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Discovery of structurally simplified analogs of colchicine as an immunosuppressant

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ABSTRACT

We have discovered a new class of colchicine-derived therapeutic agents for immune diseases including rejection of organ-transplantation and autoimmune disease. Compound **2**, which had been developed to overcome poor pharmacokinetic properties of compound **1**, a first-generation colchicine analog, turned out to show toxicity such as intestinal toxicity and loss of weight during *in vivo* tests. The deletion of 7-carboxamide group and middle ring-truncation in colchicine allowed us to have structurally simplified analogs with strong immunosuppressive activity. Herein, we report non-alkaloid tricyclic compound **7** and **12** as immunosuppressants which exhibited a strong immunosuppressive *in vivo* efficacy on the T-dependent antibody response, the Zymosan A-induced arthritis model and the Carrageenan-induced edema model. Compound **7** and **12** revealed less toxicity than the previous lead compound **2**, and their minimum lethal doses (MLD) were proved to exceed 100 mg/kg.

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Since the success of cyclosporine A (CsA) in treating organ rejection in liver transplants of the first patient[1] and subsequent approval for use in 1983, it has been used against transplant rejection, psoriasis, and autoimmune diseases such as chronic urticaria and rheumatoid arthritis. Other blockbuster immunosuppressants, tacrolimus (also known as FK506) and sirolimus (also known as rapamycin) were first approved for liver and kidney transplants in 1994 and 1999, respectively. These three drugs are still the most prescribed immunosuppressive drugs in the market.[2, 3] However, current immunosuppressants including these three show serious side effects such as renal toxicity, neurotoxicity, hypertension and pulmonary toxicity.[4-6] Despite intensive efforts to overcome rejection in organ transplants and autoimmune diseases, there still remains the need for more effective and safer immunosuppressants.

Our group has discovered a new class of immunosuppressant derived from the natural product colchicine, which inhibits microtubule polymerization by binding to tubulin.[7, 8] Colchicine (**3**) was approved as a therapeutic agent for the treatment of acute gout flares, prophylaxis, and familial Mediterranean fever. Compound **1**, a first-generation colchicine analog (Figure 1) exhibited potent activity on *in vitro* MLR and *in vivo* skin-allograft model but revealed poor pharmacokinetic properties (F~0%) *in vivo*.[9] Compound **2**, a second-generation colchicine analog (Figure 1), which was developed by the study of structure-activity relationships and solubility-activity relationships between the first generation analogs, exhibited improved pharmacokinetic properties (F = 67.3%) and effective *in vivo* activity on the Zymosan A-induced arthritis model, Carrageenan-induced edema model and the local lymph node assay (LLNA).[10] However, compound **2** turned out to have severe toxicity including intestinal toxicity, loss of spleen weight suggesting immunotoxicity, and loss of weight during *in vivo* tests. (Supporting information, Figure 1) Thus, we needed to discover less toxic compounds that retained activity compared to the previous lead compound **2**. To achieve this goal, we attempted the lead optimization of compound **2** to produce less toxic analogs. Unfortunately, as shown in our previous structure-activity relationships of colchicine analogs, alteration of the 7-membered tropolone ring with 10-thiomethoxy group induced significantly decreased activity or loss of activity. (data not shown) Furthermore, it was undesirable to alter and optimize the 2-methylcyclopropane-1-carboxamide group at C7-position because it had been selected to improve aqueous solubility and PK profiles.[10] Based on previous SAR studies and preliminary optimization of compound **2**, we decided to design structurally different compounds from colchicine analogs which have 7-carboxamide groups as a side chain. In our efforts to develop a novel series of immunosuppressants from colchicine, all of our analogs had retained the 7-carboxamide group which included aliphatic, aromatic and pseudo-aromatic groups. For this study, we investigated whether the deletion of the 7-carboxamide group or further simplified analogs which have no middle 7-membered ring would exhibit high activity with reduced toxicity compared to compound **2**. (Figure 2) Herein, we describe the synthesis and biological evaluation of structurally simplified compounds from colchicine as an immunosuppressant.

As depicted in scheme 1, Ketone **5** was readily available from deacetylthiocolchicine **4** using the formation of Schiff' base followed by anionic equilibration and hydrolysis procedure developed by Rapoport et al.[11, 12] Reduction with $NaBH_4$ produced racemic alcohol **6** which afforded a deamidated alkene **7** on subsequent methanesulfonylation followed by elimination with DBU. Hydrolysis of colchicine in 10% sulfuric acid gave a 10-hydroxy free amine **9** which was converted to quaternary ammonium salt **10** with methyl iodide. It afforded compound **11** as a mixture with its tropolonic regioisomer on Hoffman degradation procedure with Pd on C and sodium hydroxide.[13] Then, methylation of free alcohol followed by substitution with sodium thiomethoxide afforded the desired compound **12** void of 7-amide group. Scheme 2 describes the synthesis of ring-truncated analogs. The key precursor **16** of ring-truncated analogs was prepared from 5-iodotropolone, which was developed by Ebisawa et al,[14] by O-methylation with MeI and K_2CO_3 in acetone. **19**, **20**, **23** and **24**, which are ring-truncated analogs with 2 or 3 carbon-linkers, were synthesized by continuous double cross coupling reactions. Stille cross coupling reaction of **17** (or **21**) with vinyl (or allyl) tributyltin produced intermediates with terminal alkenes. They afforded desired ring-truncated analogs on Heck reaction with **16**. Cross coupling of **25** with **16** by $Pd(OAc)_2$ and PPh_3 in presence of TBAF[15] gave compound **26** which was readily converted to compound **27** by substitution with sodium thiomethoxide.

First, we evaluated the inhibitory activity of analogs on LPS-induced B cell- and Con A-induced T cell proliferation. As shown in figure 3, compounds (**7** and **12**) eliminating 7-carboxamide group exhibited high inhibitory activity on both B- and T cell proliferation in dose-dependent manners. Compound **26** and **27**, which are ring-truncated analogs with a simple biphenyl structure, showed a moderate inhibitory activity but those are less active than tricyclic **7** or **12**. However, ring-truncated compounds **19**, **20**, **23** and **24** with a carbon linker exhibited the loss of activity as described in figure 2 of the supporting information. We did not try to synthesize the analogs with 10-thiomethoxy group of these inactive compounds on the basis of our previous SAR report.[9] This series of analogs have a similar tendency in the activity as compared to the previous lead compound **2**, which is that B cell proliferation is more strongly suppressed than T cell.[10] Nitric oxide (NO) is known to contribute to the acute rejection of the organ-tranplantation[16] and deemed a tissue damaging molecule by activated macrophages in autoimmune diseases.[17] We compared their inhibitory effects of NO production on bone marrow-derived macrophages which are primary macrophages, and transformed RAW 264.7 cells. (Figure 4) **7** and **12** suppressed NO production on RAW 264.7 cells but not bone marrow-derived macrophages, suggesting the compounds (especially **12**) just suppress NO production of proliferating cells. NO is produced from diverse NO synthases (NOSs) which have been found in macrophages, dendritic cells, NK cells, B- and T cells. NOSs are regulated by cytokines such as IL-12 and IFN-γ.[18] Both **7** and **12** showed dose-dependent suppression of IL-12 on bone marrow-derived dendritic cells without affecting cell viability. (Figure 4)

Next, we further investigated the *in vivo* immunosuppressive efficacy of **7** and **12** which are the most potent compounds in this study. In the adapted immune system, naïve B lymphocytes, which have not yet recognized an antigen via their B cell receptors, are activated by many protein antigens called T-dependent antigens. After that, they proliferate, differentiate, and mount an antibody response against T-dependent antigens.[19] The spleen cells from mice were immunized with SRBCs and treated with compound **7** or **12**, and then, the total specific (anti SRBC) antibody formation was accessed.[20] Both compounds exhibited dose-dependent suppression of *in vivo* T-dependent antibody response, which implies they strongly suppress B cell-mediated immune response and it's exactly correlated with the *in vitro* data. (Figure 5a) We also evaluated their immunosuppressive effects on the arthritis and edema model as we tested compound **2** in a previous report.[10] The C57BL/6 mice were treated with **7** and **12** after subcutaneous injection of Zymosan A into the hind footpad

for asthma inflammation or Carrageenan into the left hind paw for a paw edema inflammation.[21, 22] Both **7** and **12** reduced arthritis- and edema inflammation in dose-dependent manners. **12** exhibited slightly better activity than **7**, which shows significant correlation with *in vitro* tests. (Figure 5b and 5c)

During the *in vivo* tests, we assessed the preliminary toxicity of **7** and **12** by measuring the loss of body weight. Intravenous injection of **7** or **12** on C57BL/6 mice (1 mg/kg, once a day for 4 days) did not show significant changes of body weight. (Figure 6a and b) The dose-dependent treatment of compounds in 0.1 to 3 mg/kg, which was the dosage for *in vivo* efficacy tests, also did not exhibit changes in body weight. (Figure 6c) Anatomical analysis revealed that **7** and **12** did not induce any intestinal toxicity such as dilation and inflammation in contrast to compound **2**. We next evaluated the intravenous single-dose acute toxicity of **7** and **12** on ICR-SPF mice.[23] Mice were administered with 10 mg/kg to 100 mg/kg of **7** or **12**. Then, mortalities, clinical signs including changes of body weight and autopsy were observed for 14 days after administration. (Table 1) Compared to the vehicle control group (G1), both **7** and **12** exhibited minimum lethal doses (MLD) of over 1oomg/kg, and we could not observe any death, loss of body weight nor anatomical abnormality on this toxicity test.

In summary, we have focused on discovering structurally simplified chemical entities from colchicine as new immunosuppressive agents. The deletion of the 7-carboxamide group or ring-truncation strategy generated several active analogs which were identified *in vitro*. Our results seem to afford some information about the pharmacophore of colchicine showing immunosuppressive activity, although its exact mode of action remains undetermined. Activity of **26** and **27** strongly suggests that the biphenyl structure with trimethoxyphenyl and 7-membered tropolone ring is an essential part to induce immunosuppressive activity. Nevertheless, compound **7** and **12** exhibited stronger activity than **26** and **27**, which implies that a specific positioning by a constrained ring system in 3-dimentional space, in addition to the biphenyl structure, might induce stronger activity. Of active analogs, **7** and **12** have been identified as the most potent analogs having similar activity to the previous lead compound **2** in *in vivo* tests including the Zymosan A-induced arthritis model and the Carrageenan-induced edema model. Despite similar activities, they turned out to be less toxic, and we speculate that it comes from the deletion of the 7-carboxamide group. Based on our results, we hypothesize that the deletion of the 7-carboxamide group might reduce toxicity of colchicine and make its own immunosuppressive activity stand out in bold relief. To confirm this hypothesis, we are trying to figure out the mechanism of immunosuppressive activity for these analogs. And we are undergoing *in vivo* heart-transplantation experiments on mice and an in-depth toxicology study for the further development of **7** and **12**.

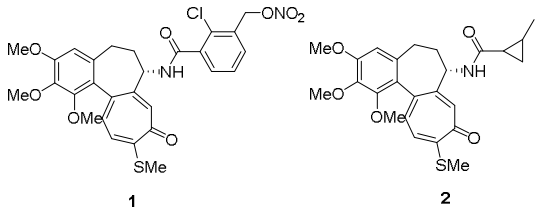


Figure 1. Structures of the previous lead compound **1** and **2**.

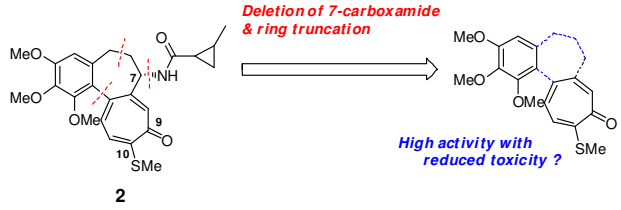
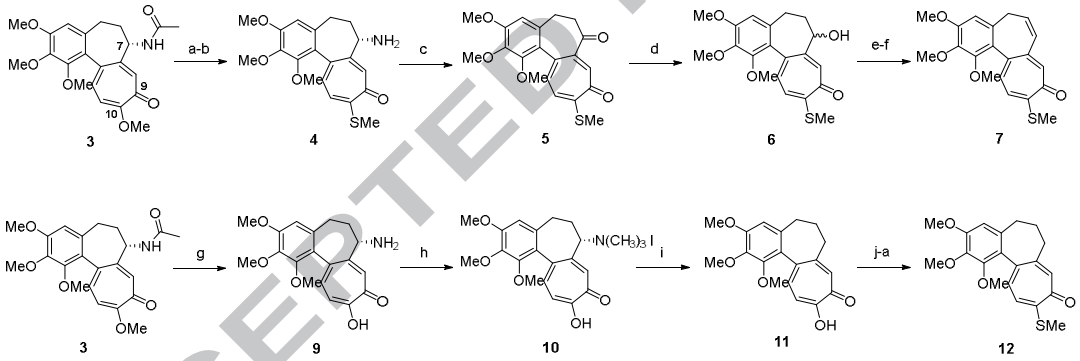
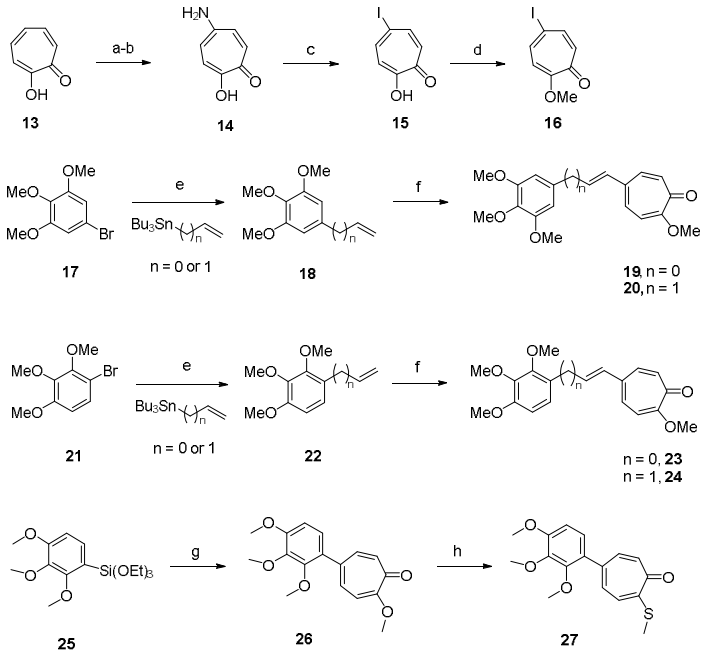


Figure 2. Strategy for structurally simplified analogs from compound **2**



Scheme 1. Reaction conditions and reagents: (a) NaSMe, THF/water (1:1); (b) 2N-HCl, MeOH, reflux, 67% for 2 steps; (c) 4-formyl-1-methylpyridinium benzenesulfonate, CH_2Cl_2/DMF (1:1), rt, 12 h, then DBU, oxalic acid, rt, 60%; (d) $NaBH_4$, MeOH/ CH_2Cl_2, rt, 95%; (e) methanesulfonyl chloride, pyridine, rt, 73%; (f) DBU, toluene, rt, 81%; (g) 10% H_2SO_4 in acetic acid, 80 ^oC, 66%; (h) MeI, 1,4-dioxane, reflux, 52%; (i) Pd on C, NaOH, water, 0 ^oC to rt, 39%; (j) MeI, K_2CO_3, MeOH, rt, 40%.



Scheme 2. Reaction conditions and reagents: (a) $NaNO_2$, HOAc, water, 58%; (b) PtO_2, H_2, MeOH, 81%; (c) $NaNO_2$, H_2SO_4, water, 0 °C, then KI, 55 °C, 60%; (d) MeI, K_2CO_3, acetone, 60 °C, 44%; (e) $Pd(PPh_3)_4$, DME, 80 °C, 59%; (f) 16, $Pd(OAc)_2$, PPh_3, $AgCO_3$, THF, 19: 57%, 20: 50%, 23: 45%, 24: 39%; (g) $Pd(OAc)_2$, PPh_3, TBAF, THF, 66%; (h) NaSMe, THF/water (1:1), 72%.

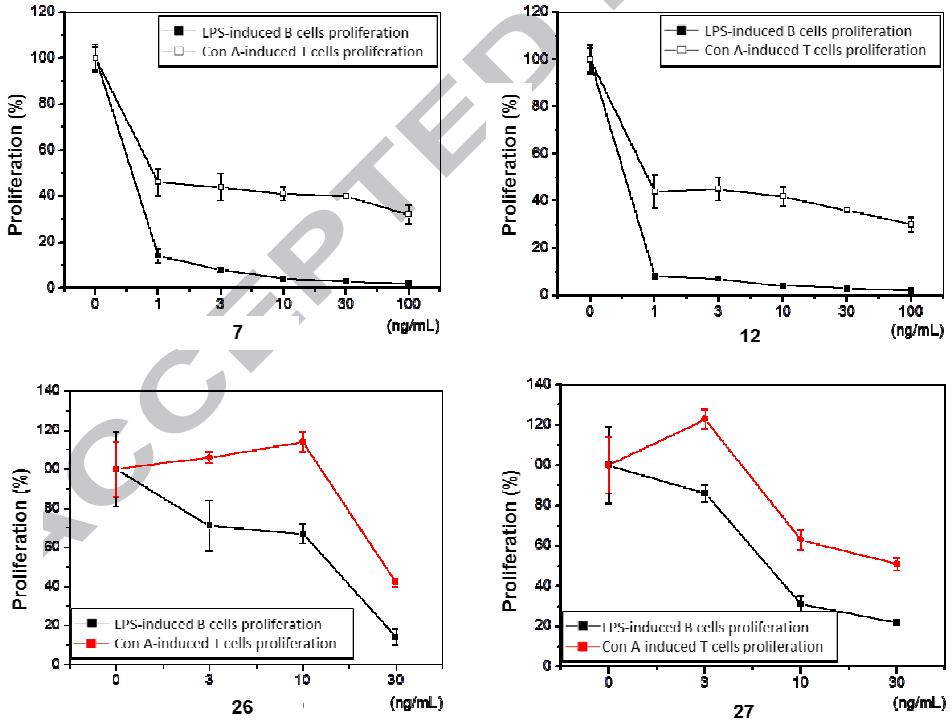


Figure 3. Inhibitory activity of compound **7**, **12**, **26** and **27** on LPS-induced B cell- and Con A-induced T cell proliferation.

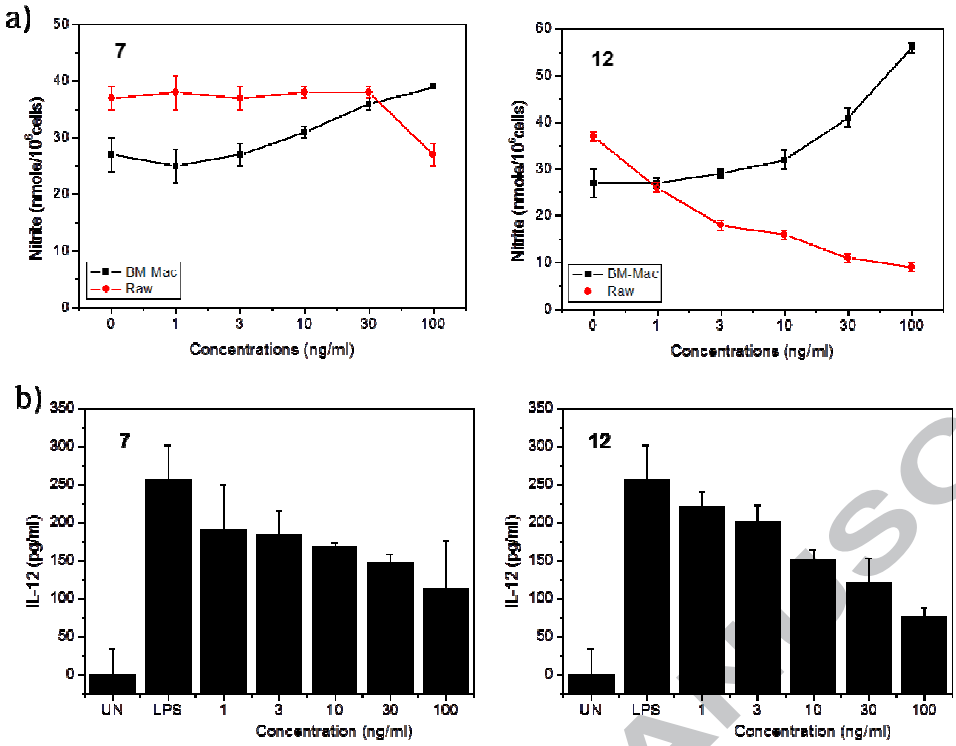


Figure 4. Inhibition of NO and IL-12 production by compound **7** and **12**. a) Inhibition of NO by **7** and **12** on bone marrow-derived macrophages and RAW 264.7 cells, b) Inhibition of IL-12 by **7** and **12** on bone marrow-derived dendritic cells (1 µg/mL of LPS).

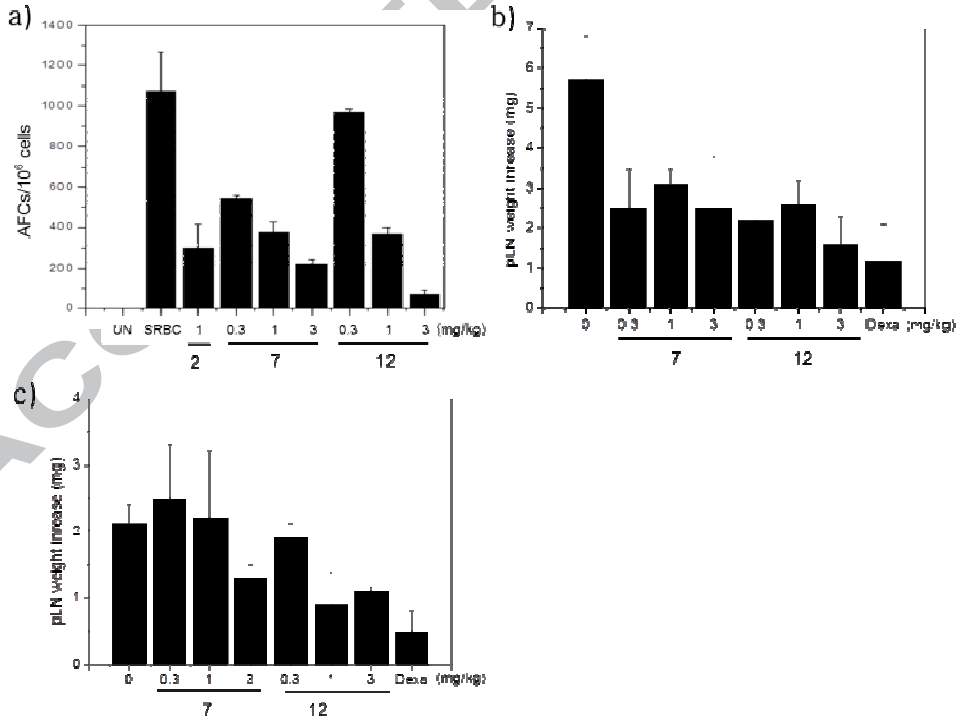


Figure 5. *In vivo* efficacy of compound **7** and **12**. a) Effect on T-dependent antibody response, b) effect on Zymosan A-induced arthritis model, c) effect on carrageenan-induced paw edema model.

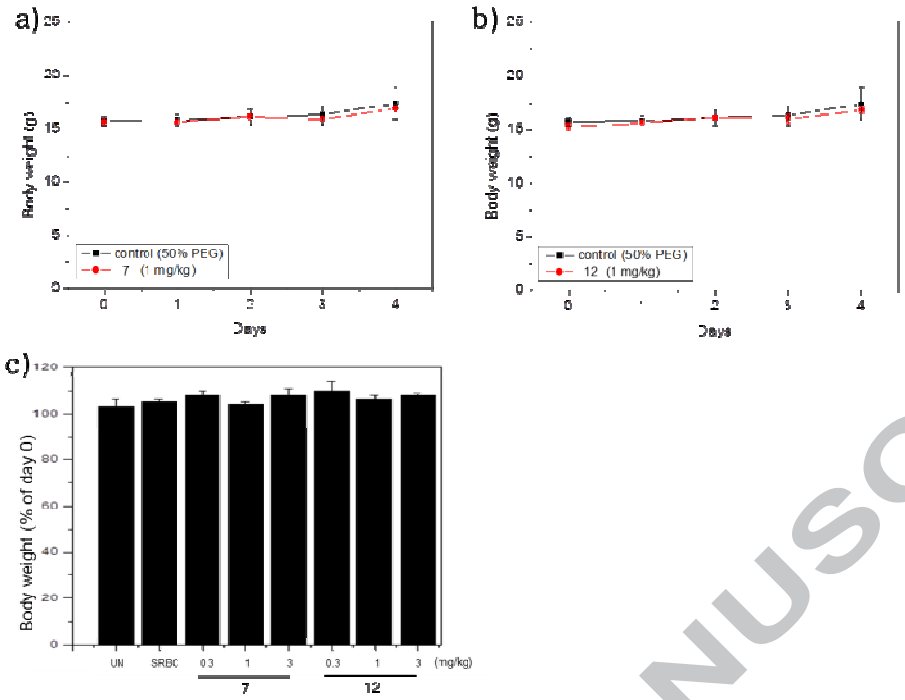


Figure 6. Preliminary toxicity test of compound **7** and **12**. a) Change of body weight during 4 days after treatment of **7**, b) change of body weight during 4 days after treatment of **12**, c) change of body weight at 4 days after dose-dependent treatment of **7** or **12**.

Table 1. Intravenous single-dose acute toxicity of compound **7** and **12**.

Group	Sex	# of mice	mg/kg	dead/tested	
				7	**12**
G1	Male	5	0	1/10	1/10
	Female	5	0		
G2	Male	5	10	0/10	0/10
	Female	5	10		
G3	Male	5	30	0/10	0/10
	Female	5	30		
G4	Male	5	100	0/10	0/10
	Female	5	100		

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Supplementary Material

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