Effect of Self-healing Encapsulation on the Initial Burst Release from PLGA Microspheres Containing a Long-Acting Prostacyclin Agonist, ONO-1301

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The purpose of this study was to perform self-healing encapsulation of ONO-1301, a long-acting prostacyclin agonist, into poly(lactide-co-glycolide) (PLGA) microspheres using the oil-in-water (o/w) emulsion solvent evaporation method in order to try to limit the initial burst release of drug. Adequate self-healing of PLGA seemed to be achieved by stirring during the evaporation of solvent at 40°C close to the glass transition temperature (Tg) of the polymer (40.1°C). The plasticizers dimethylphthalate (DEP) or tributyl O-acetylcitrate (TBAC), at concentrations of 0.1–1.0%, to the internal oleogeneous phase in the o/w emulsion system was effective in restricting the initial burst release of the prepared microspheres. The combination of a self-healing at Tg of the polymer and the addition of 1% of each plasticizer was ultimately found to be the most effective in restricting the initial burst release. It is suggested that this is due to the synergistic effect of smooth surface morphology promoted by self-healing at Tg of the polymer and a decrease of the Tg of PLGA caused by the addition of plasticizers.

Key words self-healing; burst release; plasticizer; microsphere; glass transition temperature

The concept of ‘self-healing’ was first described by White et al.1) and seems to be a unique characteristic of polymers. The term is used to describe the autonomic process by which damaged structures, such as cracks or pores, are repaired by re-arrangement of the polymer chains. In the pharmaceutical field, especially relating to microencapsulation techniques, there have been a number of articles on this phenomenon. Desai and Schwendeman prepared spontaneous hollow microspheres with many pores on the surface.2) They then put a model water-soluble peptide into the internal space and closed the pores by self-healing. One merit of this method is the avoidance of the use of solvents which may damage or reduce the potency of active peptides or proteins. The self-healing process may be applicable to encapsulation of not only peptides or proteins but also non-protein compounds. In particular, the process could be useful for the encapsulation of pharmaceutical active ingredients, as the surface of poly(lactide-co-glycolide) (PLGA) microspheres prepared by self-healing would be expected to be smooth, with fewer surface pores, thereby limiting the initial burst release. Nevertheless, the encapsulation of non-proteins using the self-healing concept has not been reported up to now.

In the present study, we used ONO-1301 as a model compound to investigate the possibility of using self-healing to encapsulate non-protein drugs in PLGA microspheres. ONO-1301 was developed as a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity.3) ONO-1301 has a carboxylic acid and a lipid-soluble functional group that activates the prostacyclin receptor, but it does not have prostanoid structures (a five-membered ring or allylic alcohol), which allows it to exhibit long-lasting prostacyclin activity. Unlike prostacyclin, ONO-1301 has a 3-pyridine radical, which is known to inhibit thromboxane synthase through interaction with carboxylic acid via a hydrogen bond. In animal studies, ONO-1301 has been reported to improve pulmonary hypertension,4,5) pulmonary fibrosis6) and arterial obstructive disease,7) due to its vasodilatory, antplatelet, and bronchodilatory properties. The use of prostacyclin itself has been limited by the fact that it causes a reflex increase in heart rate, rise in myocardial oxygen consumption and worsening of angina,8) and when ONO-1301 plasma levels are increased rapidly, a similar picture emerges. Therefore, it is important not to have a large initial burst release for this drug, as for many others.

We reported previously that slow-release ONO-1301-loaded PLGA microspheres were effective in reducing peripheral neuronal disorders in diabetic rats.9) However, the extent of the initial burst release from these microspheres was such as to suggest that it might increase the risk of adverse effects such as hypertension in the rats. The extent of the initial burst release was therefore a critical factor and we searched for a way to control it. The self-healing concept seemed to offer a promising approach to regulating and/or restricting the initial burst release of ONO-1301.

In the present study, encapsulation of ONO-1301 into PLGA microspheres with self-healing was achieved by the oil-in-water (o/w) emulsion/solvent evaporation process. ONO-1301 is not highly soluble in water and can provide a model for the encapsulation by self-healing of a drug with comparatively low solubility.

In the self-healing of polymers such as PLGA, re-arrangement to produce a smooth microsphere surface is likely to be most efficient at temperatures near the glass transition temperature (Tg). Thus, at this temperature, the re-arrangement of the PLGA matrix should be most efficient in limiting the initial burst release of ONO-1301 from the microspheres.

The aim of the present study was therefore to perform self-healing encapsulation of ONO-1301 into PLGA microspheres

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using the o/w emulsion/solvent evaporation method, and to evaluate the effect of this methodology on the initial burst release of ONO-1301 from the microspheres.

Firstly, conditions such as the temperature and time required for the evaporation of solvent (CH₂Cl₂—methanol, 5:1) were evaluated as a function of the morphology of the microspheres using the in vitro release test, in order to determine the optimal preparative conditions for smooth microspheres. Secondly, the effect of adding plasticizers such as dimethylphthalate (DEP) and tributyl O-acetylacetate (TBAC) to the internal oleaginous phase on the initial burst release rate from the microspheres was examined.

Combinations of these preparative conditions, i.e., self-healing conditions and addition of plasticizer, were then studied in order to determine the optimal conditions for limiting the initial burst release of drug. Finally, we evaluated the synergistic effects of self-healing and use of plasticizer under various conditions.

**Experimental**

**Materials**  ONO-1301 was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). PLGA, with a co-polymer ratio of DL-lactide to glycolide of 75:25 (PLGA 7505, average molecular weight 5000; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as the substrate of the microspheres. Polyvinyl alcohol (PVA) and Tween 80 (both Nacalai Tesque Ltd., Kyoto, Japan) were used as dispersants for the production of PLGA microspheres. High-quality reagent-grade acetone, acetonitrile, methanol and ethanol (Wako Pure Chemical Industries, Ltd.) were used as good solvents for PLGA and Japanese Pharmacopoeia-grade purified water was used as a poor solvent. The hydrophobic plasticizers DEP and TBAC were also purchased from Wako Pure Chemical Industries, Ltd.

**Preparation of Self-healing ONO-1301-Loaded PLGA Microspheres**  Various batches of ONO-1301-loaded PLGA microspheres were prepared using the o/w emulsion/solvent evaporation method as described in a previous report.¹⁰ The preparative procedures can be summarized as follows: an aqueous solution of PVA (a poor solvent of PLGA) was placed in a glass vessel. A dichloromethane–methanol solution of PVA (a poor solvent of PLGA) was evaporated off by stirring at various temperatures (25, 30, 35 and 40°C) and for various lengths of time (6, 12, 24 and 48h). After centrifugation and washing, ONO-1301-loaded PLGA microspheres were isolated by lyophilization. The preparative conditions for each formulation are summarized in Table 1 (formulations 1–25).

**Particle Size Measurement**  Volume diameters of the wet particles sampled prior to lyophilization were measured using a Multisizer™3 Coulter Counter® (Beckman Coulter). The particles sampled prior to lyophilization were measured using the procedure described in a previous study.⁶ The theoretical loading % was 16.7% for all formulations.

**Drug Loading and Encapsulation Efficiency**  Dimethyl sulfoxide (DMSO) containing n-propyl-4-hydrobenzoate served as an internal control to determine encapsulation efficiency, and the solution was homogenized using a sonicator.

The concentration of ONO-1301 in this solution was determined using the procedure described in a previous study.⁶ Drug loading and encapsulation efficiency were defined as:

- Obtained loading efficiency (LE) (%) = encapsulated drug / microparticle weight
- Theoretical loading efficiency (TLE) (%) = drug used for encapsulation / (drug + polymer used for encapsulation)
- Encapsulation efficiency (EE) (%) = LE/TLE

**Release of ONO-1301 from ONO-1301-Loaded PLGA Microspheres**  ONO-1301-loaded PLGA microspheres were suspended in phosphate-buffered saline (0.067 mol/L salt concentration, pH 6.8) containing 0.2% Tween-80 and the concentration of ONO-1301 adjusted to 100 mg/mL. Aliquots of this solution (1 mL) were incubated at 37°C. At various time intervals, an aliquot was centrifuged at 12000 rpm for 5 min, and the supernatant was discarded. The pellet was dissolved in DMSO, and the amount of ONO-1301 remaining was analyzed by HPLC. The drug release rate was calculated by measurement the drug residual rate in pellet when the residual rate at 0 h was 100%. The obtained release profile was analyzed using the non-linear least-squares method and the apparent first-order rate constant was calculated. The remaining drug %...
at 0.5, 1, 6, 24 h were fitted by based on the first-order release kinetics.

**Scanning Electron Microscope (SEM)** The surface morphology of microspheres was examined by taking SEM images using a JEOL JSM-6390LA SEM (JEOL, Tokyo, Japan). Briefly, microspheres were fixed previously on a brass stub using double-sided adhesive tape and then were made electrically conductive by coating, in a vacuum, with a thin layer of gold. Images of the microsphere surfaces were taken at an excitation voltage of 10 kV.

**Differential Scanning Calorimetry** The \( T_g \) of self-healing ONO-1301-loaded PLGA microspheres, with and without hydrophobic plasticizers (DEP or TBAC), was measured in a differential scanning calorimeter (SHIMADZU DSC-60, Kyoto, Japan). PLGA microsphere samples were sealed in aluminum hermetic pans and thermograms were determined by first cooling the sample to \(-40^\circ\text{C}\), then heating to \(80^\circ\text{C}\) at a rate of \(10^\circ\text{C}/\text{min}\) under a purged nitrogen atmosphere. The start point of the second run was used for \( T_g \) calculation.

**Determination of Specific Surface Areas of Self-healing ONO-1301-Loaded PLGA Microspheres** The specific surface areas of the self-healing ONO-1301-loaded PLGA microspheres were determined by the gas (Kr) adsorption method (3Flex, Micromeritics, Norcross, U.S.A.). Specific surface area was calculated with the Brunauer–Emmet–Teller (BET) equation\(^{11–13} \) from Kr adsorption at \(-196^\circ\text{C}\).

**Statistical Analysis** The results are expressed as mean\(\pm\)standard deviation (S.D.) \((n=3–4)\) with error bars. The data was evaluated for statistical significance using the Bonferroni test for differences between the groups. The statistical significance for the first-order rate constant was evaluated using Tukey’s test, while the overall significance was determined in repeated measures of one-way ANOVA. A \( p \)-value of \(<0.05 \) was considered to be statistically significant.

**Results**

**Optimization of Preparative Conditions for ONO-1301-Loaded PLGA Microspheres**

**Effect of Varying Temperature and Solvent Evaporation Time**

The effect of varying the temperature (25–40°C) and time (6–24 h) for evaporation of solvent \((\text{CH}_3\text{Cl}_2:\text{methanol}, 5:1)\) was examined in order to determine the optimum conditions for self-healing of ONO-1301-loaded PLGA microspheres.

Firstly we determined the optimal temperature for stirring the emulsion during the evaporation of solvent. As shown in Table 1 (formulations 1–4), the temperatures used for evaporation of solvent varied between 25 and 40°C. The time for evaporation of solvent was fixed at 6 h. ONO-1301-loaded PLGA microspheres obtained under these different temperatures, showed an average diameter ranging from 26.3 to 34.8 \(\mu\text{m} \). Actual loading was over 13.6% under all conditions, with drug loading efficiency over 81.9%.

In the *in vitro* release test, the initial burst release effect decreased as the temperature of the preparative process increased from 25 to 40°C as shown in Fig. 1 (formulations 1–4 in Table 1). The glass transition temperature of PLGA 7505 is 40.1°C (Table 2). The initial burst release of self-healing ONO-1301-loaded PLGA microspheres evaporated at 40°C was the lowest of the four batches. The surface morphology of ONO-1301-loaded PLGA microspheres prepared by evaporation at 25, 30, 35 and 40°C, was also smoothest at the highest temperature, 40°C, as shown in Fig. 2.

Secondly, we examined the effect of varying the time allowed for dispersion of the suspension (evaporation time) during the o/w emulsion/solvent evaporation method on initial burst release (in 24 h). Stirring (evaporation) times tested were 6, 12, 24 and 48 h, and ONO-1301-loaded PLGA microsphere prepared at 40°C (formulations 9, 10, 11 and 12 in Table 1, respectively) were compared with those prepared at 25°C (formulations 5, 6, 7 and 8 in Table 1, respectively). The results are shown in Fig. 3.

The initial burst release of ONO-1301 from PLGA microspheres prepared with self-healing at 40°C for 6, 12, 24 or 48 h was significantly suppressed compared with those prepared at 25°C. ONO-1301-loaded PLGA microspheres obtained after different evaporation times showed average diameters ranging from 28.4 to 33.8 \(\mu\text{m} \). Actual loading was over 11.8% and drug loading efficiency was over 71.1%, as shown in Table 1. The smooth surface morphology of ONO-1301-loaded PLGA microspheres prepared by self-healing (i.e., at 40°C) such as shown in Fig. 2(D) (formulation 4 in Table 1) was confirmed.*

**Table 2.** The \( T_g \) of ONO-1301-Loaded PLGA Microsphere Prepared with or without Various Concentrations of DEP or TBAC, Evaluated by Differential Scanning Calorimetry

| Plasticizer (%) | \( T_g \) (°C) |
|-----------------|--------------|
| DEP 0.0 (No. 13) | 40.10±0.10   |
| 0.1 (No. 14)    | 39.28±0.24** |
| 0.5 (No. 15)    | 37.35±0.25** |
| 1.0 (No. 16)    | 36.80±0.17** |
| 5.0             | 36.18±0.28** |
| 10.0            | 34.54±0.28** |
| TBAC 0.0 (No. 13)| 40.10±0.10   |
| 0.1 (No. 17)    | 38.05±0.11** |
| 0.5 (No. 18)    | 37.11±0.06** |
| 1.0 (No. 19)    | 36.04±0.90** |
| 5.0             | 32.35±0.08** |
| 10.0            | 18.52±1.21** |

Data are given as mean\(\pm\)S.D. \((n=3)\). **\( p<0.01 \) versus ONO-1301-loaded PLGA microspheres without hydrophobic plasticizers (formulation 13 in Table 1) (Bonferroni test/ANOVA).
Effect of Adding Hydrophobic Plasticizers to ONO-1301-Loaded PLGA Microspheres

Previous articles have described some plasticizers as useful in restricting burst release whereas others enhance burst release. On the basis of the available literature, we selected DEP and TBAC as hydrophobic plasticizers and prepared ONO-1301-loaded PLGA microspheres using different concentrations of these plasticizers in the oleogeneous phase. Six formulations (formulations 13–19 in Table 1) were prepared.

The initial burst release of ONO-1301 from PLGA microspheres prepared in the presence of hydrophobic plasticizer (DEP or TBAC) were significantly suppressed compared with ONO-1301-loaded microspheres without plasticizers, in a dose-dependent manner as shown in Fig. 4. ONO-1301-loaded PLGA microsphere with added DEP also showed a smoother surface morphology, as observed in SEM images (shown in Fig. 5). The surface morphology of ONO-1301-loaded PLGA microspheres prepared in the presence of TBAC was also improved compared with microspheres prepared in the absence of plasticizer (SEM images not shown).

The \( T_g \) of ONO-1301-loaded PLGA microspheres with added hydrophobic plasticizers at different concentrations are shown in Table 2. The addition of hydrophobic plasticizers significantly decreased the \( T_g \) of the microspheres in a dose-dependent manner.

Combined Effect of Temperature and the Addition of Hydrophobic Plasticizers on Initial Burst Release of ONO-1301-Loaded PLGA Microspheres

The combined effect of the temperature required for self-healing and the addition of plasticizer was examined in four formulations of ONO-1301-loaded PLGA microspheres (formulations 22–25 in Table 1). The initial burst release rate of ONO-1301 from PLGA microspheres prepared in the presence of 1.0% hydrophobic plasticizer (DEP or TBAC), in which the \( T_g \) of the polymer was 36°C, was the lowest of the four formulations, as shown in Fig. 6 (A or B, respectively). This suggests that the presence of a plasticizer not only reduces the temperature required to achieve self-healing but also reduces the initial burst release rate. Thus, a synergistic effect was demonstrated between the

Fig. 2. SEM Image of Self-healing ONO-1301-Loaded PLGA Microspheres Prepared by Evaporation at 25°C (A), 30°C (B), 35°C (C) and 40°C (D)

A, B, C and D correspond to formulations 1–4 listed in Table 1, respectively.

Fig. 3. In Vitro Drug-Release Profile of ONO-1301-Loaded PLGA Microspheres Prepared under Self-healing or Non-self-healing Conditions Using Different Evaporation Times

Figures 3 (A)–(D) show the results for formulations (as listed in Table 1) prepared with evaporation times of 6h (Nos. 5 & 9), 12h (Nos. 6 & 10), 24h (Nos. 7 & 11) and 48h (Nos. 8 & 12), respectively. Closed symbols (●) and open symbols (○) represent PLGA microspheres prepared at 40°C (self-healing conditions) or 25°C (non-self-healing conditions), respectively. All values are the mean±S.D. *p<0.05, **p<0.01 versus ONO-1301-loaded PLGA microspheres prepared without self-healing for each respective evaporation time (Bonferroni test/ANOVA).
addition of a plasticizer and the heating at \( T_g \) of the polymer for PLGA self-healing.

The surface areas of the four types of microsphere (formulations 20–25 in Table 1) are given in Table 3. The differences in surface morphology seen in SEM images of formulations 22–25 were not as large as the differences between ONO-1301-loaded PLGA microspheres prepared in the presence of plasticizers (DEP or TBAC), with and without PLGA healing (data not shown). This suggests that self-healing has a greater effect on the surface morphology of the microspheres than the differences in presence of plasticizers.

**Kinetic Study of Drug Release from ONO-1301-Loaded PLGA Microspheres**

The drug-release profiles of ONO-1301-loaded PLGA microspheres, with and without hydrophobic plasticizers or self-healing (formulations 20–25 from Table 1), were fitted to first-order release kinetics. The calculated first-order rate constants of formulations 20–25 were 0.069±0.002 \( (r=0.835\pm0.013) \), 0.060±0.002 \( (r=0.889\pm0.010) \), 0.041±0.003 \( (r=0.941\pm0.010) \), 0.036±0.001 \( (r=0.955\pm0.005) \), 0.049±0.002 \( (r=0.953\pm0.004) \) and 0.034±0.001 \( (r=0.940\pm0.008) \), respectively.

The first-order rate constants of self-healing microspheres were significantly lower than those of non-self-healing microspheres (formulation 20 versus 21, 22 versus 23 and 24 versus 25; \( p<0.01 \), \( p=0.07 \) and \( p<0.01 \), respectively). The first-order rate constant of self-healing PLGA microspheres prepared with hydrophobic plasticizer was significantly decreased compared with microspheres without plasticizer (formulation 20 versus 22–25; all \( p<0.01 \)). These results suggest synergistic effects of plasticizer and PLGA self-healing on the suppression of initial burst release.

**Discussion**

**Optimization of Preparative Conditions for ONO-1301 Loaded PLGA Microspheres**

The glass transition temperature of PLGA 7505 is 40.1°C and self-healing encapsulation of the microspheres was accomplished efficiently when the emulsion was kept at 40°C for at least 6 h stirring time. On SEM examination, as shown in Fig. 2, we confirmed that the number of pores on the surface of ONO-1301-loaded microspheres decreased as the preparative temperature increased. Similarly, the surface area of the prepared microspheres decreased as the preparative temperature increased, as shown in Table 2 (formulations 20 & 21 in Table 1).

The self-healing effect observed in the microspheres prepared in the present study was consistent with that described in previous reports, i.e., as a unique characteristic of polymers in which damaged structures are healed at the glass transition temperature of the polymer.\(^{14,15}\) The rough surface of ONO-1301-loaded PLGA microspheres prepared by evaporation at 25°C was improved by self-healing and evaporation of solvent at or around 40°C, i.e., near the \( T_g \) of PLGA.

Six hours seemed to be an adequate length of time for evaporation of solvent and to accomplish complete PLGA healing, as shown in Fig. 3. In a study by Wang et al.,\(^{16}\) it was reported that the pores on the microsphere surface disappeared when microspheres were incubated under self-healing condi-
composite, the decrease the initial burst release. In a TiO2–polystyrene nanospheres in the preparation of PLGA microspheres, and thereby plasticizers were shown to affect the stability of water–oil interfaces in the preparation of ONO-1301-loaded PLGA microspheres, not only the decrease of drug-release rate. In a report by Kim et al., hydrophobic plasticizers decreases the T_g caused by polymer. Thus, the addition of hydrophobic plasticizer seems to have two advantages, lowering the T_g of PLGA and stabilizing the water–oil interface.

In the present study, we also confirmed that the addition of ONO-1301 itself did not affect the T_g of ONO-1301-loaded PLGA microspheres containing various concentrations of plasticizer (data not shown).

Finally, the initial burst release of ONO-1301-loaded PLGA microspheres with added 1.0% plasticizers prepared with PLGA self-healing was determined and found to be significantly suppressed, as shown in Fig. 6. As shown in Table 3, the surface area of microspheres prepared with self-healing was less than that of microspheres prepared without self-healing (formulations 20 versus 21, 22 versus 23 and 24 versus 25, as listed in Table 1). There was not such a high correlation between the surface area of microspheres prepared under self-healing conditions with (formulations 23 and 25) and without (formulation 21) plasticizers and the extent of the corresponding initial burst release. It may be that some additional mechanism is also involved in restricting the initial burst release, not only the decrease of T_g caused by self-healing and the stabilization of the oil–water interface by plasticizers.

Regarding the mechanism of the initial burst release, there may be two different routes for drug-release: diffusion from the polymer matrix and diffusion through pores. The self-healing process seems to limit drug diffusion due to effects on both of these routes. Plasticizers may also affect both routes, as they are not only involved in the oleogeneous phase but also decrease the T_g of PLGA.

Table 3. The Specific Surface Areas of ONO-1301-Loaded PLGA Microspheres with and without Hydrophobic Plasticizers Were Calculated Using the BET Equation

| Microsphere formulation No. | Surface area (m^2/g) |
|-----------------------------|---------------------|
| 20                          | 2.04                |
| 21                          | 1.47                |
| 22                          | 0.56                |
| 23                          | 0.48                |
| 24                          | 1.69                |
| 25                          | 1.62                |

The specific surface areas are given as the mean of two observations.

Fig. 6. The Synergistic Effect of Self-healing Conditions (40 or 36°C) and Addition of Hydrophobic Plasticizers Dimethylphthalate (DEP: Fig. 6A) and Tributyl OAcetylcitrate (TBAC: Fig. 6B) on in Vitro Release Profiles of Drug from ONO-1301-Loaded PLGA Microspheres (A) and (B) show ONO-1301-loaded PLGA microspheres prepared in the absence of plasticizer without PLGA self-healing (○) or with PLGA self-healing (▲), and in the presence of 1.0% DEP (TBAC) without PLGA self-healing (△) or with PLGA self-healing (●), corresponding to formulations 20, 21, 22 (24) and 23 (25) in Table 1, respectively. All values are mean±S.D. (n=3). **p<0.01 versus ONO-1301-loaded PLGA microspheres without PLGA self-healing (formulation 20 in Table 1). *p<0.01 versus ONO-1301-loaded PLGA microspheres prepared in the presence of 1.0% plasticizer (DEP/TBAC) without PLGA self-healing (formulations 22–24 in Table 1, respectively) (Bonferroni test/ANOVA).
Formulation No. 1, 5, 13, 20 were same formulations but different serial number prepared at another day as shown in Table 1. There were some differences in the release rate at 0.5, 1, 6, 24 h between formulation No 1/No. 5 group and formulation No. 13/No.20. We considered that the differences between two groups was due to the particle size as shown in Table 1. The average of particle size were as follows, No. 1, 5, 13 and 20 were 28.2±2.5, 33.8±1.6, 23.4±0.1 and 22.3±0.2 µm, respectively. We expect that difference of particle size between No. 1/No. 5 group and No. 13/No. 20 was mainly caused by difference on experimental condition such as environment temperature and/or humidity during preparative procedure.

Conclusion

The initial burst release of self-healing ONO-1301-loaded PLGA microspheres prepared in the presence of 1.0% plasticizers with a 6-h evaporation of solvent at or around the $T_g$ of PLGA, was significantly suppressed compared with microspheres prepared without plasticizers or self-healing. We suggest that this suppression is caused by an optimization of the microspheres due primarily to a reduction in the number of pore and voids. This finding may be of particular relevance in the development of formulations in which the acute effects of pharmaceutical active ingredients must be limited.

Conflict of Interest The authors declare no conflict of interest.

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