CO-NH-Ar) as compared to **9a-g**, provide more potent CRAC channel blockers. Thus, the IC_{50} of the most potent blockers in this series, **12d** and **12a**, is 0.67 and 1.51 μM, respectively. The SAR results reveal that the Ar moiety profoundly affects the calcium blocking activity. Potent compounds are obtained from the Ar moieties of the 2,6-difluoropheyl groups (**12a**), the 3-fluoro-4-pyridyl (**12d**). In addition, the 2-chloro-6-fluorophenyl (**12b**, IC_{50}: 3.23 μM) and 2,4-difluoropheyl groups (**12e**, IC_{50}: 2.33 μM) afford slightly less

active inhibitors, while 3,5-difluoro-4-pyridyl (**12g**) and 3-methyl-4-pyridyl (**12f**) groups do not lead to active inhibitors. This finding is consistent with the known CRAC channel blockers **1, 2, 4,** and **6** that contain the same moieties (**Figure 1**).

We further expanded our SAR study to a series of pyrazoles fused to form a 5-, 6-, or 7-membered saturated carbon cycles (**15a-i**, **Table 2**), which all contain the same 3-carboxamide liker (-CO-NH-Ar). In agreement with the findings in the indazole series, the Ar moiety of 3-fluoro-4-pyridyl afforded highly potent Ca^{2+} blockers from the 5- and 6-fused pyrazoles (**15c** and **15f**). We were unable to determine the activity of the 7-fused analog **15i** due to its low solubility. Consistent with the indazole analog **12k**, the Ar moiety of 3-methyl-4-pyridyl in the fused pyrazoles **15a** and **15g** did not afford active blockers (the activity of **15e** was not determined due to low solubility). In the indazole series, the derivative substituted with the Ar moiety of 3,5-difuoro-4-pyridyl group (**12g)** is only weakly active (46% inhibition at 30 μM). In contrast, the 5- and 6-fused pyrazoles **15b** and **15d** with the same Ar moiety of 3,5-difluoro-4-pyridyl are highly active in blocking Ca^{2+} influx. The IC_{50} of **15b** is 0.65 μM, rendering it one of the most active blockers among our compounds. The 7-fused pyrazole **15h** with the same Ar moiety is however nearly 20-fold less active than **15b**.

Table 2. SAR results of fused pyrazoles



(15)

Compound	**Ar**	**n**	**IC_{50} (μM)** [a]
15a	3-Methyl-4-pyridyl	1	>30

15b	3,5-Difluoro-4-pyridyl	1	0.65
15c	3-Fluoro-4-pyridyl	1	0.99
15d	3,5-Difluoro-4-pyridyl	2	1.34
15e	3-Methyl-4-pyridyl	2	N.T. [b]
15f	3-Fluoro-4-pyridyl	2	1.53
15g	3-Methyl-4-pyridyl	3	>30
15h	3,5-Difluoro-4-pyridyl	3	12.8
15i	3-Fluoro-4-pyridyl	3	N.T.

Note: [a]: SD: <35%. [b]: N.T.: not tested due to limited solubility

We selected **12d** from the indazole-3-carboxamides series as the representative CRAC channel blocker to demonstrate its capacity in inhibiting effector functions of activated MCs. **12d** was chosen because of its high potency in blocking calcium influx (IC_{50}: 0.67 μM), as well as its moderate lipophilicity indicated by cLogP of 4.76 (ChemAxom V14.8.18.0). In comparison, the cLogP of compounds **12a** and **15b** is 6.18 and 4.65, respectively.

Activated MCs secrete numerous pro-inflammatory mediators in two phases, the immediate release of preformed mediators stored in cytosolic granules, and the initiation of *de novo* syntheses of pro-inflammatory cytokines and growth factors. We first tested **12d** to determine the inhibition of MC degranulation by measuring the release of pre-stored β-hexosaminidase (β-hex) upon MC activation. In the absence or presence of various concentrations of compound **12d**, RBL-2H3 cells were activated with the treatment of 1 μM thapsigargin in Ca^{2+} free culture. Thirty (30) minutes after assay media were replenished with extracellular Ca^{2+}, supernatants and cell lysates were analyzed for β-hex concentrations by ELISA. The ratio between the β-hex in supernatants and the total amount of β-hex (in supernatant plus cell lysates) indicated **12d** significantly and dose-dependently inhibited the release of β-hex (**Figure 2**). In the absence of a CRAC inhibitor, 40% of β-hex was released, while **12d** showed nearly complete

inhibition of β-hex release at the highest concentration tested. From the dose-response curve, we calculated the EC_{50} of **12d** to be 0.86 μM, which corresponded well with its potency in blocking the CRAC channel activity under similar assay conditions.

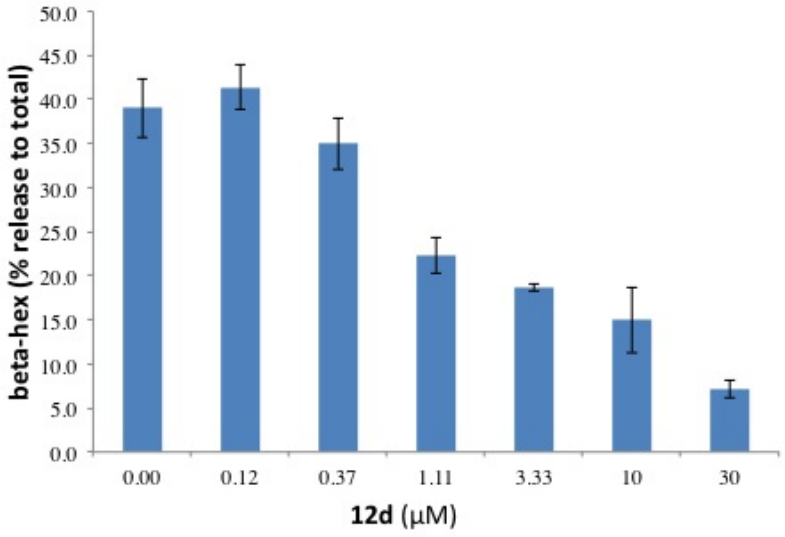


Figure 2. Dose-dependent inhibition by **12d** of β-hex release from activated mast cells. Released β-Hex in cell culture supernatant were measured and compared with total β-Hex in cell lyses (reported as %). The error bars represent standard error.

We next determined the inhibition of nuclear translocation of the nuclear factor of activated T-cells (NFAT) by **12d** in activated MCs. The nuclear factor NFAT is a master regulator of numerous cytokines including TNFα Cytosolic NFAT is dephosphorylated by the phosphatase calcineurin, which leads to the nuclear translocation of NFAT and subsequent gene activations for the expression of the corresponding cytokines. The nuclear translocation of NFAT is Ca^{2+}-dependent and mediated by the CRAC channel [38, 39]. RBL cells were first treated with 1 μM thapsigargin in Ca^{2+} free culture in the

absence or presence of various concentrations of compound **12d**, which was followed by replenishing with extracellular Ca^{2+} for 30 minutes. Nuclear fraction was prepared from the cells by subcellular protein fractionation, and the nuclear NFAT-c1 content was measured by ELISA. The fold increases of nuclear NFAT in activated MC as compared to that in resting MCs indicate the levels of MC activation. In the absence of CRAC channel blockers, we observed a 5-fold increase of nuclear NFAT in activated MCs, and the CRAC blocker **12d** significantly and dose-dependently reduced the nuclear fraction of NFAT-c1 in activated RBL cells (**Figure 3**). Further, at 10 μM and higher concentrations, the CRAC blocker **12d** was able to restore the levels of nuclear NFAT to that of resting MCs. The calculated IC_{50} of **12d** is 1.60 μM, which is comparable to its IC_{50} in blocking Ca^{2+} influx and β-hex release under similar assay conditions.

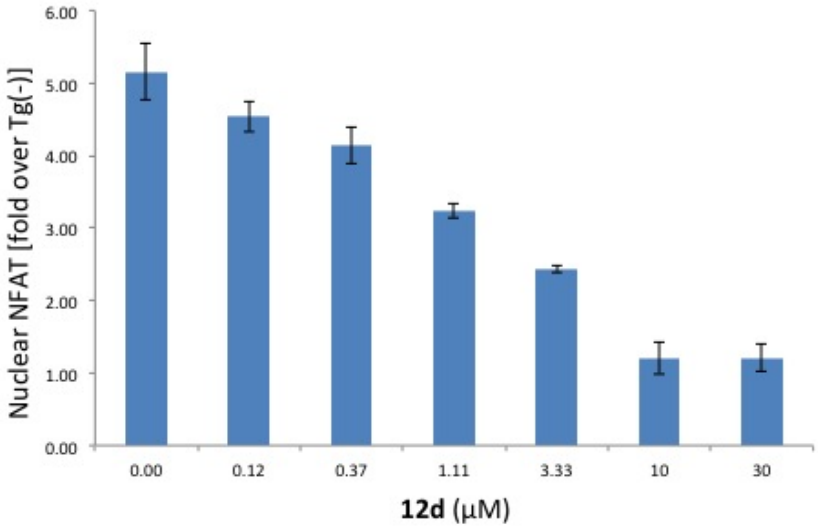


Figure 3. Dose-dependent inhibition by **12d** of nuclear translocation of NFAT in activated mast cells. The error bars represent standard error.

Lastly, we selected representative potent CRAC channel blockers from the indazole-3-carboxamides and demonstrated they dose-dependently inhibited the production of TNFα protein by activated MCs. Mast cells can secrete pre-stored TNFα immediately upon activation, as well as de novo synthesized TNFα that takes a few hours to produce. RBL cells were activated similarly as described above, in the presence of various concentrations of a CRAC channel blocker. 4 Hours after RBL cells were re-exposed to Ca^{2+}, secreted TNFα (which accounted for the combined protein from pre-stored and de novo synthesized TNFα) in the supernatants were measured by ELISA. All the CRAC channel blockers showed dose-dependent inhibition of TNFα protein secretions. As seen in **Table 3**, blocking calcium influx by the indazole type CRAC channel inhibitors is associated with significant of TNFα protein secretion. Among them, compound **12d**, with an IC_{50} of 0.28 μM, is the most potent inhibitor of TNFα production among our series. **12a-b**, and **12e** all demonstrated potent inhibition of TNFα production by activated MCs with sub-μM IC_{50}.

Table 3. Inhibition of TNFα secretion by lead compounds

Compound	IC_{50} (μM) [a]	
	Ca^{2+} influx	TNFα
12a	1.51	0.47
12b	3.23	0.74
12d	0.67	0.28
12e	2.33	0.64

Note: [a]: SD: <20%.

In this report of our preliminary SAR studies, we described the characterizations of a series of novel 1-(2,4-dichlorobenzyl)-indazole-3-carboxamides as potent CRAC channel blockers in activated mast cells. We observed a number of key structural attributes that contribute to the inhibitory activity of calcium influx via the CRAC channel. Most strikingly, the -CO-NH-Ar amide linker regiochemistry is required for activity, which is unique to the indazole type of CRAC channel blockers and opposite to most of the reported amide types of CRAC inhibitors that contain the 'reversed' –NH-CO-Ar linker (**Figure 1**). In addition, the Ar group (of the arylamines) significantly affects the activity, with the compounds containing the 2,6-difluorophenyl and the 3-fluoro-4-pyridyl groups among the best inhibitors. We further demonstrated that the representative indazole **12d** [40] potently inhibits mast cell degranulation, as well as the release of pre-stored pro-inflammatory cytokines and their *de novo* syntheses. Similarly, the Ca^{2+}-dependent nuclear translation of NFAT is reduced by **12d** in Tg-stimulated RBL cells. Therefore, blocking Ca^{2+} influx by CRAC channel inhibitors may be a viable approach to attenuate the inflammatory responses conferred by activated MCs. The 1-(2,4-dichlorobenzyl) moiety affords derivatives with excellent activities. It however also contributes to the increase of the lipophilicity of the derivatives. We will report in due course our ongoing lead optimizations aiming to reduce lipophilicity and improve systemic pharmacokinetic properties.

Acknowledgement

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[40] Analytical data for N-[1-[(2,4-dichlorophenyl)methyl]indazol-3-yl]-3-fluoro-pyridine-4-carboxamide (**12d**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.27 (br s, 1H, amide), 8.58 (t, *J* = 6.4 Hz, 1H), 8.48 (s, 1H), 8.42 (d, *J* = 8.0 Hz, 1H), 8.40 (s, 1H), 7.36-7.50 (m, 4H), 7.14 (dd, *J* = 2.0, 8.4 Hz, 1H), 6.75 (d, *J* = 8.0 Hz, 1H), 5.75 (s, 2H). ^{13}C NMR: δ 160.6, 152.2, 149.7, 147.0, 139.3, 137.2, 133.5, 133.4, 133.3, 133.2, 133.1, 131.1, 130.5, 130.4, 128.3, 124.1, 119.9, 117.3, 111.4, 50.1, MS (ESI+) m/z calc. for $C_{20}H_{14}Cl_2FN_4O$ $[M+H]^+$: 415.05; Found 415.5. HRMS calc. for $C_{20}H_{14}Cl_2FN_4O$ $[M+H]^+$: 415.0529; Found: 415.0522.

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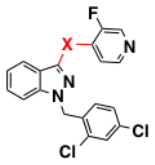

	X: -CO-NH-	-NH-CO-
IC_{50} (μM) - Ca^{2+}:	0.67	>100
- β-Hex:	0.86	
- TNFα:	0.28	
- NFAT:	1.60	