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Design, synthesis and biological evaluation of enzymatically cleavable NSAIDs prodrugs derived from self-immolative dendritic scaffolds for the treatment of inflammatory diseases

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ABSTRACT

It has been reported that delivery systems based on dendritic prodrugs of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) improved the properties of drug molecules and reduced the side effects and irritation on the gastric mucosa. To find a more effective way in NSAIDs dendritic prodrugs, in this paper, three different dendritic scaffolds of enzymatically cleavable naproxen conjugates have been synthesized in a convergent approach and well characterized by NMR and MS techniques. These self-immolative dendritic NISADs prodrugs programmed to release multiple molecules of the potent naproxen after a single enzymatic activation step, and in 50% human plasma, the drug released from the compound T3 reaching 47.3% after 24 h in vitro assay. Moreover, all prodrugs were also found to maintain more significant anti-inflammatory activity, no significant cytotoxicity against HEK293 cells and less degree of ulcerogenic potential in vivo than their monomeric counterpart naproxen. These results provided an effective entry to the development of new dendritic NSAIDs prodrugs.

1. Introduction

Nowadays, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical to treat arthritis, fever and relieve pain.[1–3] However, the NSAIDs have been reported to cause a series of side effects, gastrointestinal toxicity in particular, which has an interwovenness connection with their pharmacological activities.[4–7] Many drug delivery systems (DDSs) were designed to circumvent this drawback meanwhile preserving their satisfactory pharmacological activities. Among all the DDSs that improved the anti-inflammatory effect of small molecule NSAIDs, nontoxic prodrugs based on drug carriers were focused widely.[8–12] With the development of materials-based approaches, the dendritic prodrugs have been considered as a promising entry to find prodrugs on the basis of drug delivery.[13–18] Dendrimers, a kind of synthetic artificial macromolecules, are highly branched tree-like polymers with multiple end-groups. With the features of monodispersity, spherical macromolecules with well-defined three dimensional structures and functionalized end-groups, the dendrimers have many unique properties that significantly differ from those corresponding linear polymers.[19–23]

In traditional approaches of dendrimer-based drug delivery, the bioactive substances were covalently attached to termini and thereby independent cleavages were required for their release. The activities of drug molecules were typically attenuated or eliminated when they incorporated into the delivery system.[24,25] One example is PAMAM based NSAIDs dendritic prodrugs reported by D'Emanuele and co-workers.[26,27] Conjugation of naproxen with G0 PAMAM dendrimer increased the permeability and biocompatibility appreciably, enhanced the potency and diminished the gastrointestinal side-effects of naproxen at the same time.[28–31] Although the preliminary results were so exciting, the release properties of dendritic prodrug in vitro and potency in vivo were a challenge for us. As for the current research, NSAIDs dendritic prodrugs were mainly focused on the PAMAM, not extending to more scaffolds. Therefore, a distinct release profile might be found when different dendritic chains or scaffolds were conjugated to a model drug molecule.

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Another update is that, recently an emerging class of dendrimers has been built which allows for the simultaneous release of all end-groups upon a single triggering event.[32,33] Shabat and coworkers provided an efficient entry to obtain such special molecules with excellent results: a self-immolative dendritic linker acted as a molecular amplifier and conjugate was designed for activation by a triggering unit driven by proteins, enzyme or other bioactive molecules in biological system.[34–36] This strategy not only increased the loading capacity of drug molecules per [a] polymer molecule by two times or more than that of the classic conjugate, but also enhanced the ability to receive and translate a biological signal into an amplified response if the targeted or secreted enzyme that initiates drug release was present at a relatively low level in the malignant tissue and lead to the specific drug delivery.[37,38] Thus, the above-mentioned challenge could be overcome by using self-immolative dendritic prodrugs which allowed the release of several drug units after a single enzymatic activation step.[39,40] In this case, we tried to design and synthesize three different dendritic naproxen scaffolds with a single triggering and self-immolative linkers, and compare drug release profiles in vitro with different dendritic structures. The cytotoxicity in vitro, anti-inflammatory and ulcerogenicity in vivo of the new dendritic prodrugs were evaluated by the MTT method, xylene-induced mice auricle tumefaction model and acute ulcerogenesis assay respectively. Through a series of experiments, we hope to find effective dendritic prodrugs used in NSAIDs DDSs.

2. Results and discussion

2.1. The design of dendritic prodrugs

In our previous work, NSAIDs prodrugs with aspartate or succinate based dendrons were studied: a sustained release enhancement profile was found when the different chains were conjugated to a model drug molecule.[29] Thus, we decided to study the different dendritic prodrugs T1–T3 designed for the selective targeting of more naproxens. The design of our dendritic prodrugs was based on three classic types of dendrons: polyamide–ester, polylysine and Newkcome type dendron. All of the dendritic compounds were connected to a triggering group (that can be activated either chemically or enzymatically) and enzymatic cleavable bonds served as the 'handle' for attachment of an end-unit (naproxen). The release of the end-unit could be initiated by the removal of the triggers and then a spontaneous enzymatic cleavage reaction took place, leading to the release of the end-unit. In this case, one benzyl ester or amide which presented an enzyme substrate of arylesterase[41] in human plasma was designed as a triggering group. In order to verify our assumption, a peptidase, cathepsin B which was always used in the enzymatic release test,[42] was taken as a control compared with the arylesterase in human plasma. In addition, different dendritic scaffolds such as polyester, polyamide and polyamide–ester were conjugated to the model drug naproxen. We preliminarily planed to investigate the release capacities of the different bond-type dendritic drugs in vitro and screen outstanding dendritic carriers, which will lay a foundation for further molecular design.

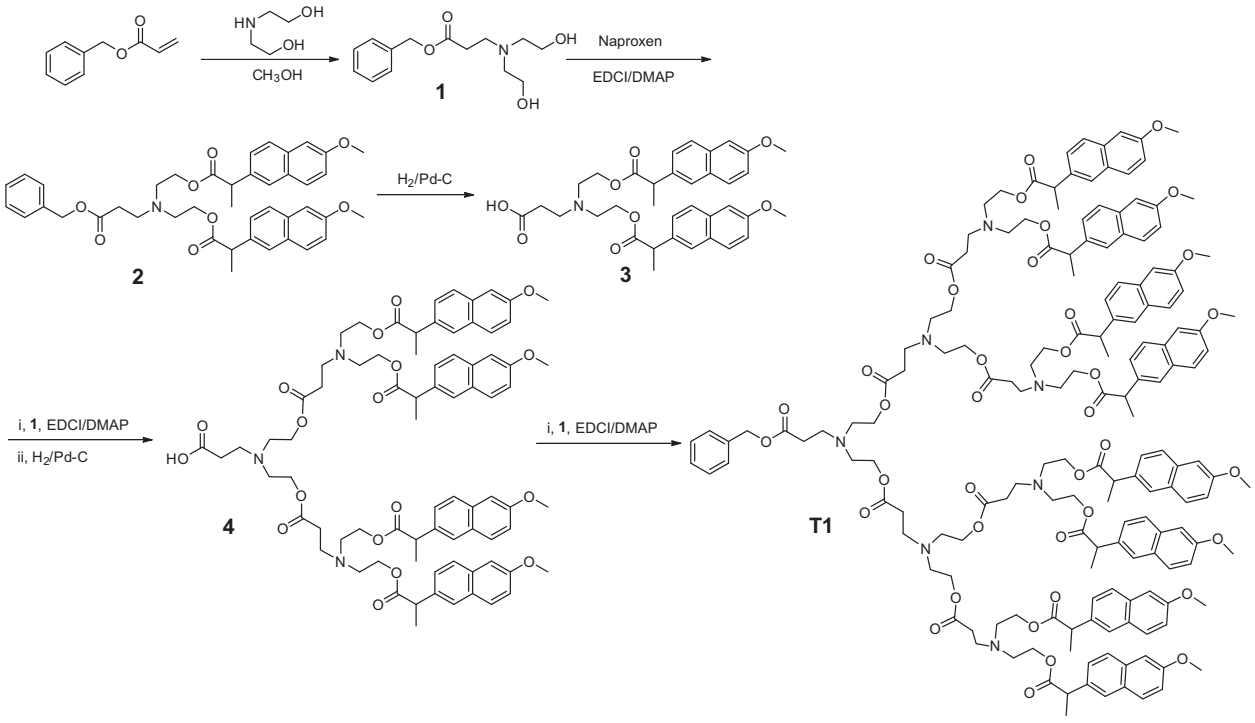
2.2. Chemistry

The synthetic procedure of the target molecules T1 could be outlined by Scheme 1. First, benzyl acrylate was conjugated to diethanolamine through a simple Michael addition and obtained the intermediate **1** with free hydroxyls. Second, the compound **2** was obtained by a condensation reaction of compound **1** with naproxen. After the removal of the benzyl group by catalytic hydrogenolysis, compound **3** was coupled with compound **1** in the presence of EDCI and HOBT. At last, the polyester dendron T1 was obtained through repeating the convergent synthesis method, as described above. In the condensation reaction with naproxen, condensing agent such as DCC or EDCI could promote the completion of the reaction accompanied by the catalysis of DMAP, so we used EDCI/DMAP as the coupling reagents in most of the ester bond condensation reactions considering the water-solubility of EDCI, the convenience of post-processing and better yield (>80%). Due to the congestion degree of the structures and the steric hindrance effect, the reaction activity of the third generation dendritic compound T1 was relatively low and the *Rf* value of different generations of naproxen dendrons in TLC layer tended to decrease, so we used different polar eluents (petroleum ether/ethyl acetate, petroleum ether/acetone) to purify different generations of naproxen dendrons by silica gel column chromatography to obtain compound T1 with complete structures.

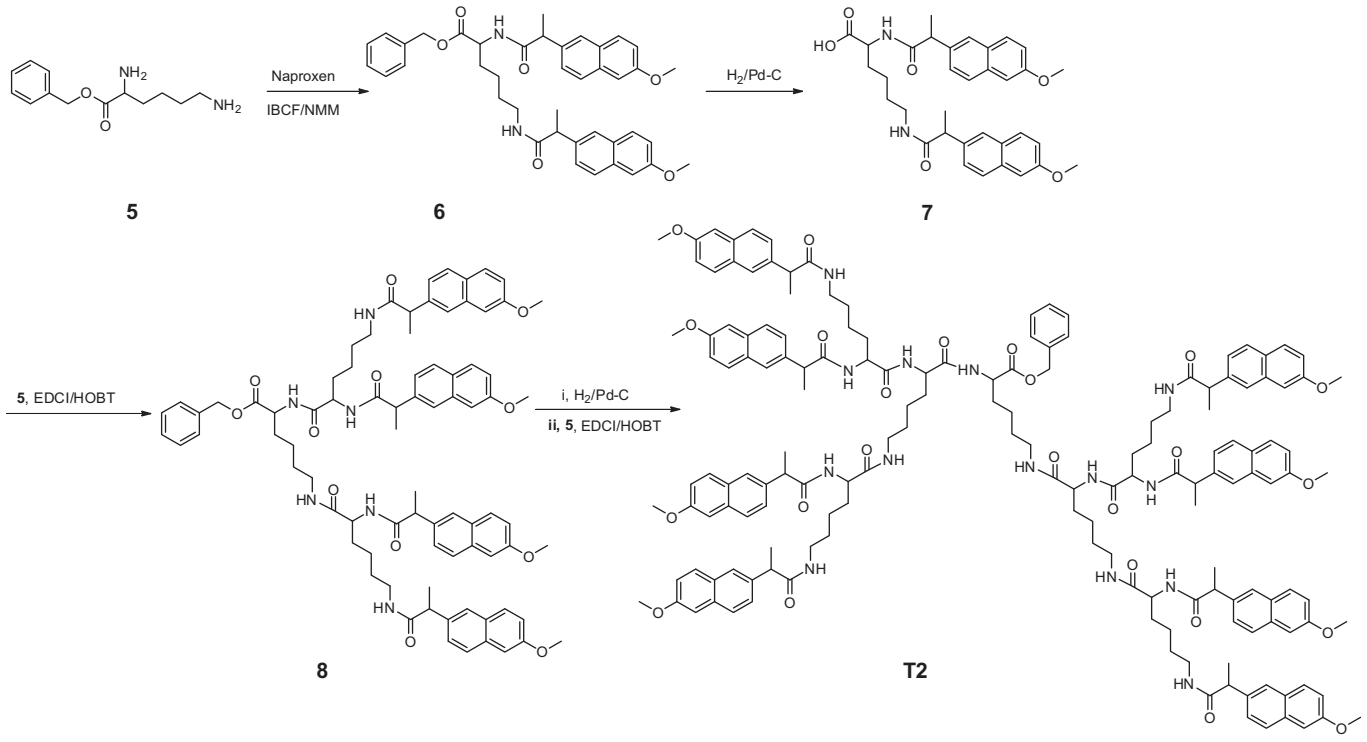
The synthesis route of target molecule T2 was outlined in Scheme 2. First, naproxen was directly conjugated to Lys-OBzl with IBCF/NMM to obtain the intermediate **6**. Then, after a series of repeating deprotection and condensation steps, we finally obtained compound T2 by convergence method. To prepare first generation intermediate **6**, we found that a variety of peptide synthesis methods could be applied, among which IBCF/NMM showed a better yield. Mixed anhydride method (IBCF/NMM as the representative coupling reagent) had many advantages in the reaction, such as less side reactions, easier to operate and the shorter reaction time. However, using this method could not obtain the second generation compound **8**, suggesting that mixed anhydride method was very sensitive to the steric hindrance and the congestion degree of the structures of these compounds. Therefore, we used EDCI/HOBT as the coupling reagents, and finally succeeded in obtaining the second generation compound **8** and the third generation target molecule T2.

The synthesis route of the target molecule T3 was outlined in Scheme 3. First, naproxen was directly conjugated to Cbz-protected tris through an ester bond condensation to obtain the intermediate **10**. After the removal of the protecting groups by catalytic hydrogenolysis, intermediate **10** experienced an amide bond condensation reaction with succinic acid monobenzyl ester to produce compound **12**. Then, after the removal of protecting groups, compound 12 reacted with Cbz-protected tris through a multi-branched condensation reaction to obtain the target molecule T3. The purposes of introducing succinate chains were not only to add more cleavable chemical bonds as a self-immolative moiety, but also to extend the length of carbon chains and reduce the difficulties of the reaction. In the synthetic process of these dendritic prodrugs, the steric hindrance was still the greatest difficulty we encountered, and we ultimately obtained the target product by EDCI/HOBT with acceptable yield after a variety of condensing agents had been tried.

The high degree of symmetry in these dendrons enabled facile confirmation of both structure and purity by NMR techniques. For example, in the ^{1}H NMR spectrum of dendron T3, the naproxen protons observed the resonance signals at 1.52 (d), 3.72 (q), and 3.88 (s) ppm were clearly distinguishable from the resonances arising from the cores (tris protons) at 2.10 (m) and 4.00 (m) ppm. Integration of the respective areas of the core protons and methoxy protons of naproxen confirmed the complete coupling of the central core and the model drugs. Furthermore, the structures of these dendrons were further verified by ESI-MS (electrospray ionization mass spectrometry), all of the ESI-MS spectra displayed a very prominent peak corresponding to the dendrons combined with protons or sodium cations. Moreover, the result of elemental analysis was also in good agreement with those of the signed structures.



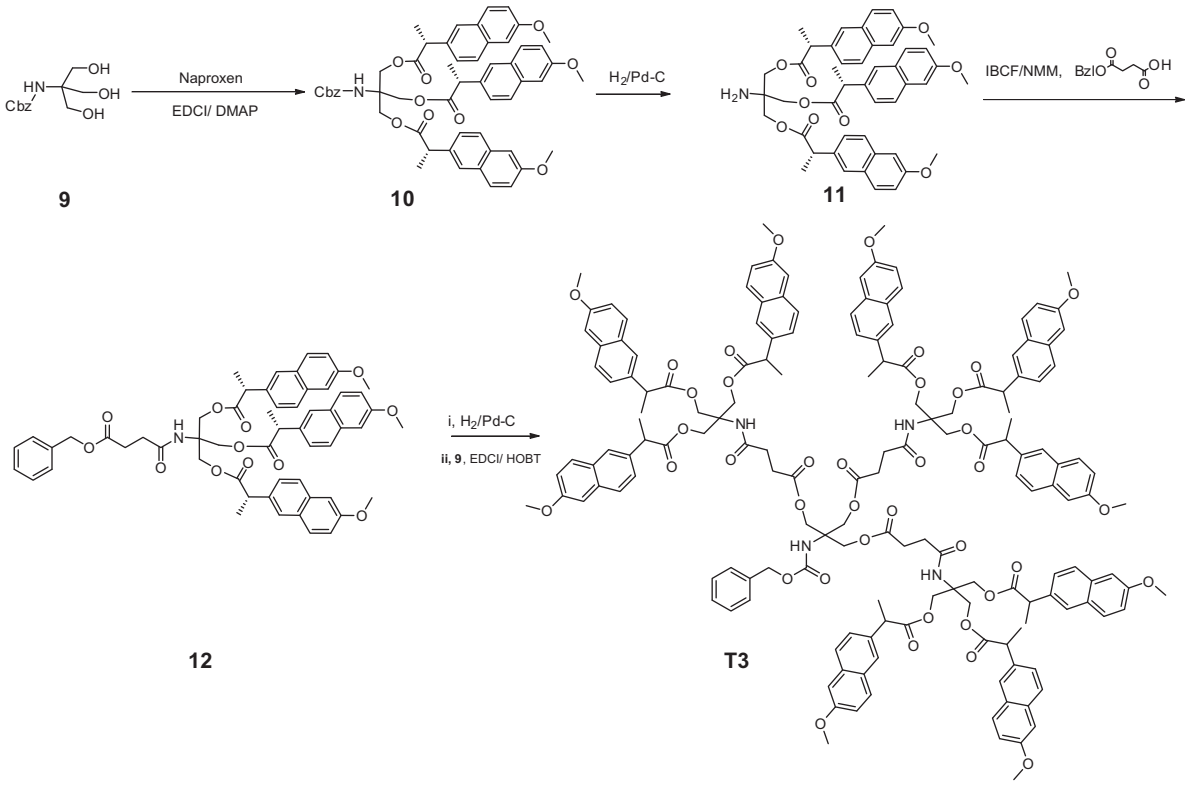
Scheme 1. The synthesis of target dendritic molecule T1.



Scheme 2. The synthesis of target dendritic molecule T2.

2.3. Biological evaluation

One of the essential prerequisites for the use of dendritic prodrugs is that they should be stable in the physiological pH, such that we can get more accurate data of their release in different microenvironment.[43] The chemical stability of prodrugs was investigated in a 0.02 M phosphate buffer at pH 2, 7, and 8 (37 °C). The evolution of the mixture was followed by HPLC over a period of 10 days. Table 1 shows the percentage of conjugate remaining after hydrolysis of the amide bond in compound T2 and the ester or amide–ester bonds in compounds T1 and T3. The stabilities of all conjugates were high with approximately >95% of the conjugates remaining at all pH



Scheme 3. The synthesis of target dendritic molecule T3.

Table 1
Chemical stability of dendritic conjugates at pH 2, 7 and 8 (37 °C)

Compound	% Conjugate remaining[a]					
	pH 2		pH 7		pH 8	
	48 h	240 h	48 h	240 h	48 h	240 h
T1 (ester)	95.7 ± 1.2	72.3 ± 4.8	96.2 ± 0.4	95.9 ± 0.7	97.3 ± 1.9	79.4 ± 7.4
T2 (amide)	97.1 ± 0.7	93.1 ± 0.4	97.6 ± 0.9	96.1 ± 1.6	91.2 ± 0.8	89.4 ± 2.6
T3 (amide–ester)	97.2 ± 1.3	89.9 ± 2.1	99.1 ± 0.2	98.4 ± 2.5	94.45 ± 3.1	79.4 ± 4.1

[a] Data are represented as mean ± SD.

values after 48 h. The direct linkage of naproxen to the T2 dendron resulted in a very stable amide prodrug under all pH conditions and even at pH 8 more than 89% of the conjugate remained intact after 10 days. The two ester or amide–ester conjugates T1 and T3 after 10 days of incubation at all pH values were less stable than T2 possibly because the less chemical stable of the ester bonds. However, most importantly, both ester conjugates showed good stability under physiological conditions (pH 7.4 and 37 °C) after 10 days of incubation. So, they may still be considered to be a sufficiently stable ester in enzymatic drug release assay.

The drug release profile was one of the most important factors to be considered for the conjugates. Since the conjugates contained different covalent bonds between the drugs and the dendritic scaffolds, they should be enzymatically and/or hydrolytically degradable to release the drug molecules to inflammatory tissue[44] and the different release capabilities in vitro of the three types of naproxen prodrugs can be compared. The precipitate (T1–T3) was incubated in the presence of cathepsin B at pH 7 or 50% (v/v in PBS) human plasma (with a small amount of Twain as hydrotropy agent) at 37 °C. All compounds are dissolved in solvent with cleaning state and the hydrolytic release of naproxen in the solution was monitored by RP-HPLC.

The results from these in vitro assays (Fig. 1) suggested several trends. First, the three different types of prodrugs were able to release the parent drugs in vitro. Specifically, the drug released rapidly from the compound T3 in 50% human plasma, reaching 57.1% after 48 h and almost 80% within five days, whereas the drug release in cathepsin B was much slower, implying 4.6% and 16.1% in the same period, respectively. Second, in 50% human plasma, the drug released from the compound T3 reaching 47.3% after 24 h. The release property of compound T3 was much better than compounds T1 and T2. For compound T1, it might be due to the C–N bond's relative stability under various environments causing the release performance decline. Indeed, T1 showed only ca. 18% of activation after 120 h of incubation in human plasma.

The trends observed with in vitro drug release data would suggest that the three linkages were relatively stable in cathepsin B but labile under human plasma conditions to allow the transport of the prodrug and effective release of the free active drug from the carrier. Prodrug T3 with benzyl ester or amide had a more rapid activation pathway in human plasma, possibly because a benzyl ester or amide linkages were better substrates of aryl ester hydrolase in human plasma and as more suitable triggers, the eliminate process was accelerated by a self-immolative disassembly pathway

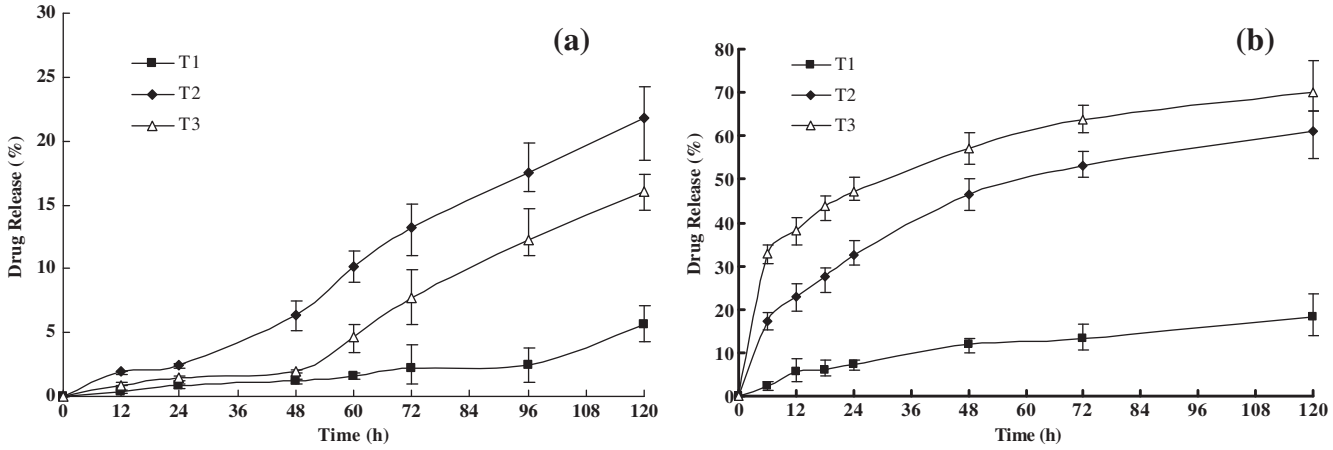


Figure 1. Release of Naproxen from compounds T1–T3 in different conditions (a) in cathepsin B at pH 7; (b) in human plasma at pH 7. The percentage of drug release was deduced from the difference between the initial amounts of compounds and that of regenerated drug Naproxen. Error bars represented the mean and standard deviation of three independent experiments.

or the ester bond was more instable than the amide bond. A plausible mechanism for the drug release could be explained as follows: when incubated with the enzyme, prodrug T3 was rapidly cleaved leading to the release of intermediate with benzyl removed, while cathepsin B might not be very good to initiate this reaction.[45] Prodrugs disappeared concomitantly with the appearance of naproxen in 120 h. Furthermore, a LC–MS analysis of dendritic prodrug T3 after 120 h at 37 °C in human plasma at pH 7.0 was taken. The solution was evaluated by an Agilent 6430 LC–MS system. The mobile phase was a mixture of methanol and 0.2% formic acid aqueous solution (linear gradient beginning with 20:80 v/v, reaching 60:40 v/v within 5 min.). From the results monitored by HPLC–MS, 120 h after the addition of human plasma, tris (M.wt. 121), succinic acid (M.wt. 118) and naproxen were detected in the mixture (see Supporting Information). The results shown in LC–MS study provided a proof that tris and succinic acid linkages were the final eliminated products after enzymatic cleavage of dendritic T3 (Fig. 2).

After in vitro drug release, several pharmacodynamics experiments were taken. First, cytotoxicity is one of the most important factors to be considered in selecting dendritic prodrugs for biomedical applications. The toxicity of compounds T1–T3 against HEK293 cells was evaluated by MTT assays and naproxen was taken as a positive control. The results are shown in Figure 3. The assays demonstrated that neither all dendritic conjugates nor naproxen was significantly toxic against HEK293 cells in the concentration range of 2–32 μg/ml. With the augment of tested concentration, the cytotoxicity of these compounds increased only by a slight degree. However, we did not observe a significant difference in the toxicity of dendrons with positive control naproxen. So, not any obvious toxicity was found in cell culture experiment, as we expected.

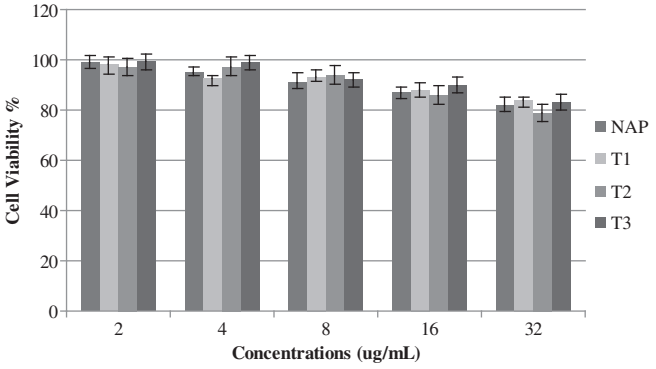


Figure 3. Cytotoxicity of dendrons against HEK293 cells over a 24 h incubation period determined by MTT assay. Cell viability was expressed as a percentage of the control cell culture. Values are represented as mean ± SD (n = 3).

Finally, Table 2 revealed the in vivo acute anti-inflammatory activity of the target conjugates at a dose of 10 mg/kg q.d. in xylene-induced mice auricle tumefaction model.[46] Compared with saline group, entire dendritic prodrugs exhibited good anti-inflammatory activity with the inhibition percentage of auricle tumefaction ranging from 39.62% to 49.64%. It was worth pointing out that

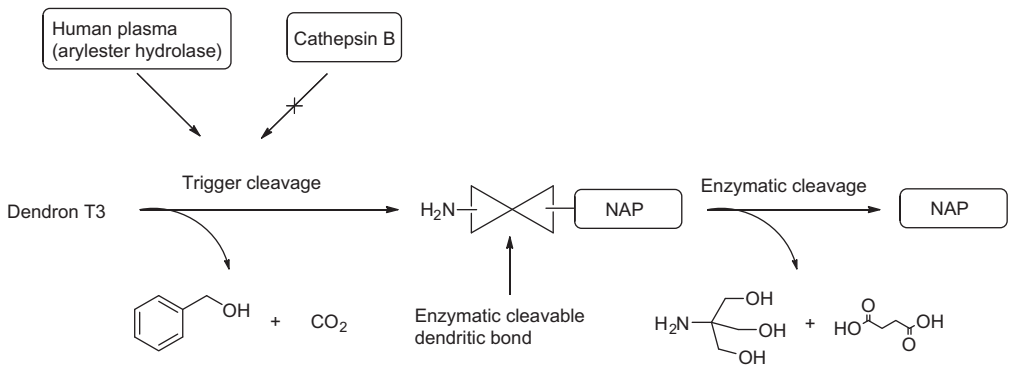


Figure 2. Human plasma-catalyzed drug release mechanism.

Table 2
In vivo inhibitory activity of dendritic prodrug in xylene-induced mice auricle tumefaction assay and ulcer index

Compound	n[a]	Dosage (μM/kg)[b]	Degree of tumefaction	Inhibition percentage[c]	Ulcer index[d]
Blank	10	––	15.67 ± 2.59	––	––
Naproxen	10	200, q.d.	10.87 ± 1.73	30.63	8.26
T1	10	25, q.d.	9.46 ± 3.74*	39.62	6.93
T2	10	25, q.d.	8.12 ± 3.07*	48.18	6.21
T3	10	22.2, q.d.	7.89 ± 2.66*	49.64	5.94

[a] Values are the means of the indicated number of experiments (n).
[b] Mice were treated with 0.9% NaCl solution, naproxen and test compounds one time a day for five days, respectively. The administration of the dendritic prodrug is same molar amount of native naproxen.
[c] Percentage of inhibition = (1 − tumefaction degree of blank group/tumefaction degree of test group) × 100%.
[d] Ulcerogenic activity was evaluated after oral administration of test compounds at the dosage of 200 mg/kg.
* $P < 0.05$ comparing with blank group.

significant anti-inflammatory activity was achieved for T1 (39.62%), T2 (48.18%) and T3 (49.64%) with potent inhibition percentage higher than the contrast drug naproxen (30.63%). On the other side, the most common side effects associated with the long-term administration of NSAIDs are gastrointestinal erosions, ulcer formation, and sometimes severe bleeding. It was therefore essential to evaluate the potential in vivo ulcerogenicity of prodrugs T1–T3 in comparison to the corresponding contrast drug naproxen. The severity of gastric damage, assessed using an ulcerogenicity assay, was expressed as an ulcer index (*UI*) and the *UI* for each test compound is calculated by adding the total length (L, in mm) of individual gastric lesions in each stomach and averaging over the number of animals in each group ($n = 10$): $UI = (L1 + L2 + L3 + L4 + L5 + L6 + L7 + L8 + L9 + L10)/10$. As shown in Table 2, the results indicated that these compounds T1–T3 (ulcer index ranges from 5.94 to 6.93) cause less gastric ulceration and disruption of gastric epithelial cells as compared to naproxen (ulcer index 8.26). The results from the preliminary in vivo assay indicated that the dendritic prodrugs, especially compound T3, exhibited improved anti-inflammatory activities with reducing gastric ulcerogenicity which were consistent with our in vitro release experimental results. We speculated that the dendritic prodrugs might make the drug half-life longer and would form a continuous and stronger effect compared with native naproxen so exhibited better anti-inflammatory activity.

3. Conclusions

In conclusion, we developed a new self-immolative dendritic NISADs prodrug programmed to release multiple molecules of the potent naproxen after a single enzymatic activation step. Upon human plasma activation, the dendritic prodrug is much easier to release than its positive counterpart as well as cathepsin B at the same concentration. The preliminary in vivo biological activities of these compounds evidenced our rational design of improved anti-inflammatory prodrugs with reduced gastric toxicity (ulcerogenicity). This targeting system could represent an important step toward the quest for the magic bullet in the anti-inflammatory therapy. Further developments in this direction including preliminary study of biodistribution and pharmacokinetic analysis of these conjugates in vivo are now underway in our laboratory.

4. Experimental

4.1. Chemistry

General: all reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC): silica gel plates GF_{254}; compounds were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (20 wt% in ethanol) followed by heating. Column chromatography was performed by using silica gel with eluent given in parentheses. ^{1}H NMR analysis was performed using $CDCl_3$ or DMSO-d_6 as a solvent at room temperature. The chemical shifts were expressed in relative to TMS (= 0 ppm) and the coupling constants J in Hz. The purity of compounds screened in biological assays was determined to be ⩾97% by HPLC (Agilent 1100 HPLC system) analysis with a photodiode array detector, An Atlantis C18 (150 × 4.6 mm, i.d. 5 μm) (Waters, Milford, Mass, USA) was used with a gradient elution of methanol and HPLC-grade water as mobile phase at a flow rate of 1 ml/min.

4.1.1. Synthesis of compound 1

Benzyl acrylate (0.9 g, 5.5 mmol) and diethanolamine (0.85 g, 8.1 mmol) dissolved in methanol (20 ml), were stirred at room temperature overnight. The solution was concentrated under vacuum, then the residue was extracted with ethyl acetate (30 ml) and washed with brine (10 ml). The organic layer was dried over anhydrous Na_2SO_4. After concentration, the compound **1** was obtained as colorless oil 1.14 g (yield 77.7%). ^{1}H NMR (400 MHz, $CDCl_3$): 2.54 (t, 2H, J = 6.4 Hz, $COCH_2$), 2.63 (t, 4H, J = 5.2 Hz, N–CH_2×2), 2.86 (t, 2H, J = 6.4 Hz, N–CH_2), 3.59 (t, 4H, J = 5.2 Hz, CH_2–O×2), 5.14 (s, 2H, ph-CH_2), 7.34–7.37 (m, 5H, ph-H).

4.1.2. Synthesis of compound 2

To the solution of compound 1 (150 mg, 0.56 mmol) and naproxen (260 mg, 1.13 mmol) dissolved in anhydrous CH_2Cl_2 (15 ml), EDCI (233 mg, 1.13 mmol) and DMAP (14 mg, 0.013 mmol) were added at room temperature. After stirring for 24 h at rt, the solution was concentrated under vacuum. The residue was then extracted with ethyl acetate (30 ml) and washed successively with 1M HCl (10 ml), 1M $NaHCO_3$ (10 ml), and brine (10 ml). The organic layer was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica-gel column chromatography using PE-EA (5:1–3:1, v/v) as an eluent to obtain compound **2** as colorless oil 440 mg (yield 85.3%). ^{1}H NMR (400 MHz, $CDCl_3$): 1.54 (d, 6H, J = 7.2 Hz, Nap-CH_3×2), 2.30 (t, 2H, J = 6.8 Hz, $COCH_2$), 2.60 (t, 4H, J = 6 Hz, N–CH_2×2), 2.73 (t, 2H, J = 6.8 Hz, N–CH_2), 3.78 (q, 2H, J = 7.2 Hz, NapCOCH×2), 3.91 (s, 6H, NapOCH_3×2), 3.99 (t, 4H, J = 6 Hz, CH_2–O×2), 5.05 (s, 2H, ph-CH_2), 7.08–7.68 (m, 17H, NapphH + ph-H). ESI-MS (m/z): calcd for 691.8 obsd 692.2 ($[M+H]^+$).

4.1.3. Synthesis of compound 3

Compound **2 (400 mg)** was dissolved in methanol (10 ml), and 10% Pd/C (100 mg) were added into the solution. The reaction mixture was stirred at room temperature under a H_2 atmosphere. After 24 h, the mixture was passed through a membrane filter to remove

the catalyst and then evaporated under vacuum to afford compound **3** as colorless oil 250 mg (yield 91.7%). ^{1}H NMR (400 MHz, $CDCl_3$): 1.48 (d, 6H, J = 7.2 Hz, Nap$CH_3\times2$), 2.34 (t, 2H, J = 6.8 Hz, $COCH_2$), 2.62 (t, 4H, J = 6 Hz, N–$CH_2\times2$), 2.71 (t, 2H, J = 6.8 Hz, N–CH_2), 3.72 (q, 2H, J = 7.2 Hz, NapCOCH×2), 3.89 (s, 6H, Nap-$OCH_3\times2$), 4.01 (t, 4H, J = 6 Hz, CH_2–O×2), 7.09–7.48 (m, 12H, Nap-phH). ESI-MS (m/z): calcd for 601.8 obsd 600.2 ($[M-H]^-$).

4.1.4. Synthesis of compound 4

The same procedure as described above for preparation of **3** from compound **1**. Compound **4**: pale yellow oil (78% total yield in two steps). ^{1}H NMR (400 MHz, $CDCl_3$): 1.52 (d, 12H, J = 7.2 Hz, Nap$CH_3\times4$), 2.2–2.4 (m, 6H, $COCH_2\times3$), 2.70–2.90 (m, 12H, N–$CH_2\times6$), 3.79 (q, 4H, J = 7.2 Hz, NapCOCH×4), 3.96 (s, 12H, Nap-$OCH_3\times2$), 3.98–4.13 (br s, 12H, CH_2–O×6), 5.10 (s, 2H, ph-CH_2), 7.08–7.68(m, 29H, Nap-phH + ph-H). ESI MS (m/z): calcd for 1433.66 obsd 1433.26 ($[M]^+$).

4.1.5. Synthesis of compound T1

The same procedure as described above for preparation of compound **4**. Compound T1: pale yellow oil (yield: 64.4%). ^{1}H NMR (400 MHz, $CDCl_3$): 1.54 (d, 24H, J = 7.2 Hz, Nap$CH_3\times8$), 2.22–2.39 (m, 14H, $COCH_2\times7$), 2.71–2.92 (m, 28H, N–$CH_2\times14$), 3.73 (m, 14H, $COCH_2\times7$), 3.79 (q, 8H, J = 7.2 Hz, NapCOCH×8), 3.96 (s, 24H, Nap$OCH_3\times8$), 3.98–4.03 (br s, 28H, CH_2–O×14), 5.10 (s, 2H, ph-CH_2), 7.08–7.68 (m, 53H, Nap-phH + ph-H). ESI-TOF MS (m/z): calcd for 2922.3736 obsd 2923.3805 ($[M+H]^+$).

4.1.6. Synthesis of compound 6

Naproxen (0.24 g, 1 mmol) dissolved in anhydrous THF (10 ml) was cooled at −15 °C, then added NMM (0.1 g, 1 mmol) and IBCF (0.13 g, 1 mmol), stirred for 5 min. Lysine benzyl ester *p*-toluenesulfonate salt (0.3 g, 0.52 mmol) dissolved in the mixed solution NMM and THF was added to the reaction. After stirred overnight, the solution was concentrated under vacuum. The concentrated solution was taken up in ethyl acetate (30 ml) and washed with 1M HCl (10 ml), 1M $NaHCO_3$ (10 ml), brine (10 ml). The organic layer was dried over anhydrous Na_2SO_4. After concentration, The crude product was purified by recrystallization using PE-EA to obtained compound **49** as white solid 0.55 g (yield 84%). ^{1}H NMR (400 MHz, $CDCl_3$): 1.48 (d, 6H, J = 7.2 Hz, Nap$CH_3\times2$), 1.37–1.82 (m, 6H, lys$CH_2\times3$), 3.25 (br s, 2H, lysN–CH_2), 3.88 (m, 2H, NapC-OCH×2), 3.99 (s, 6H, Nap$OCH_3\times2$), 4.32 (br s, 1H, lysCH), 5.22 (m, 2H, ph-CH_2), 7.04–7.83 (m, 17H, Nap-phH + ph-H). ESI-MS (m/z): calcd for 660.3199 obsd 661.3297 ($[M+H]^+$).

4.1.7. Synthesis of compound 7

Compound **6** was dissolved in methanol (10 ml), and 10% Pd/C (100 mg) were added into the solution. The reaction mixture was stirred at room temperature under a H_2 atmosphere. After 24 h, the mixture was filtrated to remove the catalyst and then evaporated under vacuum to afford compound **7** as white waxy solid 250 mg (yield 89.1%).

4.1.8. Synthesis of compound 8

Compound **7** (0.57 g, 1 mmol) and compound 5 (0.3 g, 0.5 mmol) were dissolved in DCM (15 mL), then added EDCI (0.23 g, 1.13 mmol) and HOBt (0.15 g, 1 mmol), stirred in room temperature for 16 h. After stirred overnight, the solution was concentrated under vacuum. The concentrated solution was taken up in ethyl acetate (30 ml) and washed with 1M HCl (10 ml), 1M $NaHCO_3$ (10 ml), brine (10 ml).The organic layer was dried over anhydrous Na_2SO_4. The crude product was purified by silica-gel column chromatography using PE-EA (1:1–1:2, v/v) as an eluent to obtain compound **8 0.33 g** as colorless oil. (yield 50%). ^{1}H NMR (400 MHz, $CDCl_3$): 1.52 (d, 12H, J = 7.2 Hz, Nap$CH_3\times4$), 1.25–1.92 (m, 18H, lys$CH_2\times9$), 3.35 (br s, 6H, lysN–$CH_2\times3$), 3.87 (m, 4H, NapCOCH×4), 3.98 (s, 12H, Nap$OCH_3\times4$), 4.42 (br s, 3H, lysCH×3), 5.21 (m, 2H, ph-CH_2), 7.10–7.80 (m, 29H, Nap-phH + ph-H). ESI-MS (m/z): calcd for 1341.63 obsd 1365.2 ($[M+Na]^+$).

4.1.9. Synthesis of compound T2

According to the same procedure of preparation of **49** and **51**. After increased generation and debenzylation, compound 42 was afforded as white solid (37% yield in two steps). ^{1}H NMR (400 MHz, $CDCl_3$): 1.52 (d, 24H, J = 7.2 Hz, Nap$CH_3\times8$), 1.24–1.91 (m, 42H, lys$CH_2\times21$), 3.29 (br s, 14H, lysN–$CH_2\times7$), 3.87 (m, 8H, NapCOCH×8), 3.92 (s, 24H, Nap$OCH_3\times8$), 4.46 (br s, 7H, lysCH×7), 5.11 (m, 2H, ph-CH2), 7.10–7.80 (m, 53H, Nap-phH + ph-H). ESI-MS (m/z): calcd for 2708.4275 obsd 2709.4671 ($[M+H]^+$).

4.1.10. Synthesis of compound 10

Z-protected tris (0.1 g, 0.39 mmol) was dissolved in CH_2Cl_2 (15 ml), then naproxen (0.3 g, 1.3 mmol), EDCI (0.27 g, 1.3 mmol) and DMAP (0.02 g, 0.13 mmol) were successively added into the solution. After stirred overnight at room temperature, the solution was concentrated under vacuum. The concentrated solution was dissolved in ethyl acetate (30 ml) and washed with 1M HCl (10 ml), 1M $NaHCO_3$ (10 ml), brine (10 ml). The organic layer was dried over anhydrous Na_2SO_4. After concentration, the crude product was purified by a silica gel chromatography column using PE-EA (5:1–3:1, v/v) to obtained compound **53** as white solid 0.34 g (yield 94.4%) mp:120–122 °C. ESI-MS (m/z): calcd for 891.4 obsd 914.2 ($[M+Na]^+$).

4.1.11. Synthesis of compound 12

Succinic acid monobenzyl ester (74 mg, 0.36 mmol) dissolved in anhydrous THF (10 ml) was cooled at −15 °C, then added NMM and IBCF (0.07 ml, 0.48 mmol), stirred for 5 min, compound **11 (0.28 g, 0.36 mmol)** dissolved in THF was added to the reaction. After stirred at room temperature for 3 h, the solution was concentrated under vacuum. The residue was then taken up in ethyl acetate (30 ml) and washed with 1M HCl (10 ml), 1M $NaHCO_3$ (10 ml), and brine (10 ml). The organic layer was dried over anhydrous Na_2SO_4. After concentration, the residue was purified by silica-gel column chromatography using PE-EA (3:1–2:1, v/v) as an eluent to obtain compound **12** as white solid 150 mg (yield 40.5%). mp:115–117 °C 1H NMR (400 MHz, $CDCl_3$): 1.47 (d, 9H, J = 7.6 Hz, Nap$CH_3\times3$), 1.70–1.72 (m, 2H, $COCH_2$), 2.19–2.31 (m, 2H, $COCH_2$), 3.72 (q, 3H, J = 7.6 Hz, NapCOCH×3), 3.87 (s, 9H, Nap-$OCH_3\times3$), 4.13 (s, 6H, tris-$CH_2\times3$), 5.04 (s, 2H, ph-CH_2), 7.04–7.64 (m, 23H, Nap-phH + phH). ESI-MS (m/z): calcd for 948.1 obsd 970.2 ($[M+Na]^+$).

4.1.12. Synthesis of compound T3

According to the same procedure of preparation of **53**. Compound **45**: yield 49%. ^{1}H NMR (400 MHz, $CDCl_3$): 1.52 (d, 27H, J = 7.6 Hz, Nap$CH_3\times9$), 2.10–2.72 (m, 6H, SA-$COCH_2\times3$), 3.72 (q, 9H, J = 7.6 Hz, NapCOCH×9), 3.88 (s, 27H, Nap$OCH_3\times9$), 4.00–4.11 (m, 24H, tris-$CH_2\times12$), 7.04–7.64 (m, 59H, Nap-phH + phH). ESI-MS (m/z): calcd for 2783.1539 obsd 2784.1666 ($[M+H]^+$).

4.2. HPLC analysis, chemical stability and drug release studies

The HPLC system consisted of a photodiode array detector and a set of Model LC-10AT liquid chromatography including a manometric module as well as a dynamic mixer from Agilent 1100 HPLC system. The mobile phase is a mixture of methanol and 0.05 mol/l potassium dihydrogen phosphate (adjusted to pH 3.0 with phosphoric acid) (60:40) which was filtered through a 0.45 mm membrane filter before use. A Waters XTerra RP18 column (250 mm, 4.6 mm, 5 μm) was eluted with the mobile phase at flow rate of

1.0 ml/min. The eluate was monitored by measuring the absorption at 256 nm with a sensitivity of AUFS 0.01 at 25 °C. The retention time (*RT*) of Naproxen is 10.265 min. The retention time (*RT*) of compound T3 is 7.43 min.

The conjugates (0.1 μM) was incubated at 37 °C in 20 mM phosphate buffer at different pH 2.0, 7.0, 8.0, respectively, (1 ml). Stability in phosphate buffer was monitored by analytical HPLC conditions mentioned above.

The conjugates (0.1 μM) were incubated in 50% (v/v in PBS) human plasma (with a small amount of Twain as hydrotropy agent) at 37 °C and the hydrolytic release of Naproxen in the solution was monitored by RP-HPLC. The concentration of Naproxen was analyzed using the HPLC conditions mentioned above.

4.3. In vitro cytotoxicity assay

The cytotoxic effects of compounds T1–T3 and naproxen were determined using the standard MTT assay. Briefly, HEK293 cells (2500/well) were seeded in 96-well plates and cultured for 24 h, followed by treatment with the target compounds for another 48 h. Twenty microliter of 5 mg/ml MTT was added per well and incubated for another 2.5 h at 37 °C. Then the supernatant fluid was removed and MTT formazan precipitate was dissolved in 150 μl of DMSO, shaken mechanically for 15–20 min. The optical density of each well was measured at 570 nm, using a SpectraMAX M5 microplate spectrophotometer.

4.4. In vivo studies

4.4.1. Anti-inflammatory assay

According to the requirements of the National Act on the usage of experimental animals (PR China), the Sichuan University Animal Ethical Experimentation Committee, approved all procedures of our in vivo studies. Male Kunming mice (18–22 g) which were housed in room temperature of 25 ± 2 °C and humidity of 60 ± 5% were used through the studies. Naproxen was administered (10 mgl/kg, t.i.d.) as a positive comparator. The test compounds were administered at the same dose (10 mgl/kg, t.i.d.), respectively. Fifty mice were randomly divided into 5 groups and were treated with 0.9% NaCl solution, naproxen and test compounds for five days, respectively. The buninoid filter with diameter of 7 mm is infiltrated by xylene. After 1 h of the last administration, the filter was clung to the right ear of the mice for 30 s. After 30 min the mice were executed by decollation and the ears were slotted wafers by 7 mm hole puncher. The inhibition percent of auricle tumefaction was calculated using the following formula. Percent of inhibition (%) = $(1 - a/b) \times 100\%$, where a means tumefaction degree of control group and b means tumefaction degree of test group.

4.4.2. Acute ulcerogenesis assay

Kunming mice of either sex were divided into control and different test groups of ten animals each group (18–22 g). Ulcerogenic activity was evaluated after oral administration of test compounds at the dose of 200 mg/kg. Naproxen and the test compounds were suspended and administered in a 1% methylcellulose solution. Food and water were removed 24 h before administration of test compounds. All animals were sacrificed after 4 h of drug administration. Their stomachs were removed, immerged in 10% formaldehyde solution and 30 min later cut out along the greater curvature of the stomach, gently rinsed with water, and placed on ice. The number and the length of ulcers observed in each stomach were determined by using magnifier lenses. In this assay, the severity of each gastric lesion is measured along its greatest length (1 mm, rating of 1; 1–2 mm, rating of 2; and >2 mm, rating according to their length in millimeter). The *UI* for each test compound is calculated by adding the total length (L, in mm) of individual gastric lesions in each stomach and averaging over the number of animals in each group ($n = 10$): $UI = (L1 + L2 + L3 + L4 + L5 + L6 + L7 + L8 + L9 + L10)/10$.

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Supplementary data

Supplementary data (full synthetic procedures and characterization data, as well as methods for drug release experiments) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.006.

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