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Structure–activity relationships of pyrazole derivatives as potential therapeutics for immune thrombocytopenias

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ABSTRACT

Idiopathic or immune thrombocytopenia (ITP) is a serious clinical disorder involving the destruction of platelets by macrophages. Small molecule therapeutics are highly sought after to ease the burden on current therapies derived from human sources. Earlier, we discovered that dimers of five-membered heterocycles exhibited potential to inhibit phagocytosis of human RBCs by macrophages. Here, we reveal a structure–activity relationship of the bis-pyrazole class of molecules with –C–C–, –C–N– and –C–O– linkers, and their evaluation as inhibitors of phagocytosis of antibody-opsonized human RBCs as potential therapeutics for ITP. We have uncovered three potential candidates, **37**, **47** and **50**, all carrying a different linker connecting the two pyrazole moieties. Among these compounds, hydroxypyrazole derivative **50** is the most potent compound with an IC_{50} of 14 ± 9 μM for inhibiting the phagocytosis of antibody-opsonized human RBCs by macrophages. None of the compounds exhibited significant potential to induce apoptosis in peripheral blood mononuclear cells (PBMCs). Current study has revealed specific functional features, such as up to 2-atom spacer arm and alkyl substitution at one of the N^1 positions of the bivalent pyrazole core to be important for the inhibitory activity.

1. Introduction

Immune cytopenias are clinical disorders characterized by the production of antibodies to specific hematopoietic cells in the blood.[1–4] Under these conditions, the cells become opsonized with antibodies and are subsequently recognized by the Fcγ-receptors (FcγR) on the mononuclear phagocyte membrane. Such recognition by monocyte–macrophages (Mφ) results in the phagocytosis and intracellular destruction of these cells. This process can create serious and sometimes life-threatening complications for these patients. Examples include warm autoimmune hemolytic anemia (AIHA; involving the phagocytosis of antibody-coated red blood cells), idiopathic/immune (autoimmune) thrombocytopenia (ITP; involving the phagocytosis of antibody-coated platelets) and alloimmune hemolytic anemia (involving the phagocytosis of donor red blood cells transfused into patients having alloantibodies to the donor red cell antigens (HTR) and infants at risk of hemolytic disease of the fetus and newborn (HDFN) due to the maternal antibodies crossing the placenta). In ITP, platelets are destroyed by autologous antibodies and/or the production of platelets is very low.[5]

Current therapies for the treatment of severe cases of ITP include administration of steroids, splenectomy, Rituximab (anti-CD20), thrombopoietin receptor agonists and administration of immunoglobulins.[6,7] Intravenous immunoglobulin (IVIg) and anti-D immunoglobulin are both used with varied success to treat immune cytopenias.[8,9] However, both IVIg and anti-D are a limited resource[10] due to their acquisition from human donations. Treatment with IVIg is also more expensive than that with anti-D,[11] primarily due to the large quantity of IVIg (several grams) that is required for effective therapy; the usual induction dose is 2 g IVIg/kg body weight, which may be followed by maintenance therapy as needed. IVIg cost per individual could be as much as $15,000 or higher, and shortage of IVIg is a major concern due to the need

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0968-0896/

for blood donation by thousands of individuals. Side effects of IVIg are usually mild, with headaches, sometimes severe, being the most debilitating; however, sometimes side effects can be severe and even life threatening.[12] IVIg preparations, because of their human source material, may also carry some risk of transferring blood-borne diseases, particularly any new, emerging blood-borne infections. An alternate therapy, anti-D is required in much less quantity in comparison to IVIg for successful treatment of ITP; however, the reason for this remains unknown. Serious complications from anti-D treatments, although rare, can be more dangerous than those that may occur with IVIg and include increased morbidity due to hemolysis, sometimes so severe that it had resulted in patient mortality.[13–16] Thus, development of novel, cheaper, safer and more efficient ways of treating immune cytopenias is warranted, and blood services agencies globally are in much need of such alternate therapies.

The pathophysiology of immune cytopenias, such as ITP, is due to extravascular phagocytosis of the antibody-opsonized blood cells which results in diminished levels of clinically important cells in the circulation.[16] As the cell destruction is due to recognition of the Fc portion of the opsonizing antibody by the FcγRs on phagocytes, a potential treatment modality would be to develop inhibitors of this antibody-driven FcγR-mediated phagocytosis.

Limited efforts were expended to find replacements for IVIg over the years despite an acute need for such therapeutic agents. A set of molecules carrying sulfhydryl and disulfide functional groups were evaluated for their ability to inhibit the phagocytosis of opsonized blood cells by macrophages.[17–22] It was hypothesized that sulfhydryl and disulfide functional groups found on the small molecules interact with the sulfhydryl or disulfide groups on the surface of phagocytes and thus inhibit the phagocytosis of opsonized red blood cells (RBC).[14,23,24] Among these compounds, a pyrazole derivative **1** (Chart 1) exhibited weak activity at 1 mM concentration inhibiting phagocytosis of the opsonized RBCs.[17] A library of derivatives designed based on **1** led to the isoxazole derivative **2**, with moderate activity (IC_{50} of 250 μM), and to the discovery that the homodimers **3–6** containing a disulfide bridge were more potent inhibitors of RBC phagocytosis.[25] It was observed that the disulfide bridge is more important than a thiol or other forms of polysulfide linkers to elicit the inhibition of phagocytosis.[25] Substitution at C4 of the pyrazole moiety in the disulfide-bridged dimers influenced the potency of these inhibitors. A novel scaffold **7** with no-substitution at the C4 position, exhibiting inhibitory activity with an IC_{50} of 0.1 μM for opsonized RBC phagocytosis, was identified as a potential candidate for further investigations.[25] This also led to the hypothesis to consider designing 'drug-like' chemical structures without a disulfide bridge linking the ligands to target ITP and other similar disorders. Here, we reveal the structure–activity relationships of the pyrazole class of molecules, including compounds replacing the disulfide bridge with –C–C–, –C–N– and –C–O– linkers, and their evaluation as inhibitors of phagocytosis of antibody-opsonized RBCs as therapeutics for ITP.

2. Chemistry and molecular design

A bioisosteric methodology was employed to build structure–activity relationships due to the lack of structural information on the target or large amount of data on the ligands diversity. We considered the data accumulated so far on the thiol and disulfide carrying pyrazole derivatives such as compounds **1–7** which could be modified in a number of ways to replace the disulfide linker, as well as improve the physicochemical properties of the compounds. As a first step for the structure–activity relationships, several modifications to structure **7** were considered: (a) substitution of the disulfide bridge to understand the tolerance and the necessity of this group, (Chart 1B, –X– = $–CH_2–$, $–C_2H_4–$, $–CH_2O–$, $–CH_2NHCH_2–$, –O–, $–OC_2H_4O–$), and (b) substitutions at N^1 on one of the pyrazole moieties to explore the role of substitutions ranging from simple hydroxyl group through cyclopentyl and cyclohexyl moieties (Chart 1B, R = $–CH_2OH$, –OH, –H, $–C_2H_4NH_2$, –Ph, –cyclopentyl, $–CH_2$-cyclohexyl). Thus, compounds **14**, **15**, **18**, **20**, **24**, **28**, **32**, **37**, **41**, **47–50**, and **53** were synthesized carrying the variations described above (Schemes 1–8).

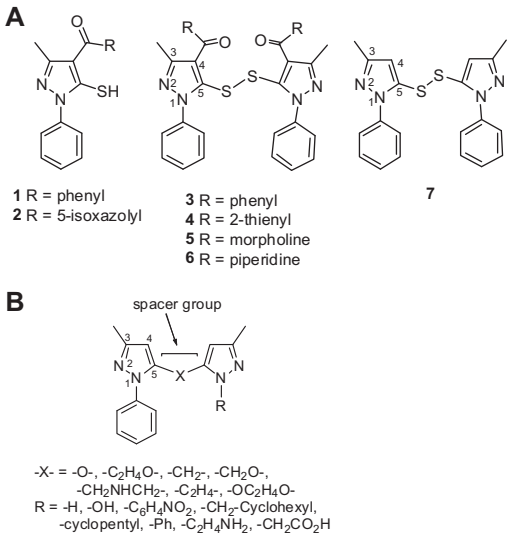
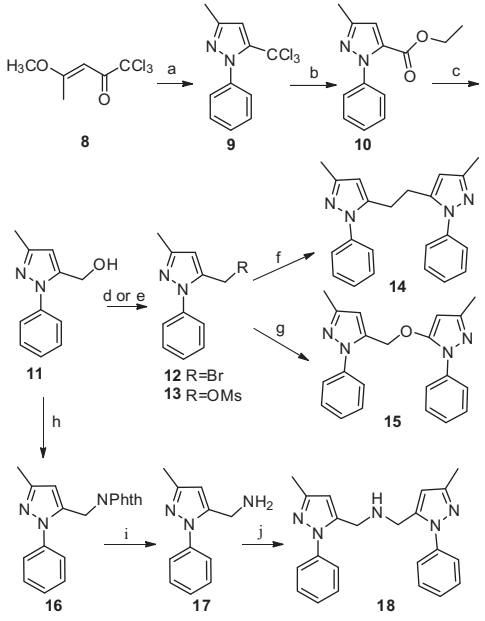
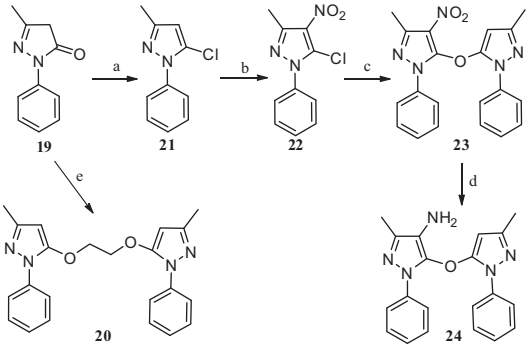


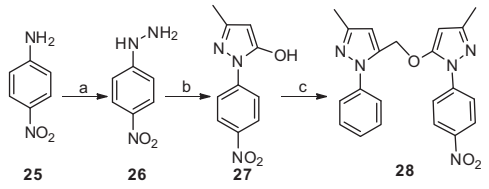
Chart 1. (A) Chemical structures of first generation derivatives inhibiting phagocytosis of opsonized human red blood cells. (B) Structural modifications incorporated into the pyrazole backbone.



Scheme 1. Synthesis of compounds **14**, **15** and **18**. Reagents and conditions: (a) Phenylhydrazine, toluene, μW at 85 °C, 5 min; (b) ethanol, pyridine, reflux, 2 h; (c) $LiAlH_4$, anhyd tetrahydrofuran, 0 °C to rt, 30 min; (d) PBr_3, toluene, reflux, 2 h; (e) methylsulfonyl chloride, Et_3N, anhydrous dichloromethane, reflux, 2 h; (f) magnesium turnings, anhyd tetrahydrofuran, μW, 100 °C, 1 h, additional stirring at rt, 42 h; (g) edaravone (**19**), Cs_2CO_3, anhydrous acetonitrile, 60 °C, 2 h, (h) PPh_3, DEAD, anhydrous tetrahydrofuran, 0 °C to rt, 48 h; (i) $NH_2NH_2.H_2O$, methanol, 0 °C to rt, 16 h; (j) compound **12**, K_2CO_3, acetonitrile, 60 °C, 2 h.

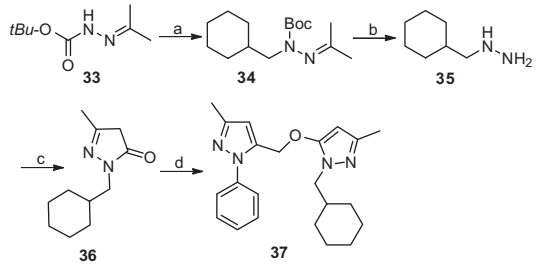


Scheme 2. Synthesis of compound **20** and **24**. Reagents and conditions: (a) edaravone (**19**), $POCl_3$, reflux, 2 h; (b) concd HNO_3, acetic anhydride, 0 °C to rt, 4 h; (c) **19**, K_2CO_3, dimethylformamide, μW, 80 °C, 12 min; (d) $SnCl_2{\cdot}2H_2O$, concd HCl; (e) 1,2-dibromoethane, Cs_2CO_3, acetonitrile, 60 °C, 16 h.

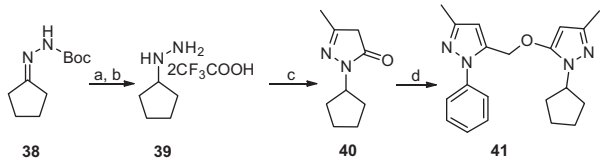


Scheme 3. Synthesis of compound **28**. Reagents and conditions: (a) $NaNO_2$, $SnCl_2{\cdot}2H_2O$, concd HCl, 0 °C; (b) ethylacetoacteate, glacial acetic acid, reflux, 4 h; (c) compound **13**, Cs_2CO_3, anhydrous acetonitrile, 60 °C, overnight.

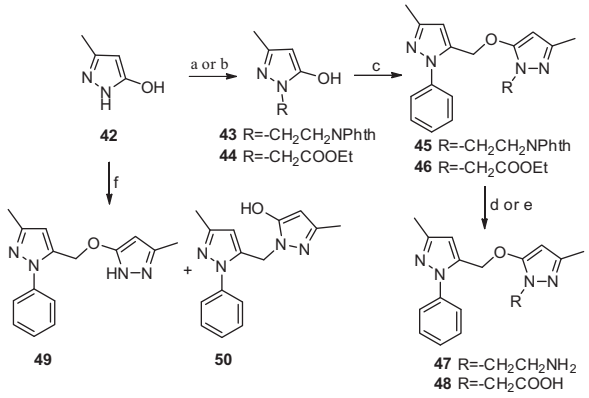
Compounds **14**, **15** and **18** were synthesized via the key intermediate **11** (Scheme 1). The vinyl ketone **8**, synthesized by acylation of the 2-methoxy propene with trichloroacetyl chloride, served as the starting material for the construction of the pyrazole moiety.[26,27] Microwave-assisted cyclocondensation of the 1,3-dienophile building block **8** with phenyl hydrazine yielded compound **9**. The trichloromethyl pyrazole derivative **9** upon refluxing with ethanol gave compound **10** in respectable yield.[28] Reduction of the ester group on compound **10** by lithium aluminum hydride (LAH) yielded the key intermediate **11**. Treatment of compound **11** with phosphorus tribromide or methanesulfonyl chloride yielded compounds **12** and **13**, respectively. Compound **12** was treated with magnesium turnings to generate the corresponding Grignard reagent in situ. The homocoupling of the resultant Grignard from **12** was accelerated by microwave heating for one hour followed by stirring at rt for 42 h to obtain the target compound **14** carrying an ethylene linker (Scheme 1). Alkylation of edaravone (**19**) with the mesylate **13** in the presence of cesium carbonate at 60 °C in anhydrous acetonitrile yielded the target compound **15** with a methyl ether linker (Scheme 1). In order to synthesize compound **18**, alcohol **11** was coupled to phthalimide to obtain the protected amino pyrazole **16**.[29] Phthalimide group was removed by treatment with hydrazine which then was subjected to N-alkylation with **12** in the presence of inorganic base K_2CO_3 to obtain the target compound **18**.



Scheme 5. Synthesis of compound **37**. Reagents and conditions: (a) KOH, Bu_4NHSO_4, toluene, (bromomethyl) cyclohexane, 80 °C, 2 h; (b) 2 N HCl, tetrahydrofuran, reflux, 2 h; (c) ethylacetoacteate, ethanol, reflux, 2 h; (d) compound **12**, Cs_2CO_3, acetonitrile , 60 °C, overnight.

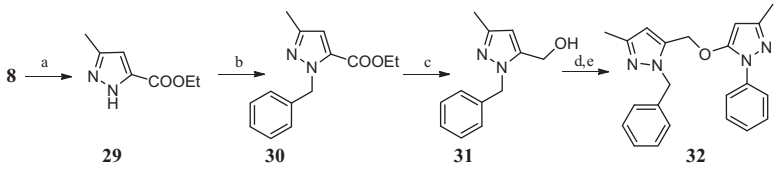


Scheme 6. Synthesis of compound **41**. Reagents and conditions: (a) $LiAlH_4$, anhyd tetrahydrofuran, 0 °C to rt, 30 min; (b) 50% trifluoroacetic acid in dichloromethane, rt, 20 min; (c) ethylacetoacetate, ethanol, reflux; (d) compound **22**, Cs_2CO_3, acetonitrile, 45 °C, 2 h.



Scheme 7. Synthesis of compounds **47–50**. Reagents and conditions: (a) *N*-(2-Bromoethyl) phthalimide, 1,4-dioxane, μW, 180 °C, 60 min; (b) ethylbromoacetate, reflux, 4 h; (c) compound **12**, Cs_2CO_3, acetonitrile, 60 °C, 16 h; (d) $NH_2NH_2{\cdot}H_2O$, methanol, 0 °C to rt, 16 h; (e) LiOH, tetrahydrofuran/methanol (1:1), 0 °C to rt, 16 h; (f) compound **13**, Cs_2CO_3, acetonitrile, 60 °C, 2 h.

Target compounds **20** and **24**, carrying ether linkers, were synthesized following the procedure in Scheme 2. Compound **20** with a four atom spacer between the two pyrazoles was synthesized by refluxing 1,2-dibromoethane with **19** under alkaline conditions (Scheme 2). For the synthesis of target compound **24**, edaravone (**19**) was treated with phosphorus oxychloride to obtain compound **21** as a yellow oil (Scheme 2).[30] Compound **21** was then nitrated at



Scheme 4. Synthesis of compound **32**. Reagents and conditions: (a) Compound **8**, ethanol, $NH_2NH_2{\cdot}HCl$, reflux, 4 h; (b) K_2CO_3, benzyl bromide and acetonitrile, 0 °C to rt, overnight; (c) LAH, THF, 0 °C to rt, 30 min; (d) methanesulfonyl chloride, Et_3N, dichloromethane, reflux 2 h; (e) edaravone, Cs_2CO_3, acetonitrile, 60 °C, 4 h.

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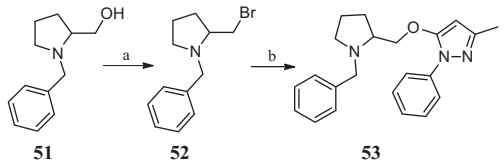


Scheme 8. Synthesis of compound **53**. Reagents and conditions: (a) CBr_4, PPh_3, anhyd tetrahydrofuran, 0 °C to rt, 16 h; (b) compound **19**, K_2CO_3, dimethylformamide, 50 °C, 16 h.

position 4 using a mixture of concd HNO_3 and acetic anhydride.[31] The electron-withdrawing character of the C4 nitro moiety of compound **22** facilitated the nucleophilic substitution at C5 by **19** to afford compound **23** under basic conditions. Nitro group was reduced to an amino moiety using tin(II) chloride to yield the target compound **24** in good yield.

Compound **28**, a nitro derivative of **15**, was synthesized starting from *p*-nitroaniline (**25**). Hydrazine derivative **26** was synthesized by conventional method of diazotization of *p*-nitroaniline (**25**) followed by tin(II) chloride reduction (Scheme 3).[32] Compound **26** was then subjected to Knorr reaction conditions, by treatment with ethylacetoacetate and glacial acetic acid to obtain the pyrazole **27**.[33] Alkylation of *p*-nitrophenyl pyrazole **27** with mesylated intermediate **13** afforded the target compound **28**.

Target compound **32** with a benzyl substitution on one of the pyrazole moieties was obtained starting from the vinyl ketone **8** (Scheme 4). The methyl pyrazole **29** was obtained by the condensation of hydrazine and vinyl ketone **8** in ethanol.[28] N^1-Benzylation of compound **29** was accomplished with benzyl bromide in the presence of K_2CO_3 to obtain benzylated pyrazole **30** which was then further reduced with LAH to obtain compound **31**. Mesylation of **31** followed by alkylation with **19** (edaravone) provided the target compound **32**. Synthesis of the cyclohexylmethyl derivative **37** required an appropriate hydrazine intermediate **35** (Scheme 5). Thus, the Boc-protected hydrazone **33** was alkylated with bromomethyl cyclohexane in the presence of potassium hydroxide and tetrabutylammonium bisulfate at 80 °C in anhydrous toluene to afford **34**.[34,35] Boc and isopropylidene groups were removed under acidic conditions by refluxing in 2*N* HCl.[34] Cyclohexylmethyl hydrazine **35** was then subjected to cyclocondensation with ethylacetoacetate in ethanol to give compound **36**. N^1-Cyclohexylmethyl substituted pyrazolone **36** was then alkylated with the bromide **12** under alkaline conditions to yield the target compound **37** (Scheme 5).

Compound **41**, with a cyclopentyl moiety, was obtained starting from cyclopentyl imine **38** (Scheme 6). The imine **38** was obtained by the condensation of *tert*-butyl carbazate with cyclopentanone.[36] Then the imine **38** was reduced with lithium aluminum hydride followed by acidic deprotection of the carbamate moiety to yield cyclopentyl hydrazine **39**. Cyclocondensation of **39** with ethylacetoacetate provided the key pyrazole intermediate, **40**, which was further alkylated with the bromide **12** to obtain the target compound **41**.

Compounds **47**–**50** were obtained from the pyrazole derivative **42** (Scheme 7). Compound **42** was obtained by the condensation of hydrazine hydrochloride with ethylacetoacetate following Knorr conditions in a microwave.[37] Compound **42** was treated with *N*-bromoethyl phthalimide at 180 °C for 60 min in dioxane under microwave environment to obtain compound **43**. Similarly, compound **44** was obtained by refluxing pyrazole **42** in ethylbromoacetate as a solvent. N^1-Alkyl substituted pyrazole derivatives **43** and **44** were alkylated with the pyrazole **12** to yield compounds **45** and **46**, respectively. Strict anhydrous conditions were maintained for the synthesis of compound **45** because any traces of moisture led to the partial hydrolysis of the phthalimide moiety. The ethyl amino group on compound **45** was fully unmasked upon treatment with hydrazine hydrate in methanol leading to the isolation of compound **47** in respectable yield. Alkaline hydrolysis of the ethyl ester **46** yielded the target compound **48**. Alkylation of compound **42** with compound **13** yielded the regioisomers, **49** and **50** (Scheme 7).

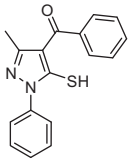
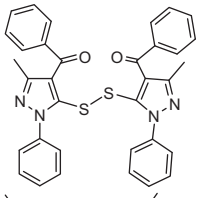
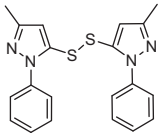
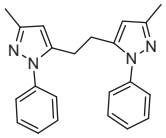
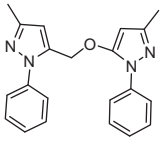
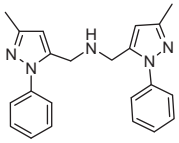
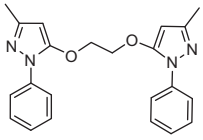
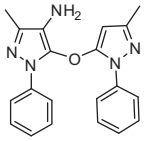
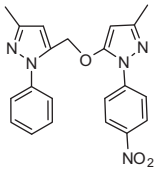
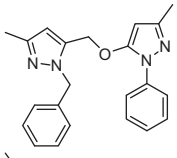
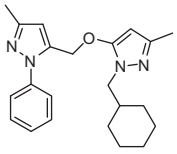
Compound **53** carrying a pyrazole and a pyrrolidine in the heterodimeric molecule was synthesized from the commercially available (1-benzylpyrrolidin-2-yl) methanol, **51** (Scheme 8). Compound **51** was first brominated using classical Appel reaction conditions by treating with carbon tetrabromide and triphenyl phosphine to yield compound **52**. This was used to alkylate edaravone (**19**) to obtain the target compound **53**.[38] All target compounds were assessed for purity, prior to testing their cellular toxicity against mammalian cells, followed by evaluation in the monocyte monolayer assay (MMA) for their ability to inhibit phagocytosis of antibody-opsonized RBCs. Physicochemical parameters, pK_a and $\mathrm{Log}\,D_{7.4}$, were derived for all compounds to investigate the role of these properties towards the inhibition of phagocytosis.

3. Discussion

Phenyl pyrazole structures such as **1**–**6** can be perceived promiscuous inhibitors, thus we approached the challenge of modifying such hit structures with caution to delineate non-specific activity versus any potential to recognize a pharmacophore that would be worthwhile pursuing additional structure–activity relationships. A recent study on the potential of promiscuous hits in HTS campaigns highlighted specific pyrazole compounds, which do not stand the test of assays outside the HTS assay.[39] While our assay is not an HTS assay, but a carefully repeated set of multiple assays using various controls, we initially arrived at pyrazole-containing molecules through identifying thiol-containing molecules,[17,18,25] followed by careful deduction of the structural features to retain the inhibition of phagocytosis by macrophages. Due to the absence of a direct receptor assay, this approach could still be susceptible to unforeseen issues in future. Thus, we are cautiously making structural changes to remove hydrophobic and frequent hit-like structural features from the early hits and improving the solubility of the compounds while retaining the inhibition of phagocytosis of RBCs by macrophages.

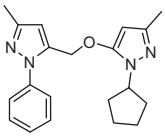
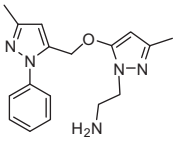
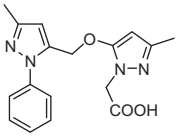
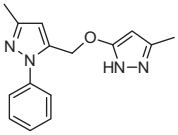
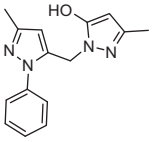
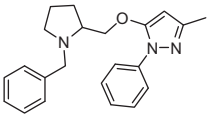
Specifically, we have shown that compound **1** is an inhibitor of RBC phagocytosis with an IC_{50} of approximately 1 mM previously, which was an early hit (Chart 1).[17,18] While this earlier work was driven by the hypothesis that thiol-containing molecules may interact with the thiols on the surface of Fcγ receptor-associated binding pockets, it was not clear if one could avoid non-specific binding of these ligands. Further investigations confirmed that **1** was in fact a mixture of the thiol and its disulfide homodimer, **3**. We discovered that the disulfide derivative **3** (Chart 1), on its own, inhibited phagocytosis of opsonized RBCs by 32% at 5 μM concentration, which is superior to its monomeric form **1** (Table 1 and Fig. 2A).[25] The isoxazole derivative, **2**, was moderately active with an IC_{50} of 250 μM against human RBC phagocytosis. An earlier structure–activity relationship study involving various acyl moieties, due to their ease to synthesize, at the C4 position on the pyrazole led to the synthesis of compounds **3**–**6**, and these compounds inhibited the phagocytosis in the order of potency: **3** (R = phenyl) = **4** (R = 2-thienyl) = **5** (R = morpholinyl) < **6** (R = piperidin-1-yl).[25] To assess the effect of presence of a C4 substitution, compound **7** devoid of any substitutions at C4 was synthesized and evaluated for its ability to inhibit phagocytosis (Fig. 1D). From these earlier investigations, compound **7** was found to be more potent than those carrying a C4 substitution that is compounds **3**–**6**.[25] This analysis indicated that a disulfide bridge with two

Table 1
Inhibition of phagocytosis of antibody-opsonized human RBCs by pyrazole derivatives

Compound	Structure	pK_a	Log $D_{(pH = 7.4)}$	%Inhibition	AI
1		2.9 ± 0.0 (B) 10.3 ± 0.4 (AH)	−0.3 ± 0.0	0 ± 10	2.0; 1.6
3		3.2 ± 0.1 (B)	8.0 ± 0.0	32 ± 7	0.8; 1.0
7		4.8 ± 0.0 (B)	3.2 ± 0.0	45 ± 4	1.0; 5.4
14		2.5 ± 0.1 (B)	1.3 ± 0.0	14 ± 8	1.0; 0.9
15		2.7 ± 0.0 (B)	2.2 ± 0.0	16 ± 11	1.1; 0.9
18		2.2 ± 0.1 (B) 5.6 ± 0.2 (B)	5.0 ± 0.0	20 ± 14	1.1; 0.9
20		2.2 ± 0.0 (B)	−1.6 ± 0.0	4 ± 3	1.0; 1.0
24		3.4 ± 0.0 (B) 7.4 ± 0.2 (B)	4.0 ± 0.0	2 ± 4	1.2; 1.0
28		2.0 ± 0.5 (B)	3.7 ± 0.0	16 ± 3	0.8; 0.8
32		2.1 ± 0.0 (B)	5.3 ± 0.0	0 ± 17	1.1; 1.0
37		3.6 ± 0.1 (B)	4.3 ± 0.0	45 ± 5	1.0; 1.1

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	pK_a	Log$D_{(pH = 7.4)}$	%Inhibition	AI
41		2.7 ± 0.2 (B)	1.7 ± 0.0	17 ± 2	0.9; 1.2
47		2.5 ± 0.3 (B) 10.9 ± 0.3 (B)	5.0 ± 0.0	33 ± 2	1.1; 1.0
48		3.4 ± 0.1 (AH) 7.3 ± 0.2 (B)	1.2 ± 0.0	10 ± 5	1.1; 1.2
49		2.4 ± 0.2 (B) 5.5 ± 0.0 (B) 12.5 ± 0.2 (AH)	2.9 ± 0.0	6 ± 4	1.0; 1.0
50		2.3 ± 0.1 (B) 8.4 ± 0.3 (AH)	4.3 ± 0.0	58 ± 4	1.2; 1.3
53		2.4 ± 0.1 (B) 7.5 ± 0.1 (B)	4.5 ± 0.0	15 ± 14	1.1; 0.9

Percent inhibition of phagocytosis in the MMA and the apoptotic index (AI) reflecting the toxicity of these compounds are specified for each compound. Inhibition is expressed as a relative percent to the +ve control with IVIg, and the concentration of each compound in the MMA was 5 μM. AI for each compound was measured at two separate concentrations (10 and 100 μM). pK_a and Log$D$ were measured at 37 °C.

pyrazole moieties, and minimal or no substitution at C4 on the pyrazole moiety provided superior activity of blocking the phagocytosis of opsonized human RBCs in the monocyte monolayer assay (MMA).

Given the scarcity of human blood cell materials required in large quantities for biological screening assays, we employed a 2-stage screening process to efficiently utilize human PBMCs and RBCs for the MMA. First, all compounds were tested for their potential to induce apoptosis in human PBMCs as a measure of toxicity at two separate concentrations, 10 and 100 μM. Only then, those compounds that did not induce severe apoptosis were considered for screening in the MMA at 5 μM concentration for their ability to inhibit phagocytosis of the opsonized RBCs by human macrophages. A representative phase-contrast microscopy image from the MMA is shown in Figure 1. Only those compounds that exhibited significant inhibition in the initial MMA were further considered for a dose–response titration. This screening strategy allowed us to employ the human blood cells most efficiently for the identification of potent inhibitors and their characterization.

In an attempt to understand the role of the disulfide bridge in the lead molecule **7**, the sulfur atoms were replaced with their bivalent bioisosteres, –CH_2– and –O– in the analogs **14** and **19**, respectively.[40] The screening results of analogs **14** and **15** were unanticipated. The bioiosteric replacement of the sulfur atoms in the disulfide bridge resulted in compromised activity (Table 1 and Fig. 2A). Next, for further investigation, compounds **18**, **20** and **24** were prepared with different spacer groups, and a spacer length of 3, 4 and 1 atoms, respectively. Compound **18** with a 3 atom spacer arm inhibited 20% of phagocytosis at 5 μM concentration. Analogs **20** and **24** comprising of 4 and 1 atom spacer arms, respectively, displayed dramatic decreases in activity at 5 μM concentration in the MMA (Table 1 and Fig. 2A). A modification of the nature and length of the spacer arm between the two pyrazole moieties of lead compound **7** showed reduced activity. Also a 2–3 atom spacer arm resulted in the weakest inhibition (10–20%) of phagocytosis in the MMA. The weak performance of these analogs (**14**, **15**, **18**, **20** and **24**) could be attributed to the varying resultant bond lengths and the torsional angles of different spacer arms between the two heterocycles (Chart 1B). Regardless of this compromised activity, compound **15** was selected for further structural modification studies. It was envisioned that the inhibition potential of the bivalent compound **15** could be improved with a suitable N^1 substitution on one of the pyrazole moieties (Chart 1B).

Previously we reported that the disulfide attached to an aromatic nitro group efficiently inhibited in vitro Fcγ receptor-mediated phagocytosis of opsonized RBCs.[17,18] In order to examine the influence of the strong electron withdrawing nitro group on the activity of the bivalent scaffold **15**, analog **28** with *p*-nitro phenyl introduced at the N^1 position of one of the pyrazole was synthesized and evaluated. However, there was no significant difference between the observed phagocytosis inhibition profile of the unsymmetrical ligand **28** and symmetrical ligand **15** (Table 1 and Fig. 2A). Compound **32** with N^1-benzyl pyrazole was ineffective at inhibiting phagocytosis. Compounds **37** and **41** with six- and five-membered saturated carbocyclic rings at the N^1 position of the pyrazole, respectively, were prepared and tested in vitro in

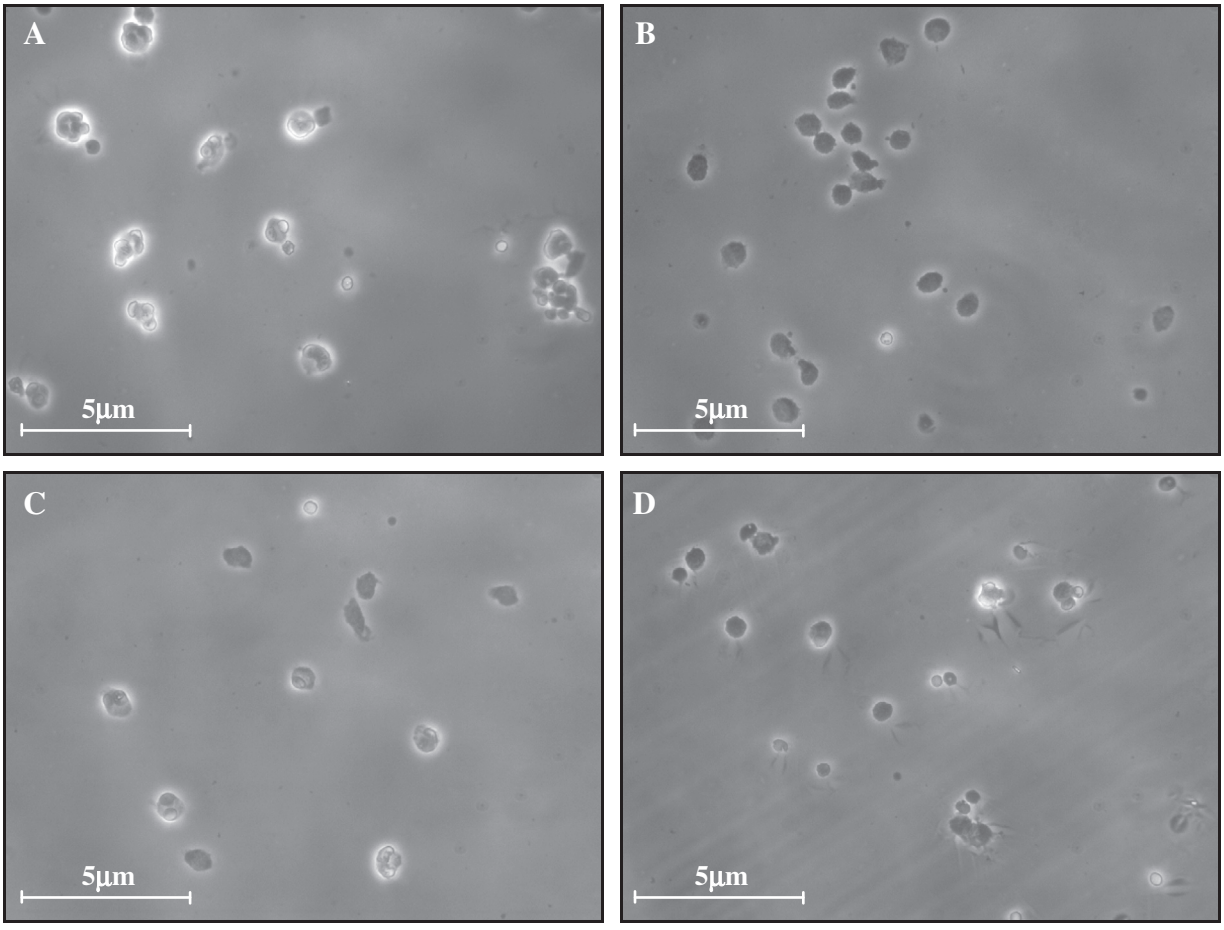


Figure 1. Representative phase-contrast microscopy images of the MMA depicting a 100% phagocytosis of opsonized R2R2 human RBCs as the control with no drug treatment (panel A), phagocytosis inhibition in the presence of IVIg, compound **50** and compound **7** (panels B, C and D, respectively).

the MMA. Compound **37** inhibited 45% of the phagocytosis of opsonized RBCs, similar to that by compound **7**. In contrast, compound **41** with a cyclopentane substitution at N^1 inhibited only 17% of in vitro phagocytosis of opsonized RBCs. Further modifications considered at the N^1 position of the pyrazole of the bivalent scaffold **15** included amino ethyl and methylene carboxylic acid. Ethyl amino derivative **47** suppressed 33% of antibody-opsonized RBC phagocytosis. However, the methyl carboxylic acid derivative **48** exhibited poor inhibition of phagocytosis at 5 μM concentration. Before investigating other substituents at the N^1 position on the pyrazole moieties of compound **15**, we considered it was worth assessing the activity of compounds devoid of mono substitution at N^1 position. Hence, compound **49** was synthesized and evaluated in the MMA. This compound however showed a poor ability to inhibit phagocytosis of opsonized RBCs (only 6%, Table 1). During the synthesis of compound **49**, its regioisomer **50** was formed which was isolated and tested in the MMA. Interestingly, a 58% decrease was observed in the phagocytosis of opsonized RBCs treated with isomer **50** (Table 1 and Fig. 2A). In order to explore the scope of potential substitutes to replace one of the pyrazole rings, the heterodimer **53** with *N*-benzyl pyrrolidine was synthesized. Phagocytosis inhibition by compound **53**-treated monocytes was 15% in the MMA, rendering it more active than the *N*-benzyl substituted pyrazole derivative **32**.

We determined the pK_a and log $D_{7.4}$ parameters of the synthesized compounds (Table 1) to identify any potential relationships of their physicochemical properties to the inhibitory potencies observed in the MMA. Compound **1** with a thiol moiety is a weak inhibitor of phagocytosis. However, **7** with no significant ionization at physiologic pH, exhibited a quite different inhibition profile (45% inhibition), and showed no evidence of inducing apoptosis at lower concentration (AI = 1.0). Most potent compounds, **37** and **50**, exhibited almost equivalent solubility at pH 7.4 based on their Log *D* profile. Compound **37** carries an ether link between the two heterocycles, whereas compound **50** carries a methylene linker; however the substitution pattern on one of the pyrazole moieties is quite different—**37** possesses a cyclohexylmethyl group, whereas **50** has a free hydroxyl moiety (a very weak acidic group with a pK_a of 8.4) on the pyrazole in addition to one carbon spacer to the second pyrazole moiety. Both compounds did not exhibit significant changes in the apoptotic indices when tested at 10 and 100 μM concentrations. Compound **47**, with an ether linker and a primary amine with a tendency to carry a +ve charge at physiologic pH, exhibited moderate potency against phagocytosis of opsonized RBCs (33%). Interestingly, for all these active compounds **7**, **37**, **47** and **50**, log $D_{7.4}$ is in the range of 3–5.

Based on these results, in search of novel small molecules that inhibit Fcγ-dependent phagocytosis of sensitized RBCs, three potential candidates, **37**, **47** and **50**, were selected for further investigations. Among these compounds, **50** is the most potent compound with an IC_{50} of 14 ± 9 μM based on the dose–response profile (Figs. 1C and 2). Current set of compounds did not provide any relationship for the inhibition of phagocytosis on the physicochemical parameters pK_a and log *D*. It is possible that the structural refinement is further needed to engage one or more of these physicochemical properties in a more meaningful way for structure–activity relationship investigations. The current study has revealed the functional features of 2-atom spacer arm and alkyl substitution

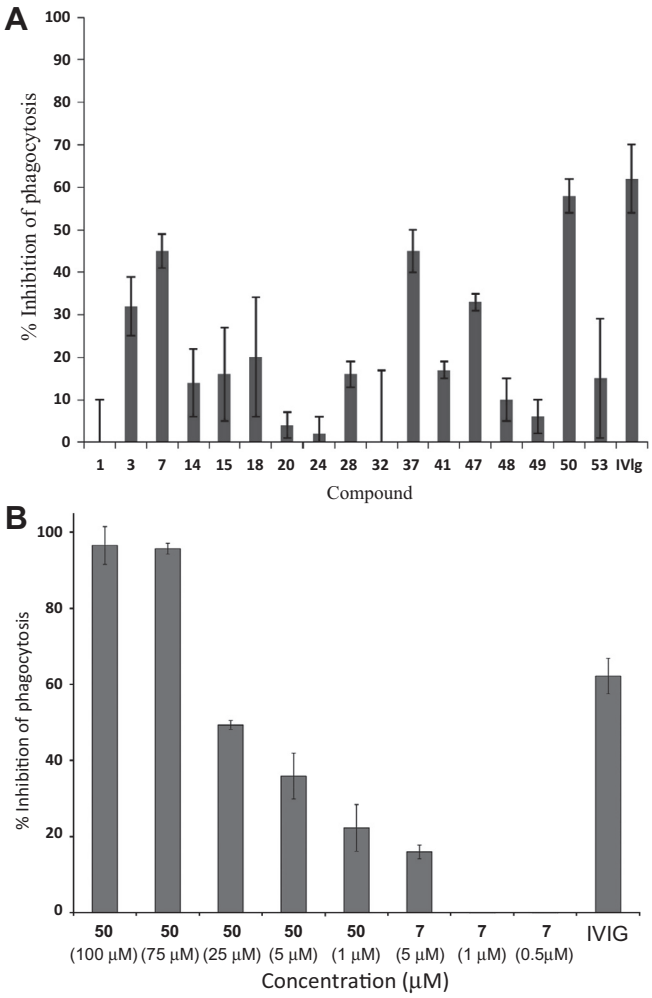


Figure 2. (A) Phagocytosis of antibody-opsonized human RBCs by pyrazole derivatives at 5 μM. (B) Dose–response for compounds **7** (earlier generation compound) and **50**, with the positive control, IVIG. Estimated IC_{50} for **50** is 14 ± 9 μM. Due to the use of human blood from various donors, multiple runs of the MMA assay results in larger standard variations than that is typically seen in standard cell line assays.

at one of the N^1 positions of the bivalent pyrazole core (compounds **37** and **47**), to be critical for the inhibition of antibody-mediated phagocytosis of opsonized RBCs (Fig. 3). An exception is compound **50** with one carbon spacer and this group of molecules will be pursued separately for further optimization.

4. Experimental section

4.1. General

All solvents and reagents were obtained from commercial sources. Column chromatography purifications were performed on Biotage flash chromatography system using normal silica gel (60 Å, 70–230 mesh) and reverse-phase (C18) cartridges. Microwave reactions were conducted in a Biotage Initiator microwave equipped with a robotic arm. Reactions were monitored by thin layer chromatography Merck. NMR spectra were recorded on Bruker spectrometer (400 MHz for ^{1}H). Chemical shifts are reported in δ ppm using residual solvent peak as the reference for the ^{1}H NMR spectra. Purity of the synthesized compounds was determined by Waters™ LC–MS system (Waters™ 2545 binary gradient module) including eluting system. Mass spectra (ESI) were recorded on a Waters™ LC/MS system equipped with a Waters™ 3100 mass detector. Edaravone (**19**) was obtained from commercial vendors.

4.1.1. (*E*)-1,1,1-Trichloro-4-methoxypent-3-en-2-one (8)

In a flame dried flask, a solution of 2-methoxy propene (1.29 g, 17.91 mmol) and pyridine (1.41 g, 17.91 mmol) was cooled to 0 °C. The ionic liquid, 1-butyl-3-methyl-imidazolium hexafluorophosphate (BMIM.PF6) (508 mg, 1.79 mmol) was added to it and was allowed to stir at 0 °C for 5 min. The above reaction mixture of enol ether, pyridine and ionic fluid were added dropwise to trichloroacetyl chloride (3.26 g, 17.91 mmol) at 0 °C. After the addition, the reaction was allowed to return to rt and was stirred for an additional 1 h. Reaction mixture was then diluted with diethyl ether, washed with water, brine and dried over Na_2SO_4. The organic layer was evaporated under reduced pressure to afford brown oil **8** (3.7 g, 16.8 mmol, yield 94%). ^{1}H NMR ($CDCl_3$) δ 2.41 (s, 3H), 3.81 (s, 3H), 6.01 (s, 1H).

4.1.2. 3-Methyl-1-phenyl-5-(trichloromethyl)-1*H*-pyrazole (9)

A microwave reaction vial containing a Teflon stirrer bar was charged with β-methoxyvinyl trichloromethyl ketone (**8**) (442 mg, 2.03 mmol), phenyl hydrazine (220 mg, 2.03 mmol) and anhydrous toluene (5 mL). The vial was capped and the reaction mixture was irradiated with microwaves at 85 °C for 5 min with cooling activated. The reaction solvent was evaporated under reduced pressure. The resulting crude was then purified by flash chromatography on silica gel using gradient 0–4% ethyl acetate in hexanes to afford a white crystalline compound **9** (190 mg, 0.7 mmol, yield 34%). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H), 6.69 (s, 1H), 7.44–7.47 (m, 3H), 7.51–7.54 (m, 2H).

4.1.3. Ethyl 3-methyl-1-phenyl-1*H*-pyrazole-5-carboxylate (10)

To a solution of compound **9** (750 mg, 2.72 mmol) in ethanol (5 mL), pyridine (8.18 mmol) was added and refluxed for 2 h. Reaction was cooled to rt and solvent was evaporated under reduced pressure. The reaction crude was then diluted with dichloromethane (20 mL), washed with 10% HCl (5 mL) and dried over Na_2SO_4. Organic layer was evaporated to obtain compound **10** (582 mg, 2.53 mmol, 93%). ^{1}H NMR ($CDCl_3$) δ 1.27 (t, J = 7.16 Hz, 2H), 2.35 (s, 3H), 4.21 (q, J = 7.16, 7.08 Hz, 2H), 6.80 (d, J = 4.00 Hz, 1H), 7.39–7.44 (m, 5H).

4.1.4. (3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)methanol (11)

To a suspension of lithium aluminium hydride (229.97 mg, 6.06 mmol) in anhydrous tetrahydrofuran, a solution of compound **10** (696 mg, 3.03 mmol) in anhydrous tetrahydrofuran was added dropwise at 0 °C. After the addition, the reaction was warmed to rt and allowed to stir for an additional 30 min. Reaction was then cooled to 0 °C and quenched with a sat Na_2SO_4 solution. The resultant precipitate was filtered under vacuum and the filtrate was evaporated to dryness to obtain a white amorphous solid **11** (515 mg, 2.74 mmol, 90%). ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 3H), 4.65 (d, J = 5.52 Hz, 2H), 6.26 (s, 1H), 7.36 (dd, J = 16.00, 8.00 Hz, 1H), 7.46 (dd, J = 16.00, 8.00 Hz, 2H), 7.58 (d, J = 7.53 Hz, 1H).

4.1.5. 5-(Bromomethyl)-3-methyl-1-phenyl-1*H*-pyrazole (12)

To a solution of compound **11** (92 mg, 0.49 mmol) in toluene, phosphorus tribromide (66.15 mg, 0.25 mmol) was added at rt. Reaction was then refluxed for 2 h, cooled to rt and neutralized with saturated $NaHCO_3$ solution. Aqueous layer was extracted with dichloromethane. Organic layer was dried over Na_2SO_4 and evaporated to yield compound **12** (104 mg, 0.41 mmol, yield 85%). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H), 4.44 (s, 2H), 6.32 (s, 1H), 7.39–7.43 (m, 1H), 7.47–7.51 (m, 2H), 7.55 (d, J = 7.76 Hz, 1H).

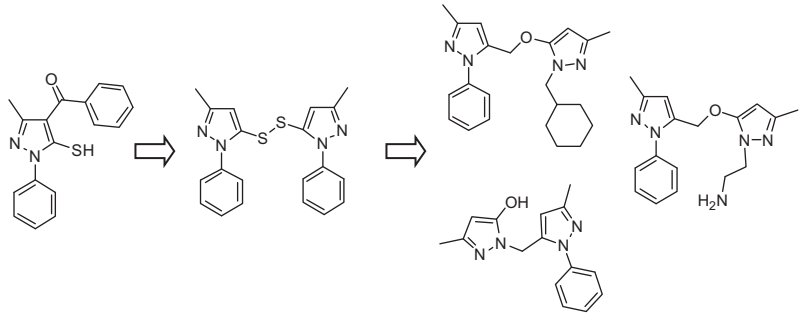


Figure 3. Structural evolution of thiol-containing phenyl pyrazole, a presumably promiscuous molecule, to a non-thiol containing early stage pharmacophore retaining the inhibitory activity against phagocytosis of opsonized RBCs by macrophages.

4.1.6. (3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)methyl methanesulfonate (13)

To a solution of compound **11** (50 mg, 0.26 mmol), triethylamine (80.59 mg, 0.79 mmol) in anhydrous dichloromethane (3 mL) methanesulfonyl chloride (45.7 mg, 0.40 mmol) was added dropwise at 0 °C. After the addition, reaction mixture was refluxed for 2 h. The reaction was then cooled to rt, diluted with dichloromethane (12 mL), washed with a sat. $NaHCO_3$ solution and dried over Na_2SO_4. Organic layer was evaporated to dryness to give compound **13** (65.4 mg, 0.24 mmol, yield 95%). Intermediate **8** was used immediately for the next step without further purification.

4.1.7. 1,2-Bis(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)ethane (14)

A microwave reaction vial containing a Teflon stir bar was charged with magnesium turnings (10.5 mg, 0.43 mmol), was capped and purged with nitrogen and anhydrous tetrahydrofuran (2 mL) was added into the vial. A solution of compound **5** (104 mg, 0.43 mmol) in anhydrous tetrahydrofuran (1 mL) was added to above suspension. The reaction mixture was irradiated with microwaves at 100 °C with cooling activated. The reaction was then allowed to stir at rt for 42 h. On completion, the reaction was quenched with a saturated solution of ammonium chloride at 0 °C. Aqueous layer was then extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The resulting crude was then purified by flash chromatography on silica gel using gradient 0–30% ethyl acetate in hexanes to afford compound **14** (33 mg, 0.09 mmol, yield 45%) as a white solid; mp 132–134 °C, ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 6H), 2.86 (s, 4H), 5.91 (s, 2H), 7.35–7.42 (m, 10 H). MS ESI (+ve) for $C_{22}H_{22}N_4$ ($M+H^+$) calcd 343.302, found 343.321.

4.1.8. 3-Methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1-phenyl-1*H*-pyrazole (15)

A suspension of edaravone (**19**) (41.6 mg, 0.24 mmol) and cesium carbonate (260 mg, 0.79 mmol) in anhydrous acetonitrile (3 mL) was allowed to stir at rt for 5 min. Compound **13** synthesized from **11** (50 mg, 0.26 mmol) was dissolved in 2 mL of anhydrous acetonitrile. The solution of **13** was then added to the above reaction suspension and heated to 60 °C for 2 h. On completion, the reaction mixture was cooled, filtered, and purified by flash chromatography on silica gel using gradient 0–30% ethyl acetate in hexanes to obtain compound **15** (12 mg, 0.03 mmol, yield 15%) as a pale yellow solid; mp 56–58 °C, ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 2.35 (s, 3H), 5.03 (s, 2H), 5.50 (s, 1H), 6.33 (s, 1H), 7.20 (m, 1H), 7.32–7.45 (m, 7H), 7.50–7.53 (m, 2H). MS ESI (+ve) for $C_{21}H_{20}N_4O$ ($M+H^+$): calcd 345.17, found 345.30.

4.1.9. 2-((3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)methyl) isoindoline-1,3-dione (16)

To a reaction mixture of compound **11** (100 mg, 0.53 mmol), triphenylphosphine (167 mg, 0.64 mmol) and phthalimide (78.12 mg, 0.53 mmol) in anhydrous tetrahydrofuran (5 mL) was added diethyl azodicarboxylate (138.62 mg, 0.64 mmol) at 0 °C. After stirring the reaction at rt for 48 h, the solvent was evaporated under vacuum and the resultant crude was purified by flash chromatography using gradient 1–30% ethyl acetate in hexanes to give compound **16** (158 mg, 0.50 mmol, yield 94%). ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 3H), 4.83 (s, 2H), 6.08 (s, 1H), 7.34–7.38 (m, 1H), 7.42–7.50 (m, 4H), 7.67–7.71 (m, 2H), 7.78–7.81 (m, 2H).

4.1.10. (3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)methanamine (17)

To a solution of compound **16** (158 mg, 0.50 mmol) in methanol (2 mL) was added hydrazine hydrate (125 mg, 2.5 mmol) at 0 °C. The reaction mixture was then warmed up to rt and stirred overnight. The reaction solvent was evaporated under vacuum and the resultant crude was suspended in methanol (2 mL). The white insoluble precipitate was filtered off and the filtrate was evaporated to afford compound **17** (79 mg, 0.42 mmol, yield 84%) as an oil. ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H), 3.83 (s, 2H), 6.13 (s, 1H), 7.30–7.33 (s, 1H), 7.38–7.43 (m, 4H).

4.1.11. Bis-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methyl)amine (18)

To a flask containing suspension of compound **17** (58 mg, 0.26 mmol) and potassium carbonate (112 mg, 0.79 mmol) in anhydrous acetonitrile (2 mL) was added a solution of compound **12** (65 mg, 0.26 mmol), in acetonitrile (1 mL) at rt. The reaction mixture was then heated to 60 °C for 2 h. The reaction mixture was then concentrated under vacuum and purified by flash chromatography using gradient 10–80% ethyl acetate in hexanes to obtain compound **18** (41 mg, 0.11 mmol, yield 43%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 2.15 (s, 3H), 2.33 (s, 3H), 2.95 (br t, 2H), 3.82 (t, J = 6 Hz, 2H), 4.96 (s, 2H), 5.31 (s, 1H), 6.32 (s, 1H), 7.34–7.48 (m, 5H). MS ESI (+ve) for $C_{22}H_{23}N_5$ ($M+H^+$): calcd 358.20, found 358.29.

4.1.12. 1,2-Bis(3-methyl-1-phenyl-1*H*-pyrazol-5-yloxy)ethane (20)

To a suspension of edaravone (**19**) (402 mg, 2.31 mmol) and cesium carbonate (1.8 g, 5.52 mmol) in anhydrous acetonitrile (5 mL) 1,2-dibromoethane (217 mg, 1.15 mmol) was added. The reaction mixture was heated to 60 °C and the reaction was continued overnight. The reaction was filtered and the filtrate was concentrated under vacuum. The crude was purified by flash chromatography using gradient 10–100% ethyl acetate in hexanes to obtain product **20** (221 mg, 0.59 mmol, yield 51%) as a yellow solid; mp 58–60 °C,

^{1}H NMR ($CDCl_3$) δ 2.26 (s, 6H), 4.32 (s, 4H), 5.50 (s, 2H), 7.17–7.22 (m, 2H), 7.27–7.32 (m, 4H), 7.60–7.63 (m, 4H). MS ESI (+ve) for $C_{22}H_{22}N_4O_2$ ($M+H^+$): calcd 375.18, found 375.30.

4.1.13. 5-Chloro-3-methyl-1-phenyl-1*H*-pyrazole (21)

To a flask containing edaravone (**19**) (2.0 g, 11.48 mmol) phosphorus oxychloride (3 mL) was added and refluxed for 2 h. The reaction mixture was poured over crushed ice, the aqueous layer was extracted with ethylacetate which was then washed with brine, dried over Na_2SO_4 and was concentrated under vacuum. The resultant crude was purified on a short column of silica gel using gradient 2–5% ethyl acetate in hexanes to give compound **21** as an yellow oil (1.8 g, 9.34 mmol, 79%). ^{1}H NMR ($CDCl_3$) δ 2.32 (s, 3H), 6.19 (s, 1H), 7.35–7.42 (m, 1H), 7.43–7.50 (m, 2H), 7.51–7.57 (m, 2H).

4.1.14. 5-Chloro-3-methyl-4-nitro-1-phenyl-1*H*-pyrazole (22)

To a round bottom flask containing compound **21** (110 mg, 0.57 mmol) was added acetic anhydride (1.5 mL) and cooled to 0 °C. Concentrated nitric acid (1 mL) was then added dropwise. The reaction mixture was warmed up to rt and stirred for 4 h. The reaction solution was then poured over crushed ice. The yellow precipitate was collected and purified by flash chromatography using gradient 1–5% ethylacetate in hexanes to obtain compound **22** (126 mg, 93%). ^{1}H NMR ($CDCl_3$) δ 2.64 (s, 3H), 7.21–7.55 (m, 5H).

4.1.15. 3-Methyl-5-(3-methyl-1-phenyl-1*H*-pyrazol-5-yloxy)-4-nitro-1-phenyl-1*H*-pyrazole (23)

In a microwave reaction vial containing a Teflon stirrer bar, compound **22** (50 mg, 0.21 mmol), edaravone (**19**) (36.6 mg, 0.21 mmol) and potassium carbonate (87 mg, 0.63 mmol) were added, and the vial was capped and purged with nitrogen. To this reaction vial, anhydrous dimethylformamide (0.5 mL) was added and the reaction mixture was irradiated with microwaves at 80 °C (high absorption settings) for 12 min with cooling activated. The crude reaction mixture was then purified by flash chromatography using gradient 1–20% ethyl acetate in hexanes to afford compound **23** as an yellow solid (65 mg, 82%); mp 76–78 °C; ^{1}H NMR ($CDCl_3$) δ 2.22 (s, 3H), 2.64 (s, 3H), 5.47 (s, 1H), 7.30–7.34 (m, 1H), 7.40–7.45 (m, 5H), 7.51–7.54 (m, 2H), 7.62–7.66 (m, 2H).

4.1.16. 3-Methyl-5-(3-methyl-1-phenyl-1*H*-pyrazol-5-yloxy)-1-phenyl-1*H*-pyrazol-4-amine (24)

To a solution of compound **23** (20 mg, 0.05 mmol) in concd HCl (1 mL), tin (II) chloride dihydrate (60.10 mg, 0.26 mmol) dissolved in concd HCl (1 mL) was added at 0 °C. The reaction was warmed up to rt and stirred for 1 h. On completion, the reaction mixture was poured over crushed ice and neutralized with a saturated $NaHCO_3$ solution. The organic layer was extracted with ethyl acetate, then washed with brine, dried over Na_2SO_4 and concentrated under vacuum. The crude was purified by flash chromatography using gradient 20–100% ethyl acetate in hexanes to obtain product **24** (8.3 mg, 45%) as a pale yellow solid; mp: 90–92 °C, ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 2.27 (s, 3H), 2.639 s, br, 2H), 5.50 (s, 1H), 7.20–7.23 (m, 1H), 7.28–7.34 (m, 3H), 7.40–7.44 (m, 2H), 7.47 (d, J = 7.84 Hz, 2H), 7.64 (d, J = 7.60 Hz, 2H). MS ESI (+ve) for $C_{20}H_{19}N_5O$ ($M+H^+$): calcd 346.17, found 346.27

4.1.17. (4-Nitrophenyl)hydrazine, hydrochloride salt (26)

p-Nitroaniline (**25**) (1.47 g, 10.6 mmol) was dissolved in concd HCl (2 mL) and cooled to 0 °C. A solution of sodium nitrite (717 mg, 10.4 mmol) in ice-cooled water (4 mL) was added drop wise to above reaction mixture and stirred for 1 h in an ice bath. Ice-cooled solution of stannous chloride (4.7 g, 20.8 mmol) in concd HCl (2 mL) was added slowly to above diazonium reaction mixture. The reaction was stirred at 0 °C for 2 h. A yellow–orange precipitate was collected and washed thoroughly with ice-cold water until the filtrate pH was neutral. The precipitate was dried overnight under vacuum to give compound **26** (640 mg, 4.18 mmol, 39%); ^{1}H NMR (DMSO-d_6) δ 4.49 (s, 2H), 6.78 (d, J = 8.78 Hz, 2H), 7.98 (d, J = 9.29 Hz, 2H), 8.41 (s, 1H).

4.1.18. 3-Methyl-1-(4-nitrophenyl)-1*H*-pyrazol-5(4*H*)-one (27)

To a solution of compound **26** (219 mg, 0.97 mmol) in glacial acetic acid (2 mL) ethyl-3-oxobutanoate (167 mg, 1.28 mmol) was added and the reaction mixture was refluxed for 3 h. The reaction mixture was cooled to 0 °C, diethyl ether (5 mL) was added to it and the stirring was continued for 1 h at 0 °C. The reaction mixture was filtered and the collected yellow–brown precipitate was washed with diethyl ether. The precipitate was then purified by flash chromatography on silica gel using gradient 10–80% ethyl acetate in hexanes to afford compound **27** (95 mg, 0.43 mmol, yield 45%). ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 3H), 3.50 (s, 2H), 8.13 (d, J = 7.78 Hz, 2H), 8.26 (d, J = 8.53 Hz, 2H).

4.1.19. 3-Methyl-5-({[3-methyl-1-(4-nitrophenyl)-1*H*-pyrazol-5-yl]oxy}methyl)-1-phenyl-1*H*-pyrazole (28)

Compound **27** (113 mg, 1.02 mmol) and cesium carbonate (334.20 mg, 0.61 mmol) were suspended in anhydrous acetonitrile (2 mL). Compound **12** (128 mg, 0.51 mmol) was dissolved in 2 mL of anhydrous acetonitrile and added drop wise to above reaction suspension. The reaction was then heated to 60 °C overnight. The solvent was removed under vacuum and the resulting crude was purified by flash chromatography on silica gel using gradient 0–50% ethyl acetate in hexanes to afford product **28** (25 mg, 0.06 mmol, 12%) as a dark yellow solid. mp 116–118 °C, ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 2.37 (s, 3H), 5.13 (s, 2H), 5.54 (s, 1H), 6.38 (s, 1H), 7.39–7.52 (m, 5H), 7.76 (d, J = 9.29 Hz, 2H), 8.19 (d, J = 9.29 Hz, 2H). MS ESI (+ve) for $C_{21}H_{19}N_5O_3$ ($M+H^+$): calcd 390.42, found 390.32.

4.1.20. Ethyl 3-methyl-1*H*-pyrazole-5-carboxylate (29)

To a stirred suspension of hydrazine mono hydrochloride (642 mg, 9.38 mmol) in ethanol (5 mL) was added β-methoxy vinyl trichloromethylketone (**8**) (1.7 g, 7.80 mmol) at rt. After refluxing for 4 h, the reaction mixture was filtered, the solvent was removed under vacuum and the resultant crude was recrystallized in a mixture of ethyl acetate and hexanes (1:1) to obtain product **29** (900 mg, 5.83 mmol, yield 75%). ^{1}H NMR ($CDCl_3$) δ 1.34 (t, J = 7.16 Hz, 3H), 2.36 (s, 3H), 4.35 (q, J = 7.08, 7.16 Hz, 2H), 6.57 (s, 1H), 9.88 (br s, 1H).

4.1.21. Ethyl 1-benzyl-3-methyl-1*H*-pyrazole-5-carboxylate (30)

To a flask containing suspension of compound **29** (313 mg, 2.03 mmol) and potassium carbonate (842 mg, 6.09 mmol) in anhydrous acetonitrile (4 mL) benzyl bromide (347.19 mg, 2.03 mmol) was added at 0 °C. The reaction mixture was then stirred at rt overnight. The reaction solvent was removed under vacuum and the resultant residue was purified by flash chromatography using gradient 1–40% ethyl acetate in hexanes to afford compound **30** (114 mg, 23%) as a clear liquid. ^{1}H NMR ($CDCl_3$) δ 1.31 (t, J = 7.08 Hz, 3H), 2.29 (s, 3H), 4.27 (q, J = 7.16, 7.12 Hz, 2H), 5.70 (s, 2H), 6.65 (s, 1H), 7.20–7.25 (m, 3H), 7.26–7.30 (m, 2H).

4.1.22. (1-Benzyl-3-methyl-1*H*-pyrazol-5-yl)methanol (31)

To a suspension of lithium aluminium hydride (53.12 mg, 1.39 mmol) in anhydrous tetrahydrofuran (3 mL) at 0 °C, a solution of compound **30** (112 mg, 0.45 mmol) in tetrahydrofuran (2 mL) was added dropwise, the reaction mixture was warmed up to rt and the stirring was continued for 30 min. The mixture was then cooled to 0 °C and quenched with a saturated solution of Na_2SO_4.

The suspension was filtered and filtrate was evaporated under vacuum to yield compound **31** (89 mg, 0.44 mmol, yield 98%). ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H), 4.51 (s, 2H), 5.34 (s, 2H), 6.04 (s, 1H), 7.12–7.15 (m, 2H), 7.26–7.33 (s, 3H).

4.1.23. 1-Benzyl-3-methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yloxy)methyl)-1*H*-pyrazole (32)

To a solution of compound **31** (64 mg, 0.31 mmol), triethylamine (95.84 mg, 0.94 mmol) in anhydrous dichloromethane (3 mL) and methanesulfonyl chloride (54.29 mg, 0.47 mmol) was added sequentially dropwise at 0 °C. After the addition, reaction mixture was refluxed for 2 h. The reaction was then cooled to rt, diluted with dichloromethane (12 mL), washed with a saturated $NaHCO_3$ solution and dried over anhydrous Na_2SO_4. The organic layers we combined and concentrated to give the mesylated derivative of **31**. The crude mesylated derivative of **31** was dissolved in anhydrous acetonitrile (2 mL) and added to a flask containing suspension of edaravone (**19**) (55 mg, 0.31 mmol) and cesium carbonate (309 mg, 0.94 mmol) in anhydrous acetonitrile (3 mL). After heating at 60 °C for 4 h, the reaction mixture was cooled at rt, the solvent was removed under vacuum and the resultant crude was purified by flash chromatography using gradient 1–35% ethyl-acetate in hexanes to afford product **32** as a white solid (40 mg, 0.11 mmol, yield 36%). mp 68–70 °C; ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 2.29 (s, 3H), 4.90 (s, 2H), 5.25 (s, 2H), 5.47 (s, 1H), 6.15 (s, 1H), 7.00–7.02 (m, 2H), 7.21–7.24 (m, 4H), 7.33–7.37 (m, 2H), 7.52–7.54 (m, 2H). MS ESI (+ve) $C_{22}H_{22}N_4O$ ($M+H^+$): calcd 359.19, found 359.29.

4.1.24. 1-(Pivaloyloxy)-2-(propan-2-ylidene) hydrazine (33)

A suspension of *t*-butyl carbazate (1 g, 7.56 mmol) and magnesium sulfate (182 mg, 1.51 mmol) in anhydrous acetone (7.5 mL) with catalytic amount of acetic acid (18.4 mg, 0.36 mmol) was refluxed for 1 h. The reaction mixture was then cooled to rt and filtered. The organic filtrate was evaporated under reduced pressure to afford white crystalline solid **33** (1.23 mg, 7.14 mmol, yield 94%). ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H), 1.74 (s, 3H), 1.93 (s, 3H), 7.51 (s, br, 1H).

4.1.25. *tert*-Butyl-1-(cyclohexylmethyl)-2-(propan-2-ylidene)hydrazine carboxylate (34)

A suspension of compound **33** (496 mg, 2.88 mmol), solid potassium hydroxide (182 mg, 3.24 mmol) and tetrabutyl ammonium bisulfate (11 mg, 0.29 mmol) in toluene (4 mL) was heated to 50 °C and the reaction was conducted for 2 h with vigorous stirring. To the above reaction mixture bromomethyl cyclohexane (639 mg, 3.61 mmol) was added dropwise and the reaction mixture was heated to 80 °C. After 2 h, the reaction was cooled to rt, diluted with ethyl acetate, washed with water, brine and dried over anhydrous Na_2SO_4. The organic layer was concentrated to yield the product **34** as a yellow oil which is a mixture of rotamers (720 mg, 2.68 mmol, 93%); ^{1}H NMR ($CDCl_3$) δ 0.84–1.07 (m, 2H), 1.08–1.32 (m, 4H), 1.44 and 1.50 (s, 9H), 1.57–1.77 (m, 5H), 1.80 and 1.86 (s, 3H), 2.03 and 2.06 (s, 3H), 3.31 (d, J = 8.00 Hz, 2H).

4.1.26. Cyclohexylmethylhydrazine (35)

To compound **34** (800 mg, 2.98 mmol), was added 2*N* HCl in THF (217 mg, 5.96 mmol) at rt. The reaction mixture was refluxed for 2 h and after cooling to rt, the solvent was evaporated under vacuum. The crude residue was dissolved in ethanol (3 mL) and cooled to 8 °C. Diethyl ether was then added slowly to precipitate dihydrochloride salt of **35** as a white solid (303 mg, 2.34 mmol, 78%); ^{1}H NMR (DMSO-d_6) δ 0.84–0.93 (m, 2H), 1.04–1.22 (m, 3H), 1.58–1.74 (m, 6H), 2.72 (d, J = 6.72 Hz, 1H), 6.90 (m, 7H).

4.1.27. 1-(Cyclohexylmethyl)-3-methyl-1*H*-pyrazol-5(4*H*)-one (36)

To a solution of compound **35** (150 mg, 0.74 mmol) in ethanol (3 mL), ethylacetoacetate (116 mg, 0.89 mmol) was added and the reaction mixture was refluxed for 2 h. The solvent was removed under vacuum and the resulting crude was purified by flash chromatography on silica gel using gradient 0–15% methanol in dichloromethane to afford compound **36** (42 mg, 29%). ^{1}H NMR ($CDCl_3$) δ 0.90–1.00 (m, 2H), 1.12–1.23 (m, 3H), 1.64–1.75 (m, 6H), 2.08 (s, 3H), 3.19 (s, 2H), 3.44 (d, J = 7.08 Hz, 2H).

4.1.28. 1-(Cyclohexylmethyl)-3-methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1*H*-pyrazole (37)

Compound **36** (40 mg, 0.20 mmol) and cesium carbonate (201 mg, 0.61 mmol) were suspended in anhydrous acetonitrile (2 mL). Compound **12** (65 mg, 0.26 mmol) was dissolved in 2 mL of anhyd acetonitrile and added dropwise to above reaction suspension. The reaction was then heated to 60 °C overnight. The solvent was evaporated under vacuum, and the resulting crude was purified by flash chromatography on silica gel using gradient 0–40% ethyl acetate in hexanes to give **37** as a pale yellow solid (20 mg, 27%); mp 86–88 °C; ^{1}H NMR ($CDCl_3$) δ 0.80–0.98 (m, 2H) 1.05–1.29 (m, 5H) 1.58–1.87 (m, 5H) 2.18 (s, 3H) 2.37 (s, 3H) 3.62 (d, J = 7.28 Hz, 2H) 4.94 (s, 2H) 5.30 (s, 1H) 6.34 (s, 1H) 7.33–7.56 (m, 5H); MS ESI (+ve) for $C_{22}H_{28}N_4O$ ($M+H^+$): calcd 365.23, found: 365.36.

4.1.29. *tert*-Butyl-2-cyclopentylidenehydrazinecarboxylate (38)

A suspension of *tert*-butyl carbazate (4.46 g, 33.77 mmol) and cyclopentanone (2.84 mg, 33.77 mmol) in hexanes (35 mL) was refluxed for 30 min. The reaction mixture was cooled and filtered. A white precipitate was collected and dried over vacuum to afford compound **38** (6.4 g, 95%); ^{1}H NMR ($CDCl_3$) δ 1.49 (s, 9H), 1.69–1.76 (m, 2H), 1.80–1.87 (m, 2H), 2.15 (t, J = 7.15 Hz, 2H), 2.45 (t, J = 7.28 Hz, 2H), 7.19 (br s, 1H).

4.1.30. Cyclopentylhydrazine (39)

To a suspension of lithium aluminium hydride (268 mg, 7.06 mmol) in anhydrous tetrahydrofuran (2 mL) at 0 °C, a solution of compound **38** (700 mg, 3.53 mmol) in tetrahydrofuran (2 mL) was added dropwise. The reaction mixture was then warmed to rt and was allowed to stir for an additional 30 min. The reaction was then cooled to 0 °C and quenched with a saturated sodium sulfate solution. The resultant precipitate was filtered under vacuum and the filtrate was concentrated. The residue was then dissolved in Dichloromethane (4 mL), cooled to 0 °C, and a mixture of trifluoroacetic acid and dichloromethane (1:1, 1 mL) was added dropwise, and the solution was stirred at rt for 20 min. Then the solvent was evaporated under vacuum to yield **39** (932 mg, 80%). Compound **39** was used in the next step without any further purification.

4.1.31. 1-Cyclopentyl-3-methyl-1*H*-pyrazol-5(4*H*)-one (40)

To a solution of compound **39** (932 mg, 2.83 mmol) in ethanol (5 mL) ethylacetoacetate (50 mg, 3.11 mmol) was added. The reaction mixture was refluxed overnight and the solvent was removed under vacuum. The crude was purified by flash chromatography on silica gel using gradient 0–50% ethylacetate in hexanes to yield the product **40** (60 mg, 13%); ^{1}H NMR ($CDCl_3$) δ 1.58–1.59 (m, 2H), 1.79–190 (m, 6H), 2.09 (s, 3H), 3.19 (s, 2H), 4.53–4.57 (m, 1H).

4.1.32. 1-Cyclopentyl-3-methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1*H*-pyrazole (41)

Compound **40** (60 mg, 0.36 mmol) and potassium carbonate (54 mg, 0.39 mmol) were suspended in anhydrous dimethylformamide (1 mL). The reactant **13** (90 mg, 0.36 mmol), was dissolved

in 1 mL of anhydrous dimethylformamide and was added dropwise to the above suspension. The reaction was continued for an additional 2 h at 45 °C. The solvent was evaporated under vacuum, and the crude was purified by flash chromatography on silica gel using gradient 0–40% ethylacetate in hexanes to obtain the product **41** as a pale yellow solid (42 mg, 34%); mp 56–58 °C; ^{1}H NMR ($CDCl_3$) δ 1.47–1.65 (m, 2H), 1.77–1.98 (m, 6H), 2.13–2.22 (m, 3H), 2.29–2.43 (m, 3H), 4.35–4.51 (m, 1H), 4.95 (s, 2H), 5.25–5.36 (m, 1H), 6.35 (s, 1H), 7.34–7.56 (m, 5H); MS ESI (+ve) for $C_{20}H_{24}N_4O$: calcd 337.203, found: 337.343 for ($M+H^+$).

4.1.33. 3-Methyl-1*H*-pyrazol-5-ol (42)

To a suspension of hydrazine hydrate (2.6 g, 20 mmol) in methanol (5 mL), 3-keto-ethyl butanoate (1.0 g, 20 mmol) was added. The reaction mixture was then refluxed for 2 h. The reaction solvent was evaporated under vacuum to afford white solid product **42** (1.92 g, 98%); ^{1}H NMR ($CDCl_3$) δ 2.08 (s, 3H), 5.20 (s, 1H), 10.07 (br s, 1H).

4.1.34. 2-(2-(5-Hydroxy-3-methyl-1*H*-pyrazol-1-yl)ethyl)isoindoline-1,3-dione (43)

Compound **42** (500 mg, 5.10 mmol) and *N*-(2-bromoethyl)-phthalimide (1.29 g, 5.58 mmol) were suspended in 1,4-dioxane (3 mL) in a microwave reaction vial containing a Teflon stir bar. The vial was capped and the reaction mixture was irradiated with microwaves at 180 °C for 1 h at normal absorption with cooling activated. The reaction solvent was evaporated under vacuum, and the crude was purified by flash chromatography on silica gel using gradient 0–10% methanol in dichloromethane to afford product **43** (335 mg, 24%); ^{1}H NMR ($CDCl_3$) δ 1.98 (s, 3H), 3.10 (s, 2H), 3.94 (s, 4H), 7.68–7.70 (m, 2H), 7.80–7.82 (m, 2H).

4.1.35. Ethyl-2-(3-methyl-5-oxo-4,5-dihydro-1*H*-pyrazol-1-yl)acetate (44)

To a flask containing compound **42** (547 mg, 5.58 mmol) was added ethyl bromoacetate (932 mg, 5.58 mmol) and refluxed for 4 h. Then, the reaction was cooled, diluted with ice-cooled water (2 mL), neutralized with cold saturated solution of $NaHCO_3$ and stirred for 15 min at rt. The aqueous reaction mixture was extracted with ethyl acetate and the organic layer was washed with brine, dried over Na_2SO_4 and concentrated. The crude was then purified by silica gel flash chromatography using gradient 0–10% methanol in dichloromethane to yield compound **44** as a white solid (138 mg, 13%); ^{1}H NMR ($CDCl_3$) δ 1.28 (t, J = 1.00 Hz, 8H), 2.15 (s, 3H), 3.26 (br s, 1H), 3.73 (br s, 1H), 4.22 (q, J = 8.00 Hz, 2H), 4.45 (s, 2H).

4.1.36. 2-(2-(3-Methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1*H*-pyrazol-1-yl)ethyl) isoindoline- 1,3-dione (45)

Compound **43** (144 mg, 0.53 mmol) and cesium carbonate (519.03 mg, 1.59 mmol) were suspended in anhydrous acetonitrile (2 mL). Compound **12** (128 mg, 0.51 mmol) was dissolved in 2 mL of anhydrous acetonitrile and added dropwise to above reaction suspension. The reaction was then heated to 60 °C overnight. The reaction solvent was removed under vacuum and the resulting crude was purified by flash chromatography on silica gel using gradient 0–50% ethyl acetate in hexanes to afford product **45** (40 mg, 18%); ^{1}H NMR ($CDCl_3$) δ 1.99 (s, 3H), 2.28 (s, 3H), 3.88–3.91 (t, J = 5.7 Hz, 2H), 4.09–4.12 (t, J = 5.2 Hz, 2H), 4.79 (s, 2H), 5.24 (s, 1H), 6.12 (s, 1H), 7.31–7.35 (m, 1H), 7.38–7.45 (m, 4H), 7.61–7.63 (m, 2H), 7.70–7.72 (m, 2H).

4.1.37. Ethyl-2-(3-methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1*H*-pyrazol-1-yl)acetate (46)

Compound **44** (40 mg, 0.21 mmol) and cesium carbonate (212 mg, 0.65 mmol) were suspended in anhydrous acetonitrile (2 mL). Compound **12** (53 mg, 0.21 mmol) was dissolved in 2 mL of anhydrous acetonitrile, and added dropwise to the above suspension. The reaction was then heated to 60 °C for 2 h. The reaction solvent was evaporated under vacuum and the resulting crude was purified by flash chromatography on silica gel using gradient 0–50% ethyl acetate in hexanes to give product **46** (24 mg, 32%); ^{1}H NMR ($CDCl_3$) δ 1.21–1.25 (t, J = 7.12 Hz, 3H), 2.18 (s, 3H), 2.34(s, 3H), 4.15–4.20 (q, J = 7.12, 7.16 Hz, 2H), 4.58 (s, 2H), 4.96 (s, 2H), 5.35 (s, 1H), 6.33 (s, 1H), 7.34–7.38 (m, 1H), 7.42–7.48 (m, 4H).

4.1.38. 2-(3-Methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1*H*-pyrazol-1-yl)ethanamine (47)

To a cold solution of compound **45** (40 mg, 0.09 mmol) in methanol (2 mL) hydrazine hydrate (102 mg, 3.18 mmol) was added. The reaction was warmed to rt and stirred overnight. The reaction solvent was evaporated to dryness and the resulting crude was purified by flash chromatography on silica gel using gradient 0–10% methanol in dichloromethane to give an oily product **47** as a colorless oil (23 mg, 82%); ^{1}H NMR ($CDCl_3$) δ 2.15 (s, 3H), 2.33 (s, 3H), 2.95 (br m, 2H), 3.81–3.84 (t, J = 5.88 Hz, 2H), 4.96 (s, 2H), 5.31 (s, 1H), 6.32 (s, 1H), 7.34–7.37 (m, 1H), 7.41–7.48 (m, 4H); MS ESI (+ve) for $C_{17}H_{21}N_5O$ ($M+H^+$): calcd 312.18, found 312.28.

4.1.39. 2-(3-Methyl-5-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methoxy)-1*H*-pyrazol-1-yl)acetic acid (48)

Compound **46** (24 mg, 0.07 mmol) was dissolved in a mixture of methanol and tetrahydrofuran (1:1, 2 mL). Lithium hydroxide (29 mg, 1.62 mmol) was added to the above reaction mixture at 0 °C. The reaction was then warmed up to rt and stirred overnight. The solvent was removed under vacuum, water (1 mL) was added and the crude mixture was neutralized with 10% HCl at 0 °C. The aqueous layer was extracted with ethyl acetate, and the organic layer was died with anhydrous Na_2SO_4. The solvent was removed under vacuum and the resultant crude was purified by short column chromatography on silica gel using gradient 1–10% methanol in dichloromethane to obtain compound **48** as a yellow solid (21 mg, 91%); mp 148–150 °C; ^{1}H NMR ($CDCl_3$) δ 2.16 (s, 3H), 2.34 (s, 3H), 4.63 (s, 2H), 4.96 (s, 2H), 5.34 (s, 1H), 6.33 (s, 1H), 7.34–7.35 (m, 1H), 7.40–7.44 (m, 4H). MS ESI (+ve) $C_{17}H_{18}N_4O_3$ ($M+H^+$): calcd 327.14, found 327.20.

4.1.40. 3-Methyl-5-((3-methyl-1*H*-pyrazol-5-yloxy)methyl)-1-phenyl-1*H*-pyrazole (49) and 3-methyl-1-((3-methyl-1-phenyl-1*H*-pyrazol-5-yl)methyl)-1*H*-pyrazol-5-ol (50)

Compound **42** (104 mg, 1.06 mmol) and cesium carbonate (346 mg, 1.06 mmol) were suspended in anhydrous acetonitrile (2 mL). Compound **12** (266 mg, 1.06 mmol) was dissolved in 2 mL of anhydrous acetonitrile and added dropwise to above suspension. The reaction was then continued 80 °C for 2 h. The solvent was removed evaporated and the resulting crude was purified by column chromatography on silica gel using gradient 0–3% methanol in dichloromethane to give products **49** (19 mg, 7%) and **50** (8 mg, 3%) as white solids. Compound **49**: mp 186–188 °C, ^{1}H NMR ($CDCl_3$) δ 2.23 (s, 3H), 2.34 (s, 3H), 5.11 (s, 2H), 5.52 (s, 1H), 6.37 (s, 1H), 7.30–7.36 (m, 1H), 7.42 (t, J = 7.91 Hz, 2H), 7.55 (d, J = 7.53 Hz, 2H); MS ESI (+ve) for $C_{15}H_{16}N_4O$ ($M+H^+$): calcd 269.14; found: 263.23. Compound **50**: ^{1}H NMR ($CDCl_3$) δ 1.92 (s, 3H), 2.28 (s, 3H), 5.00 (s, 2H), 5.40 (s, 1H), 5.97 (s, 1H), 7.32–7.58 (m, 5H); MS ESI (+ve) for $C_{15}H_{16}N_4O$ ($M+H^+$): calcd 269.14, found: 269.23.

4.1.41. 1-Benzyl-2-(bromomethyl)pyrrolidine (52)

In a flask containing carbon tetrabromide (1.7 g, 5.12 mmol) and triphenylphosphine (1.44 g, 5.49 mmol) in anhydrous tetrahydrofuran (5 mL), 1-benzylpyrrolidin-2-yl-methanol (700 mg, 3.66 mmol) was added at 0 °C. The reaction was allowed to stir

at rt for 16 h. The solvent was evaporated under vacuum and the crude mixture was purified by column chromatography 1–10% ethyl acetate in hexanes to yield compound **52** (735 mg, 79%). ^{1}H NMR ($CDCl_3$) δ 1.55–1.84 (m, 3H), 2.13 (t, J = 10.04 Hz, 1H), 2.20–2.30 (m, 1H), 2.36 (t, J = 10.42 Hz, 1H), 2.75 (d, J = 11.04 Hz, 1H), 3.10 (d, J = 8.78 Hz, 1H), 3.54 (s, 2H), 4.06–4.19 (m, 1H), 7.19–7.39 (m, 5H).

4.1.42. 5-((1-Benzylpyrrolidin-2-yl)methoxy)-3-methyl-1-phenyl-1*H*-pyrazole (53)

To a suspension of edaravone, **19** (34 mg, 0.19 mmol) and potassium carbonate (81 mg, 0.59 mmol) in anhydrous dimethylformamide (1 mL), compound **52** (50 mg, 0.19 mmol) dissolved in dimethylformamide (1 mL) was added. The reaction was heated overnight at 50 °C. On completion, the reaction was cooled to the rt and diluted with 5 mL of water. The aqueous layer was extracted with ethyl acetate and then washed with brine, dried over Na_2SO_4 and evaporated under vacuum. The resulting crude was purified by column chromatography 1–25% ethyl acetate in hexanes to afford compound **53** as an yellow oil (26 mg, 39%). ^{1}H NMR ($CDCl_3$) δ 1.24–1.74 (m, 3H), 1.96–2.01 (m, 1H), 2.24 and 2.26 (s, 3H), 2.30–2.38 (m, 2H), 2.90–3.00 and 3.49 (m, 2H), 3.53 and 3.99 (s, 2H), 3.90–4.29 (m, 1H), 5.37 and 5.44 (s, 1H), 7.17–7.47 (m, 8H), 7.66–7.75 (m, 2H). MS ESI (+ve) for $C_{22}H_{25}N_3O$ ($M+H^+$): calcd 348.21, and found 348.35.

5. Phagocytosis

5.1. Monocyte monolayer assay

The monocyte monolayer assay (MMA) was performed as previously described[17–20] with slight modifications. Briefly, human peripheral blood mononuclear cells (PBMC) containing monocytes were isolated by Ficoll-Paque density gradient centrifugation and incubated in culture medium containing 10% fetal bovine serum overnight at 37 °C under 5% CO_2 atmosphere. PBMCs (7×10^5) were then layered onto individual chamber slides (Labtek, Thermo Scientific) and incubated at 37 °C, 5% CO_2 for 1 h to allow the monocytes to adhere. Following an aspiration of non-adherent cells, test compounds, or IVIg used as a positive control for inhibition of phagocytosis,[41] were added at various concentrations to the adhered cells and further incubated for an additional hour. The compound-containing media was aspirated and the monocytes were incubated with anti-D-opsonized human RBCs at the aforementioned incubation conditions for an additional 2 h. The slides were washed, fixed in methanol and analyzed under phase-contrast microscopy to quantify RBC phagocytosis. A minimum of 100 monocytes were counted per sample and the number of RBCs that were phagocytosed were counted to generate a phagocytosis index. Percent inhibition of phagocytosis was calculated by normalizing the phagocytosis index in each treated sample to the phagocytosis index in the positive control (phagocytosis of opsonized human RBCs with vehicle only, representing 100% phagocytosis). Figure 1 depicts representative phase-contrast microscopy images of the MMA depicting a 100% phagocytosis of opsonized R2R2 RBCs as the negative control (Fig. 1A) and in the presence of IVIg as the positive control (Fig. 1B).

6. Apoptotic index (AI)

6.1. Annexin V/propidium iodide viability assay

Human PBMCs containing monocytes were isolated and incubated overnight as described above. The cells were then seeded in plastic tissue culture-treated plates and incubated for one hour under the aforementioned conditions to allow the monocytes to adhere. Following the incubation, the non-adherent cells were aspirated, and test compounds were added at a range of concentrations and incubated for an additional hour. The cells were then scraped, collected and washed. To evaluate the induction of apoptosis, cells were stained with Annexin-V conjugated to APC (BD Biosciences) following the manufacturer's instructions. Propidium Iodide (PI) (BD Biosciences) was added to 1 μg/mL, and the cells were analyzed by flow cytometry as above. Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences), and the apoptotic index was calculated based on the fold increase in the percentage of apoptotic (Annexin-V-positive, and Annexin-V-positive and PI-positive) cells in compound-treated over vehicle-treated groups. Vehicle alone showed 4–12% of the cells undergoing apoptosis. Typically, an AI in the range of 1–1.5 does not indicate significant induction of apoptosis by the compound; any AI higher than 2 implies there is enhanced apoptosis of the cells by the compound.

7. Physicochemical properties

Sirius-T3 instrument (Software Version 1.1.0.10, Sirius Analytical LTD, UK) was used to determine the pK_a and Log$D$ properties for compounds **1–11**. Assays were performed in triplicate at 37 °C using 5 μL of 10 mM solution of each sample per assay. The dissociation constant (pK_a) was determined by the Fast UV experiment in the presence of neutral buffer to stabilize the pH electrode across the pH range of 2 to 12 during the titration of the analyte, or by a pH-metric pK_a measurement. The distribution coefficient, Log$D$ was determined using the pH-metric method in which the compound was titrated for pK_a in the presence of water and octanol solvent mixture, and compared to the measured aqueous pK_a value. Log D is reported for each target compound at pH 7.4 at 37 °C, and were measured in triplicates.

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Supplementary data

Supplementary data (compounds purity data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.016.

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