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# Design, Synthesis, and Characterization of Rhein Analogs as Novel Inhibitors of Scavenger Receptor A

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## Abstract

Scavenger receptor A (SRA) has been known as an immunosuppressive factor and therefore therapeutic inhibition of SRA may be potentially exploited for cancer immunotherapy. Our previously work suggested that rhein may act as an inhibitor of SRA in reversing immunosuppression of SRA during T cells activation. Herein, three deconstruction analogs of rhein, compound **1**, **2**, and **3**, were further studied as inhibitors of SRA. These three compounds, particularly compound **1**, also known as a natural product danthron, enhanced T cells activation, indicated by increased transcriptional activation of interleukin 2 (*Il2*) gene, production of IL-2 protein, and proliferation of T cells. Additionally, the interaction between these compounds and SRA was studied by molecular modeling. Compound **1** showed a favorable binding mode with the cysteine rich domain of SRA protein compared to compound **2** and **3**. Collectively, those results would provide insight for future design and development of next generation rhein derivatives as SRA inhibitors.

## Graphic Abstract

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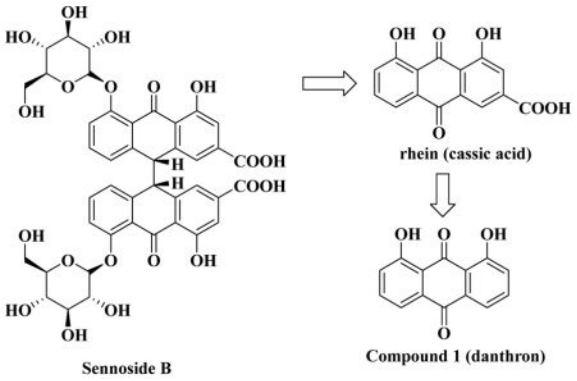
Supporting information

Brief statement in format listing the contents of the materials supplied as Supporting Information. Results of analytic parameters, experimental materials and methods for rhein analogs were available.




**Keywords**

scavenger receptor A; rhein; danthron; cancer immunotherapy

Scavenger receptor A (SRA), also namely CD204 or macrophage scavenger receptor, is mainly expressed in myeloid cells (e.g., dendritic cells, macrophages), and has multiplex structures [1]. The activity of SRA in lipid metabolism was firstly discovered by Goldstein *et al* [2], and SRA was also the first member of scavenger receptor family that was cloned [3, 4]. Due to its ligand-binding feature, the activity of SRA in lipid metabolism, host defense [5, 6], atherosclerosis [7], and pathogen recognition [8, 9] have been extensively studied. We have demonstrated that SRA plays a role as an immunosuppressive regulator in restricting dendritic cell-induced immune responses during vaccination against tumor [10–15], suggesting that development of small molecule inhibitors for SRA may provide therapeutic potential in immunotherapy of cancer.

Two natural products, namely sennoside B (Fig. 1) and tannic acid, were previously identified as SRA inhibitors by Mamula *et al* [16]. Particularly, sennoside B, a macromolecular natural product, showed dose dependent binding to the SRA [16]. We subsequently used the ‘deconstruction-reconstuction-elaboration’ approach to identify critical molecular component for its biological activity [17, 18]. Through this deconstruction strategy, a new lead compound, namely rhein or cassic acid (Fig. 1), was identified [19]. Rhein is a known natural product isolated from a traditional Chinese medicinal plant rhubarb [20]. Compared with sennoside B, rhein showed a higher inhibition activity of SRA in rescuing T cell activation.

Herein, rhein was further deconstructed in order to define the necessary functional groups in this molecule. In this process, each of three functional groups on the anthraquinone skeleton was removed one by one and three rhein analogs were obtained. One of them (compound **1**, Fig. 1) is commercially available. Compound **1** was known as a natural product, namely danthron. Danthron was also isolated from the traditional Chinese medicinal plant, rhubarb [21]. The other two compounds (compound **2** and **3**, Fig. 1) were synthesized through multi-step chemical synthesis (compound **2**, Scheme 1; compound **3**, Scheme 2). Then, the biological activity of these three analogs were tested and related docking study based on SRA cysteine rich domain was conducted.

First, to test the activity of these three analogs in rescuing T cell activation, β-Galactosidase (β-Gal) assays was used with B3Z T cells. As we reported previously [19], β-Gal was encoded by the structure of lacZ and lacZ came from *IL2* gene [22]. Once B3Z cells were activated, *IL2* gene in cells would be triggered and produce lacZ and β-Gal. In this experiment, anti-CD3/CD28 antibodies were used to induce the activation of B3Z T cells and as a mark of β-Gal increase. Besides, SRA protein was used as an immunosuppressive inhibitor of T cells. As our forward report [23], anti-CD3/CD28 antibodies could efficiently activate B3Z T cells, induce the increase of β-Gal, and the activation of T cells induced by anti-CD3/CD28 antibodies was distinctly reduced by the presence of SRA protein (Fig. 2). When B3Z T cells were treated with rhein analogs, the suppressive effect of SRA protein was evidently reversed (Fig. 2). Compared to the control, compound **1**, **2**, and **3** displayed a distinct activity of activating T cells during reversion of the suppression of SRA protein. Compound **1** showed the most significant activity. Compound **3** showed similar potency compared with that of anti-CD3/CD28 antibodies in activating T cells. Based on these, compound **1** and **3** were chosen to carry on for further studies.

Enzyme linked immunosorbent assay (ELISA) was then used to further test these two compounds in antagonizing the immunosuppressive activity of $CD11b^{+}Ly6C^{high}Ly6G^{-}$ myeloid-derived suppressive cells (MDSCs) that express SRA [24], by assaying the level of interleukin (IL-2) secreted by T cells. $CD11b^{+}Ly6C^{high}Ly6G^{-}$ cells were considered to be one kind of immunosuppressive cells derived from myeloid. It was reported that MDSCs through regulating the cytokines product by macrophages to adjust the responses of immune system, promote the growth and spread of tumor [24, 25]. Here, MDSCs were treated with or without various compounds prior to co-culture with splenocytes in the presence of anti-CD3/CD28 antibodies. As expected, MDSCs strongly suppressed T cell activation, indicated by IL-2 production. Compared to the control, both compound **1** and rhein significantly enhanced T cells activation at indicated doses (Fig. 3). It is evident that compound **1** exhibited its activity in a dose-dependent fashion. At a higher dose (20 μM), compound **1** was even more potent than the parent natural product rhein. The effect of compound **3** appeared to be modest compared to others. These results indicated that compound **1** and **3**, especially compound **1**, is able to reverse the immunosuppressive activity of $CD11b^{+}Ly6C^{high}Ly6G^{-}$ MDSCs and promote T cells activation.

To confirm the ability of the two compounds to block the immunosuppressive activity of $CD11b^{+}Ly6C^{high}Ly6G^{-}$ MDSCs, T cells proliferation was assessed using [$^{3}$H] thymidine ([$^{3}$H]TdR, 0.5 μ Ci/well) incorporation test [12]. As shown in Fig. 4, rhein, compound **1**, and **3** enhanced T cell proliferation in the presence of immunosuppressive MDSCs. Consistent with IL-2 production in Fig. 3, T cells, co-cultured with compound **1**-treated MDSCs, showed higher [$^{3}$H]TdR uptake than the other two at both doses.

Finally, we used the β-Gal assay to assess the effect of compound **1** and **3** on promoter activity of *Il2* gene in B3Z T cells. As expected, anti-CD3/CD28 antibodies effectively induced activation of *Il2* gene promoter, indicated by increased level of β-Gal (Fig. 5). Treatment of cells with SRA protein markedly decreased β-Gal level upon stimulation with anti-CD3/CD28 antibodies. When compound **1** or **3** was added, the promoter activity of *Il2* gene impaired by SRA was clearly restored during T cell stimulation.

The results above demonstrated that these rhein analogs, especially compound **1**, could act as inhibitors of SRA in rescuing T cells activation. As mentioned above, compound **1** was known as a natural product, danthron. The documented biological activities of danthron included genotoxicity [26], DNA damage and anti-cancer [27], and apoptosis [28]. It was also reported that danthron could promote the cytotoxic activity of immune cells in leukemic mouse model [29]. In the current study, we revealed that enhanced activity of immune cells following danthron treatment may be attributed to its inhibitory activity toward SRA.

In addition to biological assays, molecular modeling was applied to gain insight on the interaction between these analogs and the SRA protein in order to understand why these structurally similar compounds had different biological activities in reversing immunosuppression during T cells activation.

All three molecules were docked into the SRA model following similar procedure previously [19]. Results from clustering of the docking poses of rhein analogs showed very similar binding modes for compound **2** and **3** (Fig. 6b and 6c). The carboxylic acid group in these ligands appeared to be involved in electrostatic interactions with ARG2 of chain A. One of the carbonyl group (the one on the opposite side of hydroxyl group) showed hydrogen bonding with the backbone nitrogen of VAL1 of chain A. Both molecules also presented hydrophobic interactions with ILE16 and LEU17 of chain B.

However, compound **1** showed a different binding mode as compared to the other two (Fig. 6a). Hydroxyl groups in compound **1** displayed strong hydrogen bonding interaction networks with SER76 and SER41 of chain B. The carbonyl group on the opposite side of the ring appeared to be involved in hydrogen bonding interactions with the backbone nitrogen of VAL1 of chain A. VAL1, ILE16, and LEU17 form a hydrophobic cavity that interacted with the anthraquinone backbone of compound **1**. Thus, the residues in the binding pocket provided potential hydrogen bonding interactions and hydrophobic force to stabilize the ligand in the binding pocket. On the other hand, the COOH group and the carbonyl group next to it in compound **2** and **3** seemed to fix the ligands in a predominate pose in the protein binding site irrespective of other functional groups on the molecules. Due to this, Compound **2** and **3** bound to the protein in a way that the hydroxyl groups in both ligands were placed away from the serine residues and thus couldn’t participate in forming hydrogen bonding networks as seen in binding interactions with Compound **1**. Such result from the docking study showed that compound **1** had a beneficial binding pose with the cysteine rich domain in SRA protein compared with compound **2** and **3**.

Furthermore, the molecular interaction between three rhein analogs and SRA protein were evaluated by applying different scoring systems. Among them, GOLD and ChemPLP scoring systems have been generally used for characterizing the binding poses of ligand in the active pocket of protein based on factors such as hydrogen bonding energy, vander Waals energy, binding angle and distance [30] while HINT scoring system has been applied to understand the interaction and binding affinity between ligands and their active pocket of protein mainly based on free energy and non-covalent interactions in biological surroundings [31–33]. Interestingly, no remarkable difference was observed between the GOLD and ChemPLP scores for all three compounds (Table 1). One possible explanation for such a

similarity would be that all three compounds were deconstruction products from rhein, which shared the same rigid structural skeleton, and such a structural feature mainly decided the binding poses for all three compounds as GOLD and ChemPLP Scores indicated even in somehow different binding environments. On the other hand, the HINT scores of three compounds were clearly different with compound **1** the highest, compound **3** the second, and compound **2** the lowest. Such a result was in a good agreement with the sequence of those compounds' biological activity observed. Comparing compound **2** and **3** (both in a similar binding pocket), while the carboxylate moiety served as an anchor to recognize the basic side chain in ARG2 of the protein, the hydroxyl group on the same aromatic ring in compound **2** seemed not in favorable interaction with LEU17 which lead to reduction of its HINT score. Meanwhile compound **1** seemed to be able to settle in a nearby binding locus through a much more favorable interaction between its aromatic skeleton and a hydrophobic pocket formed mainly by VAL1, ILE16, and LEU17, which apparently contributed to its higher HINT score. Such a different binding mode may provide explanations for their biological activity as inhibitors of scavenger receptor A.

In conclusion, three rhein analogs, compound **1**, **2**, and **3**, were designed and obtained by applying deconstruction strategy and tested as inhibitors of SRA in rescuing T cells activation. The biological studies showed these analogs, especially compound **1**, were able to enhance T cell activation by inhibiting immune suppression of SRA protein, evidenced by increased production of IL-2, transcriptional activation of *Il2* gene and the proliferation of T cells. Docking study suggested that compound **1** displayed a different binding mode in the cysteine rich domain compared to compound **2** and **3**. The relative position of two hydroxyl groups and carbonyl groups in compound **1** made it in an advantageous position to bind in a hydrophobic pocket in the protein through a hydrogen bonding network. These results would guide future molecular design of rhein analogs as SRA inhibitors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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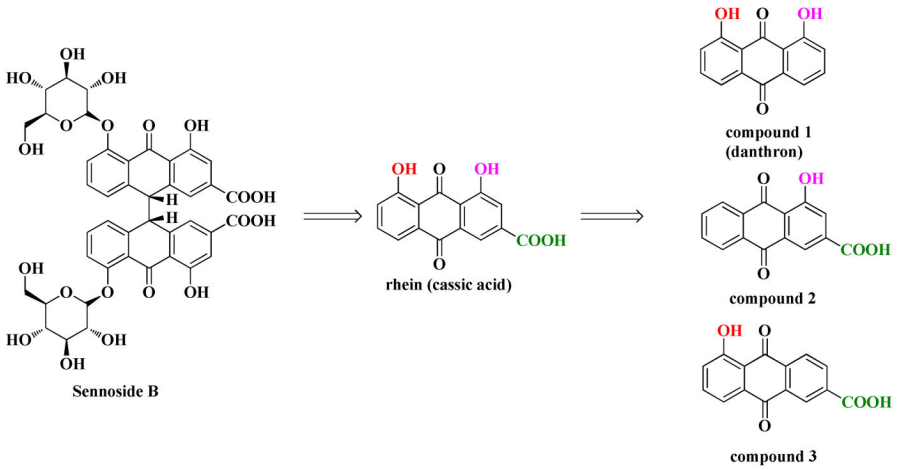



**Figure 1.**
The design of three rhein analogs.

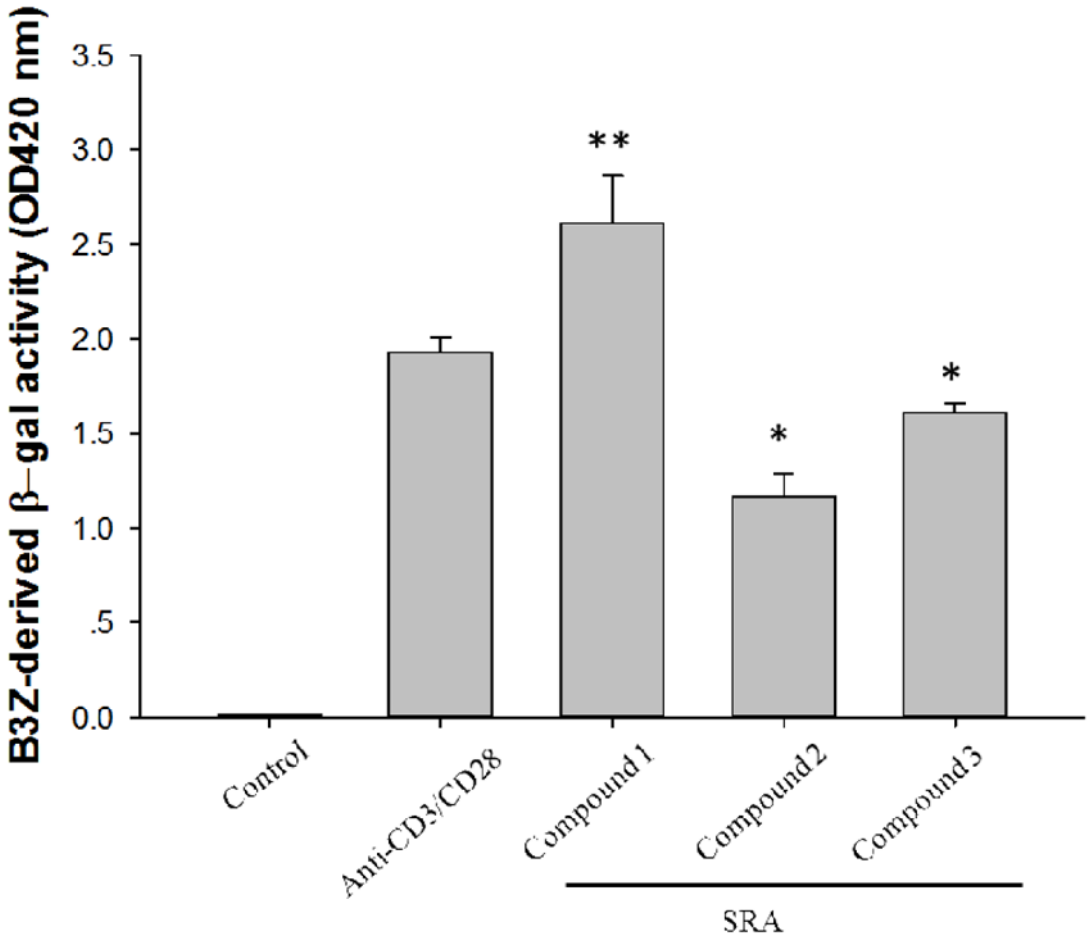



**Figure 2.**

β-Gal assay of rhein derivatives in T cell activation. B3Z T cells were seeded $2 \times 10^6$/well in 12-well plate in the presence of anti-CD3/CD28 stimulation combined with SRA protein and compound **1**, **2**, and **3** (50 μM), respectively, for 5 h. Cells without any treatment served as control. β-Gal assay was performed to detect the activation of *Il2* gene promoter after incubation at 37 °C for 1 h. The experiments were repeated at least three times with similar results. *, $p < 0.05$; **, $p < 0.01$.

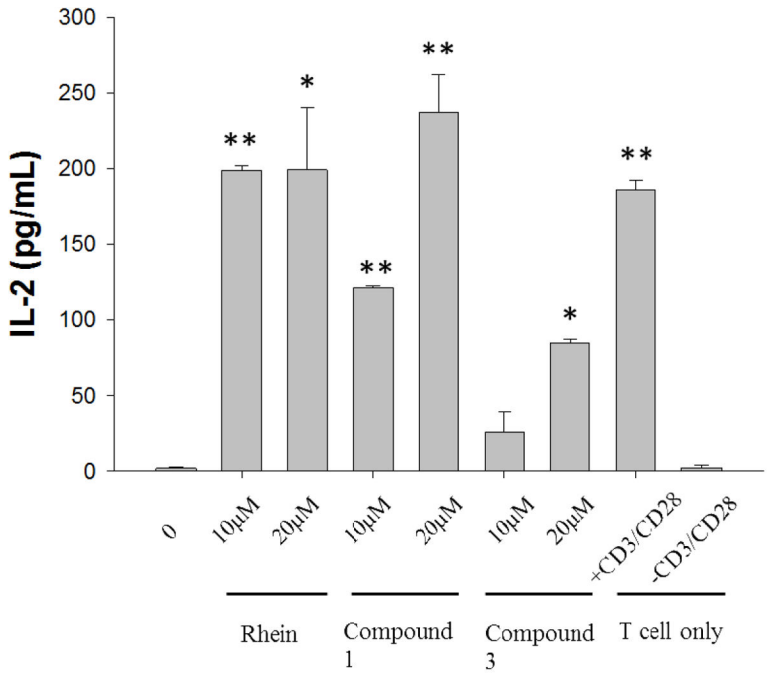



**Figure 3.**

Rhein and its derivatives reversed immunosuppressive activity of $CD11b^{+}Ly6C^{high}Ly6G^{-}$ MDSCs. Mice were subcutaneously injected $2 \times 10^5$ RM1-OVA tumor cells. Ten days later, spleen-derived $CD11b^{+}Ly6C^{high}Ly6G^{-}$ cells were sorted using a Flow cytometer. MDSCs were pretreated with rhein, compound **1** or compound **3** at indicated doses for 2 h. Splenocytes from naïve mice were co-cultured with purified MDSCs at 1:1 ratio in the presence of 1 μg/mL plate-bound anti-CD3 mAb, 0.5 μg/mL soluble anti-CD28 mAb and rhein or the other two derivatives with indicated doses in a 96-well flat-bottom plate. Supernatant was collected 56 h after culture, and levels of cytokines IL-2 was determined by ELISA. The experiments were repeated at least three times with similar results. *, $p < 0.05$; **, $p < 0.01$.

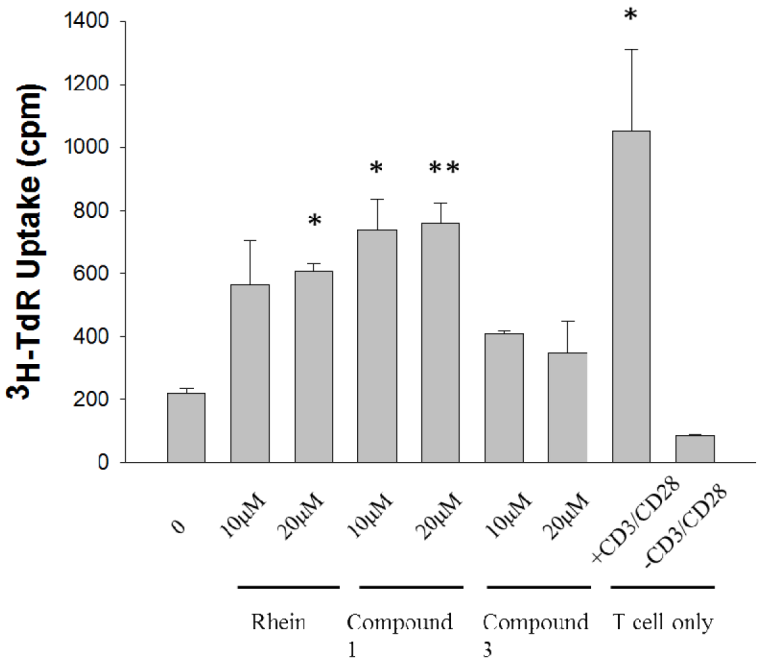



**Figure 4.**

[$^3$H]thymidine incorporation assays for the proliferation of T cells. MDSCs sorted from tumor-bearing mice were pretreated with rhein or its derivatives compound **1** or **3** at indicated doses for 2 h. Splenocytes from naïve mice were co-cultured with purified MDSCs at 1:1 ratio in the presence of 1 μg/mL plate-bound anti-CD3 mAb, 0.5 μg/mL soluble anti-CD28 mAb and rhein or the other two derivatives with indicated doses in a 96-well flat-bottom plate for 72 h. Cells were pulsed with 0.5 μ Ci/well [$^3$H]thymidine for the last 16 h of incubation. Proliferation was measured based on [$^3$H]thymidine incorporation in triplicate wells. The experiments were repeated at least three times with similar results. *, $p < 0.05$; **, $p < 0.01$.

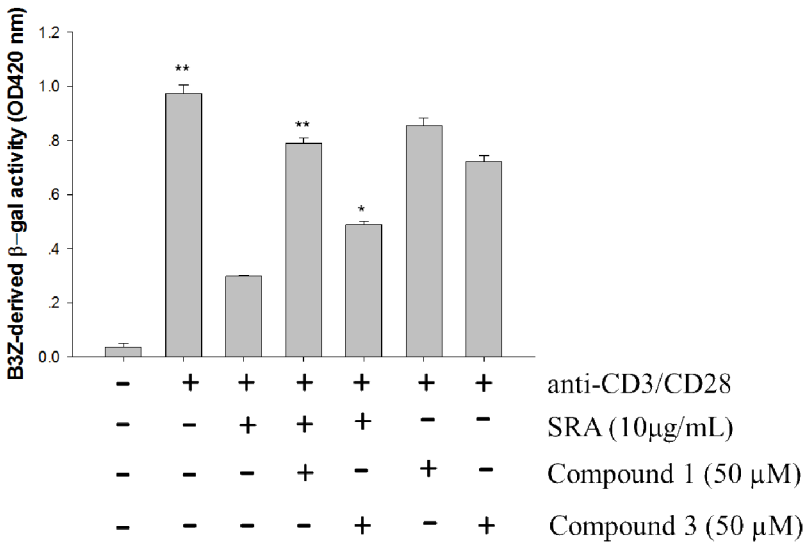



**Figure 5.**

β-Gal assay of compound 1 and compound 3 in restoring SRA-impaired transcription of *Il2* gene during T cell stimulation. B3Z T cells were seeded $2 \times 10^6$/well in 12-well plate and stimulated with anti-CD3/CD28 antibodies in the presence of SRA protein (10 μg/mL) combined with compound **1**, or compound **3** (50 μM each), respectively, for 5 h. β-Gal assay was performed to detect the activation of *Il2* gene promoter. The experiments were repeated at least three times with similar results. *, $p < 0.05$; **, $p < 0.01$.

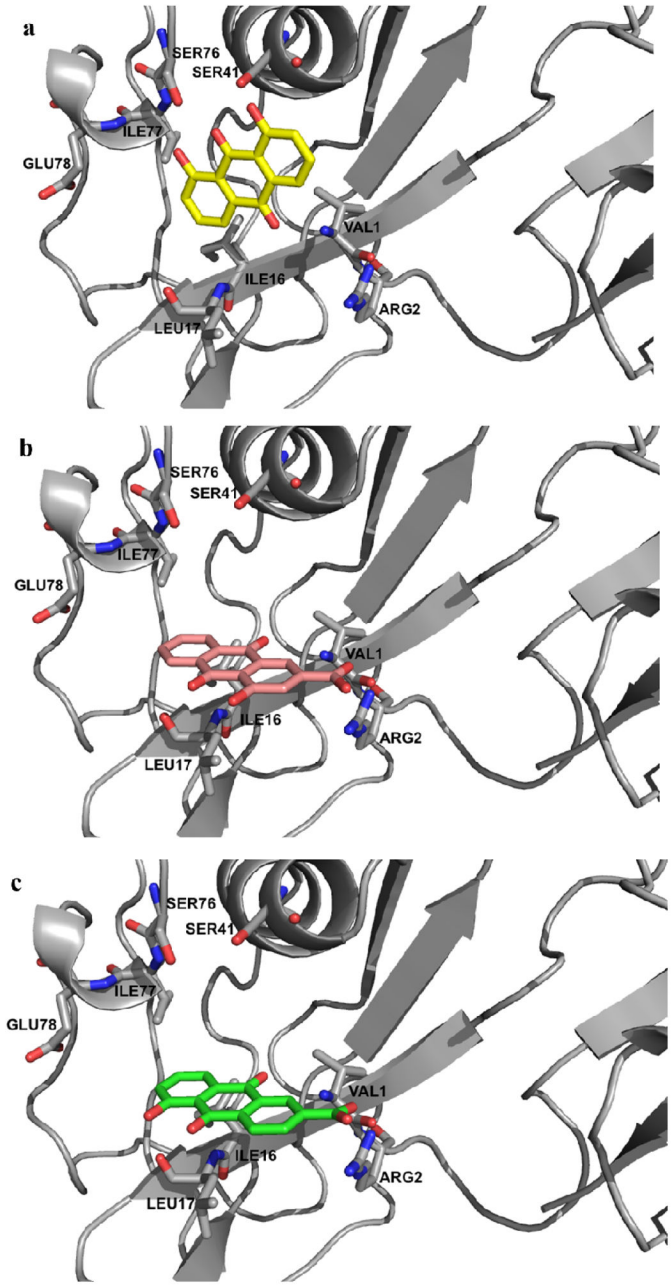
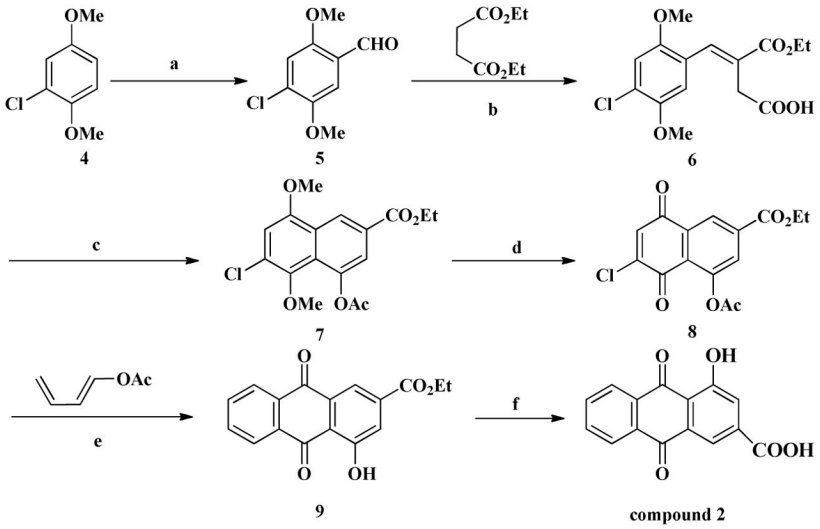


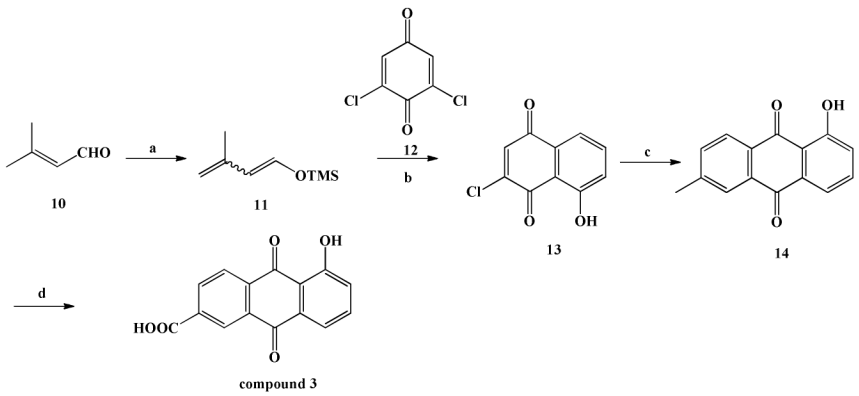

**Figure 6.**

Docking study of rhein analogs in cysteine rich domain of SRA protein. Most preferred docking mode of compound **1** (yellow), compound **2** (pink), and compound **3** (green) in the cysteine rich domain of SRA protein. Amino acid residues involved in putative interactions are shown in grey sticks representation.




**Scheme 1.**

Synthesis of compound **2**.[20] Regents and conditions: (a) TFA, Hexamethylenetetramine, reflux, 90–95 °C; (b) Diethyl succinate, NaH, Toluene, EtOH, $N_2$, 55 °C; (c) $Ac_2O$, NaOAc, $N_2$, reflux; (d) Ammonium cerium(IV) nitrate, acetonitrile, rt; (e) Buta-1,3-dien-1-yl-acetate, EtOH, reflux; (f) 10% NaOH.




**Scheme 2.**

Synthesis of compound **3**.[21, 22] Regents and conditions: (a) TMSCl, TEA, $ZnCl_2$, Hydroquinone, benzene, 4 h, 70 °C; (b) HOAc, $CrO_3$, Benzene, reflux 40 h; (c) **11**, THF, rt, 40 h; (d) i. $Ac_2O$, Pyridine; ii. $CrO_3$, HOAC, $Ac_2O$.

**Table 1**

GOLD, ChemPLP and HINT Scores for the interaction between rhein analogs and the SRA protein cysteine rich domain.

| Ligands | GOLD Score | ChemPLP Score | HINT Score |
|---|---|---|---|
| Compound **1** | 35.30 | 35.13 | 195.3719 |
| Compound **2** | 37.38 | 37.26 | 62.3885 |
| Compound **3** | 38.45 | 38.48 | 163.1161 |