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# Disulfide linked pyrazole derivatives inhibit phagocytosis of opsonized blood cells

Meena K. Purohit [a,b], Iain Scovell [c], Anton Neschadim [c], Yulia Katsman [c], Donald R. Branch [c], Lakshmi P. Kotra [a,d,e,*]

[a] Center for Molecular Design and Preformulations, Toronto General Research Institute, University Health Network, Toronto, Ontario, Canada M5G 1L7
[b] Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Rajasthan, India
[c] Research & Development, Canadian Blood Services, Toronto, Ontario, Canada
[d] McLaughlin Center for Molecular Medicine, University of Toronto, Toronto, Ontario, Canada
[e] Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada M5S 3M2

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ABSTRACT

Immune thrombocytopenia (ITP) is caused by production of an autoantibody to autologous platelets. ITP can be treated either by reducing platelet destruction or by increasing platelet production. Fcγ receptor mediated phagocytosis of the opsonized blood cells is a well-accepted mechanism for the underlying pathogenesis of ITP and inhibition of this phagocytosis process with small molecules is a potential strategy for the development of drugs against ITP. A broad screen indicated that 4-methyl-1-phenyl-pyrazole derivative (**1**) could inhibit the phagocytosis of opsonized blood cells with weak potency. We reveal here the discovery of the polysulfide products, synthesis of various 1-phenyl-pyrazole derivatives, and the biological evaluation of pyrazole derivatives as inhibitors of phagocytosis for potential use as therapeutics for ITP. Substitution at C4 of the pyrazole moiety in the disulfide-bridged dimers influenced the potency in the increasing order of **10** ≅ **11** ≅ **16** < **19** < **20**. A novel scaffold, **20** with an $IC_{50}$ of 100 nM inhibiting opsonized blood cell phagocytosis was identified as a potential candidate for further studies. Confirmation of the disulfide bridge additionally provides clues for the non-thiol or non-disulfide bridge carrying ligands targeting ITP and other similar disorders.



Thrombocytes, or platelets, are anucleate circulating blood cells involved in haemostasis. A normal platelet count for an adult human is between 150,000 and 450,000 per μL of blood. When the thrombocyte count drops below 150,000 per μL of blood, it is defined as thrombocytopenia.[1,2] The degree of severity of the thrombocytopenia is correlated to the platelet counts—a severe form of thrombocytopenia manifests at platelet counts between 10,000 and 30,000, associated with bleeding with minor trauma and at counts less than 10,000, associated with spontaneous bleeding as well as the risk of internal bleeding. Observed reduced platelet counts could occur either because of decreased platelet production such as in bone marrow failure, abnormal platelet distribution (splenomegaly), or increased platelet destruction. Platelet destruction could be caused by immune-mediated or non-immune mediated destructive pathways.[1,3,4] A common pathophysiologic mechanism leading to thrombocytopenia is the disproportionate amount of consumption or destruction of platelets in circulation compared to their rate of production.

Immunological destruction of platelets occurs in response to unknown stimuli, and is called 'immune thrombocytopenia' or 'primary immune thrombocytopenia' (ITP) which is caused by production of autoantibodies to autologous platelets.[5] These antibodies are generated against platelet membrane glycoproteins such as GP IIb/IIIa. When the antibodies bind to these glycoproteins they are marking the platelets for destruction. Fcγ receptors (FcRs) expressed on the macrophages recognize these opsonised platelets and clear them from circulation (Chart 1A).[6,7] ITP can be treated by reducing platelet destruction or by increasing platelet production. Current treatments for ITP include corticosteroids, rituximab, intravenous immunoglobulins (IVIG and anti-D), administrstion of thrombopoitin receptor (TPO) agonists and surgical measures such as splenectomy and there are no treatments that directly target the Fcγ receptors, which would directly interfere with the platelet recognition.[8] These therapies have inherent limitations and challenges, such as the toxicities associated with immunosuppressant rituximab, relapse of ITP on discontinuation of the TPO receptor agonist treatment, and possible exposure to blood-borne infections from IVIG.

Thrombopoietin (TPO) receptor agonists enhance platelet production by stimulating the TPO receptor on pluripotent hematopoietic stem cells and in megakaryocytes. For this reason, these

* Corresponding author. Tel.: +1 416 581 7601; fax: +1 416 581 7559.
*E-mail address:* lkotra@uhnres.utoronto.ca (L.P. Kotra).

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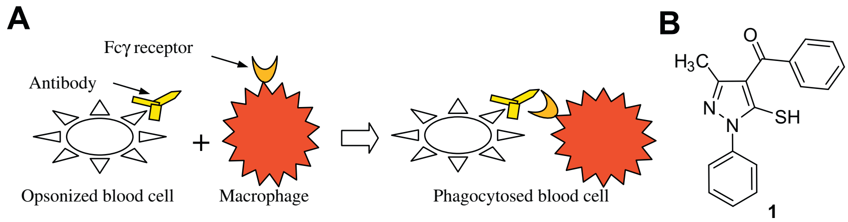


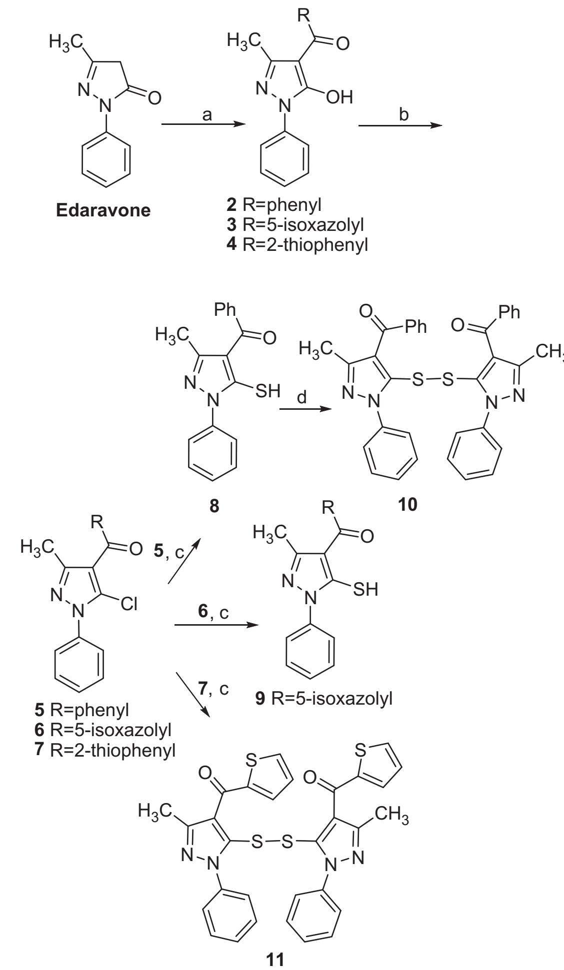

**Chart 1.** (A) Fcγ receptor-mediated phagocytosis of opsonized blood cells. (B) Structure of 1-phenylpyrazole derivative **1**.[18,19]

agonists have been sought after for the treatment of ITP.[9] Although this approach may increase the production of platelets it does not interfere with the destruction of the thrombocytes. IVIG is limited in supply since it is produced from thousands of human plasma donors and is an expensive therapy.[10] Molecular mechanisms of action of IVIG and anti-D therapies is not yet completely understood, but a number of theories have been proposed.[11–15] One of the most likely mechanisms of action for IVIG and anti-D therapy is the competitive blockade of Fcγ receptor on macrophages.[16,17] This provides an opportunity for the design of small molecule therapeutics targeting mononuclear phagocytes as a potential treatment for ITP.

Earlier, several small molecules carrying sulfhydryl and disulfide groups on aromatic and aliphatic compounds were screened for their ability to inhibit the phagocytosis of opsonized thrombocytes by macrophages.[18–23] It was hypothesized that these molecules would interact with the sulfhydryl or disulfide groups on the cell surface of human mononuclear phagocytes and inhibit the phagocytosis of opsonized red blood cells (RBC). This is based on the premise that cell surface proteins of monocyte-macrophages carry sulfhydryl and disulfide groups that play an important role in endocytic-phagocytic function, and small molecules carrying such groups would function as antagonists to the phagocytosis process.[24,25] Among these compounds, a pyrazole derivative 1 (Chart 1B) exhibited weak activity at 1 mM concentration.[18] We further explored this discovery to dissect the chemistry and structure–activity relationships of this class of molecules. Here, we reveal the synthesis, unexpected discovery of the polysulfide products, their evaluation as inhibitors of phagocytosis of the opsonized blood cells as therapeutics for ITP.

1-Phenyl-1*H*-pyrazolyl derivatives were synthesized starting from edaravone as the key starting material. For the synthesis of compounds **8**–**11**, appropriate aroyl chlorides and edaravone were first reacted in the presence of calcium hydroxide to obtain the respective substituted pyrazoles **2**–**4** (Scheme 1).[26] Compounds **2**–**4** were then chlorinated using phosphorous oxychloride to obtain compounds **5**–**7**.[27] The temperatures and reaction times for chlorination were dependent on the aromaticity and the acid stability of the heteroaryl groups at the C4 position of the pyrazole derivatives **2**–**4**. 4-Acyl-5-chloro-pyrazole derivatives **5**–**7** were then treated with sodium sulfide to obtain thiolated pyrazoles **8**–**11**: phenyl derivative **5** yielded the corresponding thiol derivative **8**, which was then refluxed in DMF for 3 h to afford the disulfide derivative **10**; isoxazole derivative **6** yielded the thiol **9**, whereas compound **7** yielded the disulfide derivative **11**. Interestingly, treatment of **5** with sodium sulfide resulted in **8** as well as a minor byproduct the disulfide **10**, which was confirmed by LCMS and could be separated by chromatography.

Unlike the aryl acid chlorides, treatment of edaravone with carbamoyl chlorides under Jensen conditions afforded O5-carbamates instead of C4-carbamoylated products. Therefore, an alternative route was used to synthesize C4-carbamoylated derivatives **16**, **17** and **19** (Schemes 2 and 3). Edaravone was first formylated at C4 followed by chlorination in situ at C5 position using




**Scheme 1.** Synthesis of compounds **8**–**11**. Reagents and conditions: (a) Edaravone, $Ca(OH)_2$, R-COCl, anhyd 1,4-dioxane, reflux, 3-4 h; (b) $POCl_3$, reflux for 2 h for R = phenyl (**2**), or at 80 °C for 50 min for R = 5-isoxazolyl (**3**), or at 50 °C for 30 min for R = 2-thienyl (**4**); (c) $Na_2S$, anhyd DMF, 60 °C, 2–3 h; (d) DMF, 100 °C, 3 h.

Vilsmeier-Haack reaction conditions (DMF/$POCl_3$) yielded compound **12** (Scheme 2).[28] The resulting aldehyde **12** was then oxidized with potassium permanganate under aqueous conditions to obtain the carboxylic acid **13**.[29] Subsequent reaction between the carboxylic acid **13** and the secondary amine (morpholine or piperidine) in the presence of HATU and DIPEA at ambient temperature yielded compounds **14** and **15** in good yields. C4-Carbamyolated pyrazole derivatives **14** and **15** were reacted with a mixture of sodium sulfide and elemental sulfur to yield compounds **16**–**17**.[30,31] Weaker activation of the carbamoyl group at C4 in compounds **14**–**15** may be responsible for the sluggish nucleophilic displacement of the chloride by the sulfide anion. An important observation was made that routine TLC and NMR characterizations for