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Novel non-trimethoxylphenyl piperlongumine derivatives selectively kill cancer cells

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ABSTRACT

Piperlongumine (**PL**) is a natural alkaloid with broad biological activities. Twelve analogues have been designed and synthesized with non-substituted benzyl rings or heterocycles in this work. Most of the compounds showed better anticancer activities than the parent **PL** without apparent toxicity in normal cells. Elevation of cellular ROS levels was one of the main anticancer mechanisms of these compounds. Cell apoptosis and cell cycle arrest for the best compound **ZM90** were evaluated and similar mechanism of action with **PL** was demonstrated. The SAR was also characterized, providing worthy directions for further optimization of **PL** compounds.

Piperlongumine (**PL**, Piplartine, 5,6-dihydro-1-[(2*E*)-1 -oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1*H*)-pyridinone, Scheme 1) is an active alkaloid isolated from the root of plant species *Piper longum* L.[1] The therapeutic potentials include anti-cancer,[2–4] anti-diabetes,[5] antiplatelet aggregation,[6] anti-inflammation,[7,8] anti-fungal activity[9,10] and neurodegenerative diseases.[11] In 2011, Schreiber group reported the **PL** selectively killed cancer cells probably due to elevation of ROS level.[3] However, the exact protein target (s) has not well characterized. Further structure-activity relationship (SAR) study showed the **PL** compounds had two key pharmacophores: C2-C3 and C7-C8 double bonds (Scheme 1, highlighted in red) where contain two classical Michael acceptors.[12] Comparing these two Michael acceptors, the C2-C3 double bond is more reactive with cysteines of the target proteins. Removal of C7-C8 double bond led to a decrease in cytotoxicity without decreasing the ROS level. In 2014, our group further extended the SARs by introducing halogen or morpholine substituents at C2 and alkyl substituents at C7 position.[13] The 2-halogenated piperlongumines (e.g. **ZM30**, Scheme 1) showed potent *in vitro* and *in vivo* anticancer activities due to its higher reactivity of C2-C3 Michael acceptor. The 2-morpholine substituted ones (IC_{50} > 100 μM) were deactivated probably due to steric hindrance. Compound **ZM30** showed better activity than **PL** against A549 cells. However, the selectivity for the cancer cells (cancer cells, A549, IC_{50} = 19.83 μM; normal cells, MRC-5, IC_{50} = 14.30 μM) was poor.

Based on above information and SAR study, the importance of the trimethoxylphenyl group has not been evaluated, which appears in lots of natural anticancer agents, such as colchicine and combretastatin A4. Herein, non-substituted benzyl rings and heterocycles were designed to replace the trimethoxylphenyl group (Scheme 1A). We also proposed to replace the C7-C8 double bond by the bioisosteric groups, such as cyclopropyl group (Scheme 1B) and triple bond (Scheme 1C). Removal of the C7-C8 olefin (Scheme 1D) is another aspect for evaluation.

Twelve analogues (Scheme 2) were obtained to evaluate the *in vitro* cytotoxicity against six cancer cell lines (human lung carcinoma A549, human colorectal carcinoma HCT116, human breast carcinoma MDA-MB-231, human Hepatic adenocarcinoma SK-Hep-1, human osteosarcoma U2OS and Saos-2) and a normal cell line (human lung fibroblast cell lines WI38) by MTT assay. **PL** was used as a reference compound. The IC_{50} values for each compound are summarized in Table 1.

Overall, most of the novel **PL** analogues showed better cytotoxicity than the parent compound **PL**, except **ZM83**, **ZM85** and **ZM89**.

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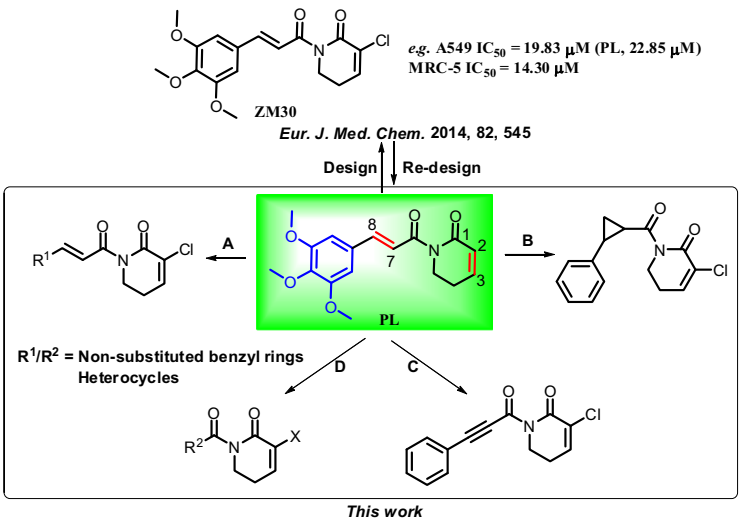
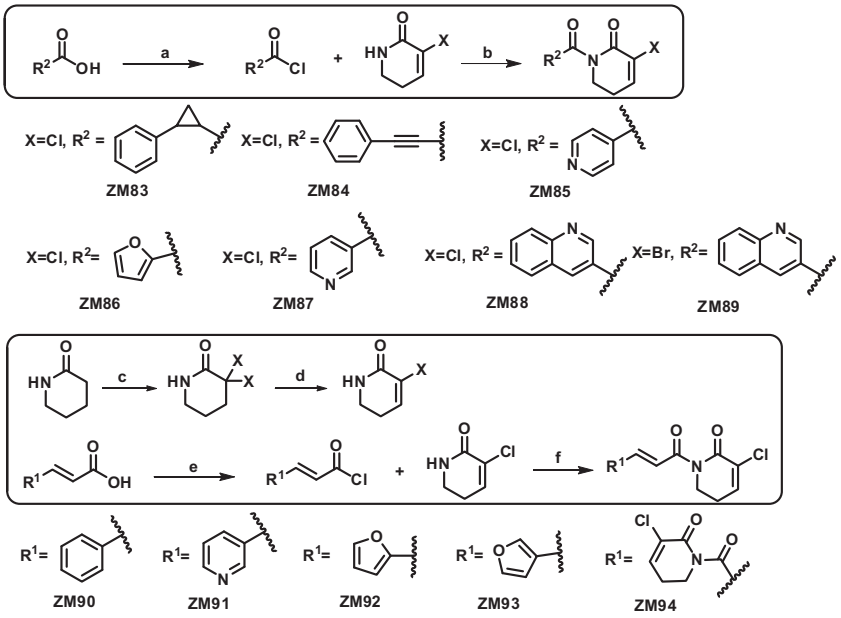


Scheme 1. The design strategy of non-trimethoxylphenyl piperlongumine derivatives.



Scheme 2. Conditions and reagents: (a) anhydrous DCM, oxalyl chloride, one drop of DMF, 3 h; (b) anhydrous THF, TEA, room temperature, overnight; (c) For chlorination: PCl_5, $CHCl_3$; room temperature, crude product for next step; For bromination, PCl_5, DCM, 0 °C, then ZnI, Br_2, room temperature; (d) Li_2CO_3, LiCl, anhydrous DMF, 130 °C, 7 h; (e) anhydrous DCM, oxalyl chloride, one drop of DMF, 3 h; (f) anhydrous THF, TEA, room temperature, overnight.

These compounds were sensitive to human osteosarcoma cells with the IC_{50} values in nanomolar or low micromolar range. To our delight, all the compounds show no apparent cytotoxicity against normal cells, recovering the selectivity of **PL**. The SAR study demonstrated the following results: (1) The trimethoxyl group was removed from **ZM30** to obtain **ZM90**. This compound showed excellent cytotoxicity against all the six cell lines, especially the SK-Hep-1 (IC_{50} = 710 nM) and Saos-2 (IC_{50} = 11 nM), with only 84.6 μM of the normal WI38 cells. This result indicated that the trimethoxyl group is not a pharmacophore for the **PL** compounds. (2) **ZM84** with triple bond showed excellent cytotoxicity against five cell lines (IC_{50} = 420–1730 nM) except A549. (3) The heterocycle-substituted **PL** compounds (**ZM91**-**94**) exhibited good activities. **ZM91** with 3-pyridinyl group was potent against the six cell lines (IC_{50} = 530–2970 nM). The activities of **ZM92** with 2-furyl, **ZM93** with 3-furyl and **ZM94** with double 2-halo-lactams were comparable with that of **PL** towards A549 cells. For the other five cell lines, they showed 2–9-fold better activities than **PL**. (4) Removal of the C7-C8 olefin to directly connect the chlorides to the lactam was unfavorable to the cytotoxicity. Compared with **ZM91** and **ZM87**, the C7-C8 olefin was removed, leading to 2–5-fold decrease in the cytotoxicity. Compounds **ZM85** and **ZM87**–**89** showed moderate cytotoxicity.

To summarize the SAR, C7-C8 olefin is of great importance to the cytotoxicity. The alkynyl was compatible to replace the double bond but not for the cyclopropyl group. The enhancement of the molecular flexibility or disruption of the conjugated system of α, β-unsaturated system were proposed to be the main reasons. Moreover, the trimethoxyl group is not a critical pharmacophore to the **PL** analogues.

It is reported that the antitumor mechanism of **PL** is to increase the level of ROS and apoptotic cell death.[3] We further evaluated the cellular ROS levels of the most potent compounds (**ZM84**, **ZM90**, and **ZM91**) using two sensitive cancer cell lines (HCT-116 and

Table 1
In vitro anticancer activity of the derivatives.

Compounds	IC_{50}[a] (μM)						
	A549	HCT-116	MDA-MB-231	SK-Hep-1	U2OS	Saos-2	WI38 (Normal cells)
PL	6.84	7.34	10.6	13.3	9.49	7.31	>100
ZM83	85.1	64.5	65.1	72.9	68.3	23.5	>100
ZM84	7.93	0.92	1.73	1.19	1.12	0.42	>100
ZM85	>100	>100	>100	>100	>100	89.9	>100
ZM86	18.6	5.89	19.2	13.6	8.60	3.86	>100
ZM87	8.64	3.84	4.83	4.83	5.67	2.55	31.0
ZM88	10.6	10.2	8.68	3.52	6.63	2.96	>100
ZM89	>100	>100	>100	75.2	97.5	26.7	>100
ZM90	4.12	1.67	1.67	0.71	1.17	0.011	84.6
ZM91	2.97	2.36	2.97	1.90	1.67	0.53	47.0
ZM92	8.30	4.49	5.52	1.27	1.47	0.28	>100
ZM93	11.0	4.37	4.97	2.78	2.26	1.15	>100
ZM94	8.60	4.20	5.04	3.96	7.29	0.82	89.5

[a] Values were measured with MTT method.

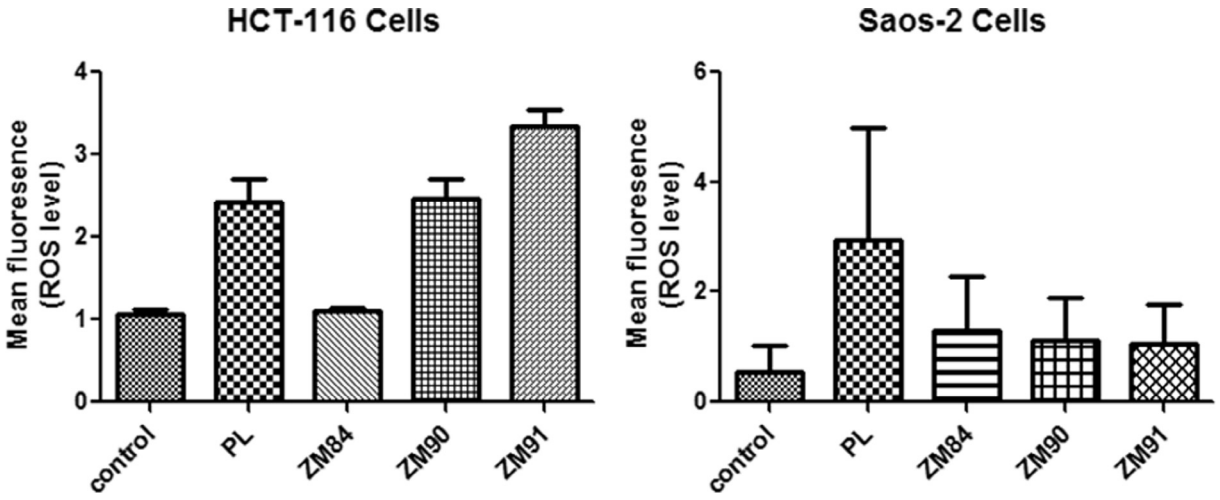


Fig. 1. Piperlongumine derivatives induced ROS elevation. HCT-116 and Saos-2 cells were treated with compounds (10 μM) or DMSO (control) for 1 h.

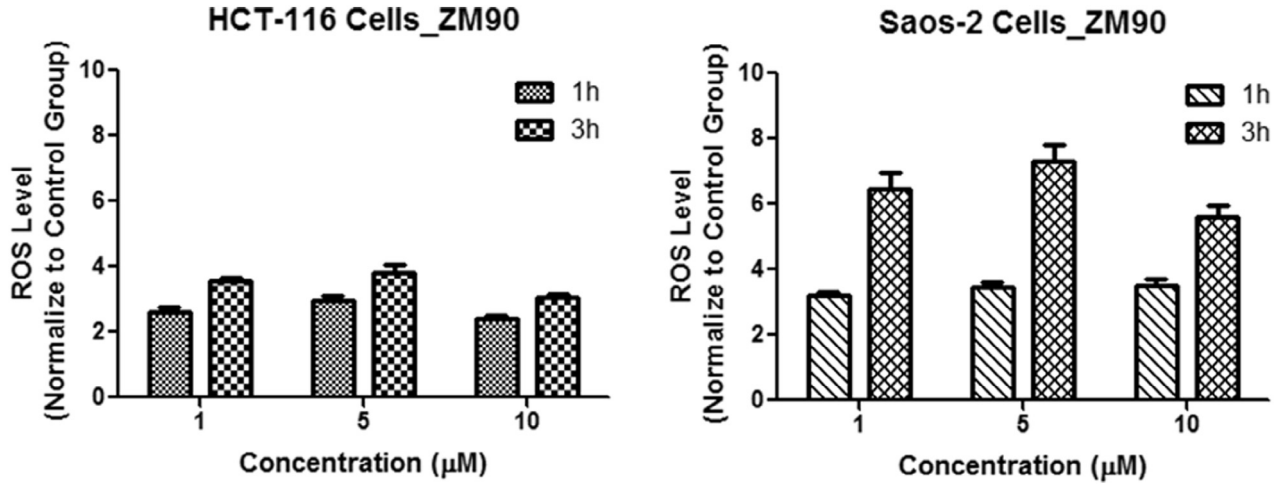


Fig. 2. Piperlongumine derivatives induced ROS elevation. HCT-116 and Saos-2 cells were treated with compound **ZM90** in dose (1, 5, 10 μM)- and time (1 h and 3 h)-response manner.

Saos-2) by fluorescence microscopy (Fig. 1). The HCT-116 cellular ROS levels were obviously increased after 1 h treatment with 10 μM of **ZM90** or **ZM91**, which was better than the **PL**. However, the **ZM84** without C7-C8 double bond showed no apparent change. In Saos-2 cells, the compounds exhibited twofold change in ROS level, but less than that of **PL**.

Further dose- and time-response manners of the active compound **ZM90** were then evaluated. The compound showed a signif-

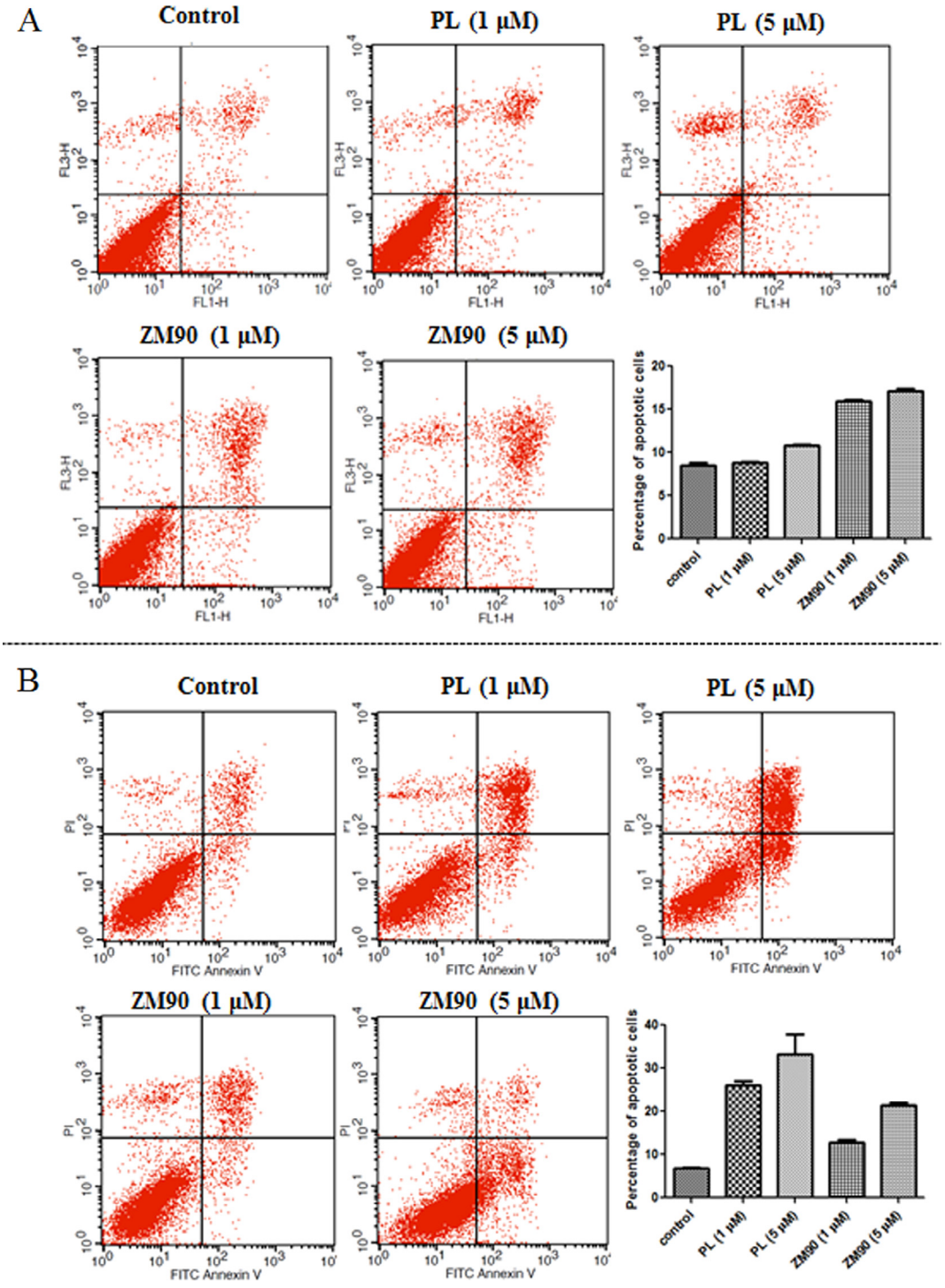


Fig. 3. Cell apoptosis induced by **ZM90** after 24 h treatment on HCT-116 (A) and Saos-2 (B) cell lines.

icant time-dependent manner in both cell lines. For the dose, the compound increased the cellular ROS levels from 1 to 5 μM. And at 10 μM, the ROS levels in two cell lines were slightly decreased (Fig. 2).

Cell morphology and fluorescence-activated cell sorting was used to evaluate the effect of compound **ZM90** on induction of apoptosis (Fig. 3). Compared with the DMSO control group, compound **PL** and **ZM90** can induce the apoptosis of both cell lines in a dose-response manner. As shown in Fig. 3A, the percentage of HCT116 apoptotic cells after **ZM90** treatment for 24 h was 16% and 17% at 1 and 5 μM, which was higher than that of **PL**. For Saos-2 cells, the percentage of apoptotic cells after **PL** treatment was 26% and 33%, where **ZM90** has 12.7% and 21.5% induction (Fig. 3B). The result suggested that the piperlongunime derivatives showed remarkable apoptotic effect in cancer cells.

In addition, flow cytometric analysis was also performed to determine the cell cycle arrest of **ZM90** (Figs. 4 and S1). After 24 h treatment of compounds in HCT-116 cells, it was obvious that **PL** resulted in an increase in the percentage of cells blocked in the S and G0/G1 phase. Similar effect was observed for **ZM90** and more cell cycle populations in G0/G1 phase were arrested in a dose-response manner (Fig. 4A). In Saos-2 cells, no significant change

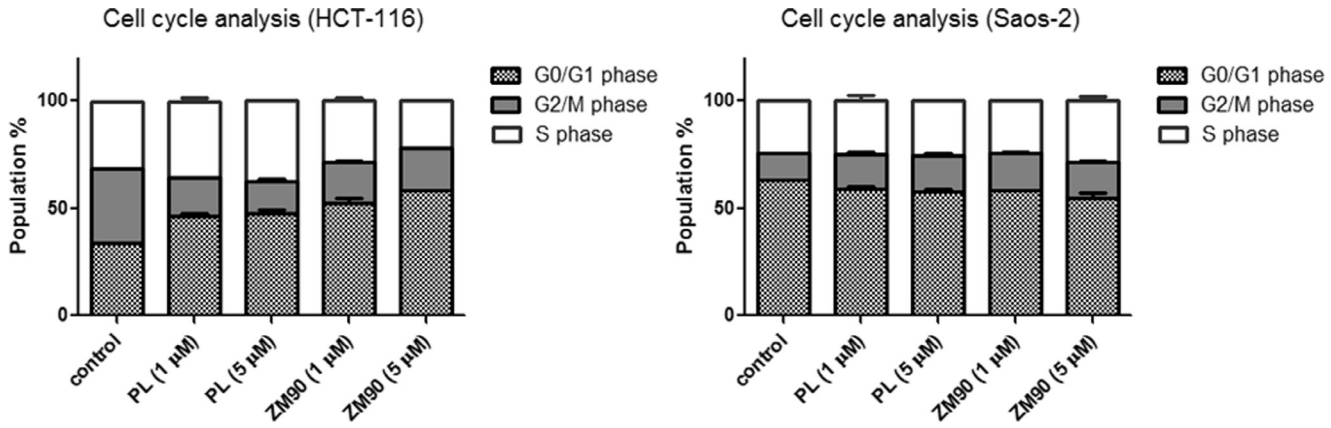


Fig. 4. The effect of **PL** or **ZM90** treatment on HCT-116 and Saos-2 cell cycle distributions for 24 h.

was demonstrated in G2/M and S phases. Slight decrease in G0/G1 phase was shown (Fig. 4B). The above results indicated **ZM90** and **PL** had a similar mechanism of action for the cancer treatment.

In conclusion, twelve **PL** analogues were designed and synthesized with non-substituted benzyl rings or heterocycles. Most of the compounds exhibited better anticancer activities than the parent **PL**. Different from the initial halogenated **PL**, these compounds had a higher selectivity towards cancer cells and normal cells. ROS elevation was one of the main anticancer mechanisms of these compounds. Cell apoptosis and cell cycle arrest for compound **ZM90** were evaluated and similar mechanism of action with **PL** was demonstrated. The SAR has been also characterized, providing worthy directions for further optimization of **PL** compounds.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.04.035.

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