Макрогетероциклы
http://macroheterocycles.isuct.ru

DOI: 10.6060/mhc181115w

Evaluation for *in vitro* Photodynamic Activity of Chlorin e_6–Artesunate Conjugates by Irradiation with Different Wavelengths of Light Source

Xiuhan Guo,[a,b] Yueqing Li,[a] Shisheng Wang,[a,b@] Liu Wang,[a] Rui Cai,[c] Guangzhe Li,[a] and Weijie Zhao[a,b]

[a]*School of Pharmaceutical Science and Technology, Dalian University of Technology, 116023 Dalian, P. R. China*
[b]*State Key Laboratory of Fine Chemicals, Dalian University of Technology, 116023 Dalian, P. R. China*
[c]*School of Chemical Engineering, Dalian University of Technology, 116023 Dalian, P. R. China*
[@]*Corresponding author E-mail: wangss@dlut.edu.cn*

In the development of new photosensitizers for cancer therapy, increasing the capacity of intracellular reactive oxygen species (ROS) production is an important strategy. In this paper, artesunate as a ROS generation group was regioselectively introduced to the chlorin e_6 scaffold to obtain four conjugates of chlorin e_6 and artesunate. By irradiation with 440 nm, 630 nm and 660 nm of light source the four conjugates exhibited significantly improved phototoxicity against HepG2 cells compared with chlorin e_6 and artesunate. Irradiation with 440 nm or 660 nm light source gave higher phototoxicity as well as intracellular ROS level.

Keywords: Photodynamic therapy, chlorin e_6, artesunate, reactive oxygen species, photoexcitation.

Оценка фотодинамической активности *in vitro* конъюгатов хлорина e_6 с артесунатом при облучении источниками света различной длины волны

Х. Гуо,[a,b] Ю. Ли,[a] Ш. Ванг,[a,b@] Л. Ванг,[a] Р. Каи,[c] Г. Ли,[a] В. Жао[a,b]

[a]*Школа фармацевтической науки и технологии, Даляньский технологический университет, 116023 Далянь, КНР*
[b]*Государственная ключевая лаборатория тонких химических соединений, Даляньский технологический университет, 116023 Далянь, КНР*
[c]*Школа химической инженерии, Даляньский технологический университет, 116023 Далянь, КНР*
[@]*E-mail: wangss@dlut.edu.cn*

Важным фактором эффективности фотосенсибилизаторов для ФДТ является их способность к генерации активных форм кислорода (АФК) в клетке. В данной работе осуществлён синтез конъюгатов хлорина e_6 с терпеноидом артесунатом, который может служить дополнительным источником АФК за счёт наличия в молекуле перекисного мостика. В опытах in vitro на клетках HepG2 фотовозбуждение проводилось при 440, 630 и 660 нм. Наилучшие результаты получены при освещении светом при 440 и 660 нм для тризамещённого конъюгата, а в случае монозамещённых производных для 13- и 17-изомеров.

Ключевые слова: Фотодинамическая терапия, хлорин e_6, артесунат, активные формы кислорода, фотовозбуждение.

Introduction

Photodynamic therapy (PDT) is based on the excitation of a photosensitizer, administered systemically or topically, with light of a specific wavelength corresponding to the absorption peak of the photosensitizer.[1] This can generate reactive oxygen species (ROS), which readily reacted with electron-rich biomolecules in surrounding unsaturated lipids, amino acids and DNA, resulting in tumor cell death by necrotic and apoptotic mechanisms. Hence, the ROS producing capacity in the cells is one of the vital indexes for the evaluation of the photosensitizers.[2,3] Chlorophyll is a kind of important pigments which have the abilities to absorb optical energy and convert it to chemical energy during plant photosynthesis. In the process of photosynthesis, the light with different wavelengths plays different role on the plant growth regulations.[4-7] Similarly, treating photosensitizers with different wavelengths of light has great effects on their PDT in antibacterial and anti-tumor application.[8-13] As degradation products of Chlorophyll *a*, chlorins possess porphine macrocyclic scaffold and high photosensitive effect. Besides, chlorins exhibit selective uptake and efficient phototoxicity in tumor tissue. Chlorins, especially chlorin e_6, have been widely used as photosensitizing agents for treatment of cancer. For the purpose of improving properties and photodynamic activities of chlorins, many researchers have focused on their structure modification and obtained some new photosensitizers.[14-18] In the present study, artesunate (ART) was introduced into the carboxyl residue of chlorin e_6 as a efficient ROS donor based on the fact that artemisinin and its derivatives can generate ROS *via* the cleavage of endoperoxide bridge resulting in the toxicity against plasmodium and tumor cells.[19-23] Herein, we report the synthesis of chlorin e_6-artesunate conjugates (**3**, **6**, **9**, **11**) and their phototoxicity by irradiation with different wavelengths of light.

Experimental

Synthesis

Chlorin e_6 and Pheophytin, the starting materials for **3**, **6**, **9** and **11**, were prepared as described by Smith *et al*.[15] The experimental details and characteristics for all the synthesized target compounds **3**, **6**, **9** and **11** were performed as previously described in our work.[14,24]

Phototoxicity and dark toxicity studies

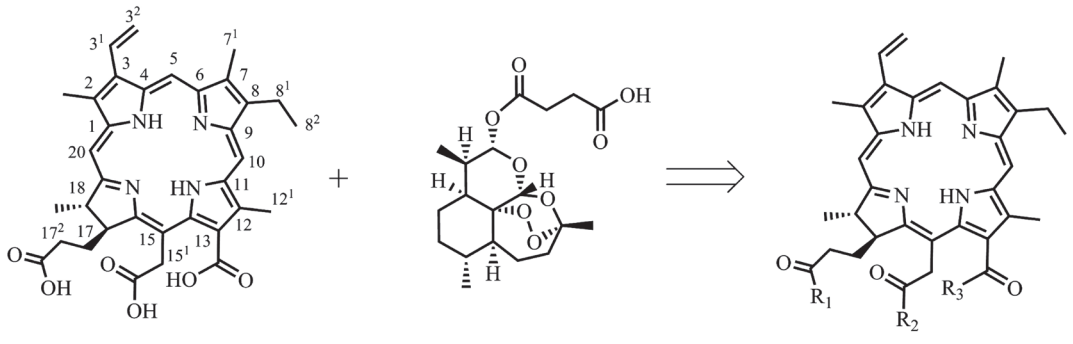
The HepG2 cells were plated at 5000 cells per well in a 96-well plate and allowed to grow for 24 h at 37 °C, 5 % CO_2, then the cells were incubated with photosensitizer for overnight at 37 ℃, 5 % CO_2. Cells were washed three times with PBS, then replaced with 100 μL of fresh culture medium. Then they were irradiated with LED light (18 W, 660 nm) for 10 min (1.7 J/cm^2 light dose). Then cells were incubated for overnight at 37 °C, 5 % CO_2. The cell viability were determinated by the MTT method (Synergy H1 Microplate Reader, Bio Tak). All tests were carried out in triplicate independent experiments. The dark toxicity assay keeps identical to the phototoxicity assay except for illumination.

Detection of intracellular reactive oxygen species (ROS)

HepG2 cells were seeded onto 24-well plates (5×104 cells/well) and cultured for 24 h at 37 °C in DMEM. Then the cells were incubated with photosensitizers (30 μM) for 10 h at 37 °C, 5 % CO_2. Culture media was gently removed, and the cells washed with PBS. Serum-free medium containing dichlorodihydrofluorescein diacetate (DCF-DA, Sigma) was added to the cells to give a final concentration of 20 μM for 20 min in the dark. Then, the cells were washed with PBS, and replaced with fresh cell culture media without phenol red. Then they were irradiated with LED light (18 W, 660 nm). Fluorescence at 530 nm in response to excitation at 495 nm was measured (Synergy H1 Microplate Reader, Bio Tak).

Results and Discussion

Three chlorin e_6 derivatives with a single ART side chain at 13^1,15^2 or 17^3 of porphine parent nucleus (**3**, **6**, **9**) and a chlorin e_6 derivatives with three ART side chains (**11**) were designed and synthesized (Figure 1). We fulfilled regioselective synthesis of chlorin e_6 derivatives **3**, **6** and **9** by utilizing different starting material and different reactivity of the carboxyl groups. The four conjugates of chlorin e_6 and ART were efficiently synthesized with chlorin e_6 or pheophytin *a* as a starting material, ethanediamine as



Chlorin e6 | Artesunate (ART) | Chlorin e6 -artesunate conjugates

3: R_1 = OH, R_2 = $NHCH_2CH_2NHART$, R_3 = OH
6: R_1 = $NHCH_2CH_2NHART$, R_2 = OH, R_3 = OH
9: R_1 = OH, R_2 = OH, R_3 = $NHCH_2CH_2NHART$
11: R_1 , R_2 , R_3 = $NHCH_2CH_2NHART$

Figure 1. Chemical structures of novel photosensitizers chlorin e_6-artesunate conjugates.

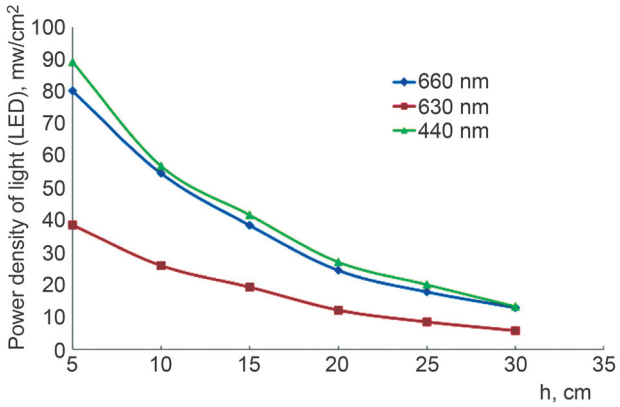


Figure 2. The relation between power density of light (LED) and irradiation distance.

a linking group, and *N*-hydroxysuccinimide ester of ART as an active ester. The detailed procedures were listed in supporting information.

The four chlorin e_6 derivatives were evaluated for their phototoxic effect against HepG2 cells under irradiation with different wavelength of light. The LED lamps for plant growing with different fixed wavelength were used as the light source of irradiation. Because the power density of light is related to wavelength and irradiation distance, we first examined the relationship between power density of light and irradiation distance at wavelength of 440, 630 and 660 nm, respectively (Figure 2). In order to ensure the equal power density of light at different wavelength, we set the irradiation condition on wavelength and distance as follows: 440 nm/22 cm, 630 nm/10 cm, 660 nm/20 cm.

As shown in Figure 3 and Table 1, chlorin e_6 derivatives **3**, **6**, **9** and **11** exhibited more phototoxicity against HepG2 cells than the parent compound chlorin e_6 under irradiation with different wavelength of light (The detailed experimental procedures were described in Supporting information). Among the four derivatives, **11** showed the most phototoxic effect at 440 nm (IC_{50} 0.4 μM), 630 nm (IC_{50} 4.0 μM) and 660 nm (IC_{50} 0.8 μM), which was about five to nine times more effective than chlorin e_6. Meanwhile, the dark cytotoxicity of derivatives **3**, **6**, **9** and **11** were also increased at different extent compared with chlorin e_6. ART showed almost the same level of dark cytotoxicity and photocytotoxicity, indicating that ART was a cytotoxic agent but not a photosensitizer. From this perspective, the significantly improved phototoxicity of **3**, **6**, **9** and **11** can be attributed to the synergic action of photodynamic effect from chlorin skeleton and cytotoxic effect from ART side chain under light irradiation conditions.

For the photodynamic effect evaluation, above mentioned three LED light sources were employed. The photosensitizers gave high photo toxicity against HepG2 cells under irradiation with 440 nm or 660 nm but showed much lower phototoxicity under 630 nm light irradiation. In order to verify whether the photodynamic effect at different wavelength is related to the intracellular ROS production, we examined the ROS level in HepG2 cells after treated with photosensitizers and irradiation at different wavelength using DCF-DA as a probe for ROS detection. As shown in Figure 4, all the photosensitizers but not ART produced much more ROS under irradiation with 440 or 660 nm light than 630 nm light, which was in accord with the photodynamic effect in HepG2 cells. The results confirmed that the photodynamic effects of photosensitizers in HepG2 cells at different wavelength were highly dependent on the intracellular ROS level. In the dark or under the same wavelength of light, compound **3** exhibited weaker dark toxicity or phototoxicity than compounds **6** and **9**, which was presumed to result from the different spatial conformation of macrocycle and side chains. According to Smith *et al*., different positions of substituents (at 13, 15 and 17) of chlorin e_6 can form different spatial conformation and subsequently influences the PDT effects. Seeing from the above results, the change of molecular conformation from substituent at position 15 in compound **3** probably decreased the dark toxicity and phototoxicity.[15]

Conclusions

We synthesized four conjugates of chlorin e_6 and artesunate (**3**, **6**, **9**, **11**) and evaluated their phototoxicity by irradiation with three different wavelength of light source. The results showed the introduction of ART side chain greatly increased the phototoxicity compared with the parent compound, which might be attributed to the cytotoxicity and ROS production capacity of ART. Furthermore, there existed much difference in phototoxicity against HepG2 cells with 440, 630 and 660 nm of light source, which was proved to be dependent on the different

Table 1. Phototoxicity and dark toxicity of chlorin e_6 derivatives against HepG2 cells.

Compounds	Dark IC_{50}/μM	Photo IC_{50}/μM		
		660 nm	630 nm	440 nm
13-ART (**9**)	36.6±2.5	1.8±0.1	4.6±0.2	1.2±0.1
15-ART (**3**)	57.0±2.9	2.4±0.2	10.4±0.4	1.4±0.1
17-ART (**6**)	29.1±1.4	1.5±0.1	3.0±0.1	1.3±0.1
13,15,17-Tri-ART (**11**)	12.9±0.6	0.8±0.1	4.0±0.2	0.4±0.1
Artesunate **(ART)**	6.5±0.6	7.7±0.1	14.0±0.6	9.9±0.4
Chlorin e_6	>100	5.3±0.2	19.0±0.8	3.5±0.1

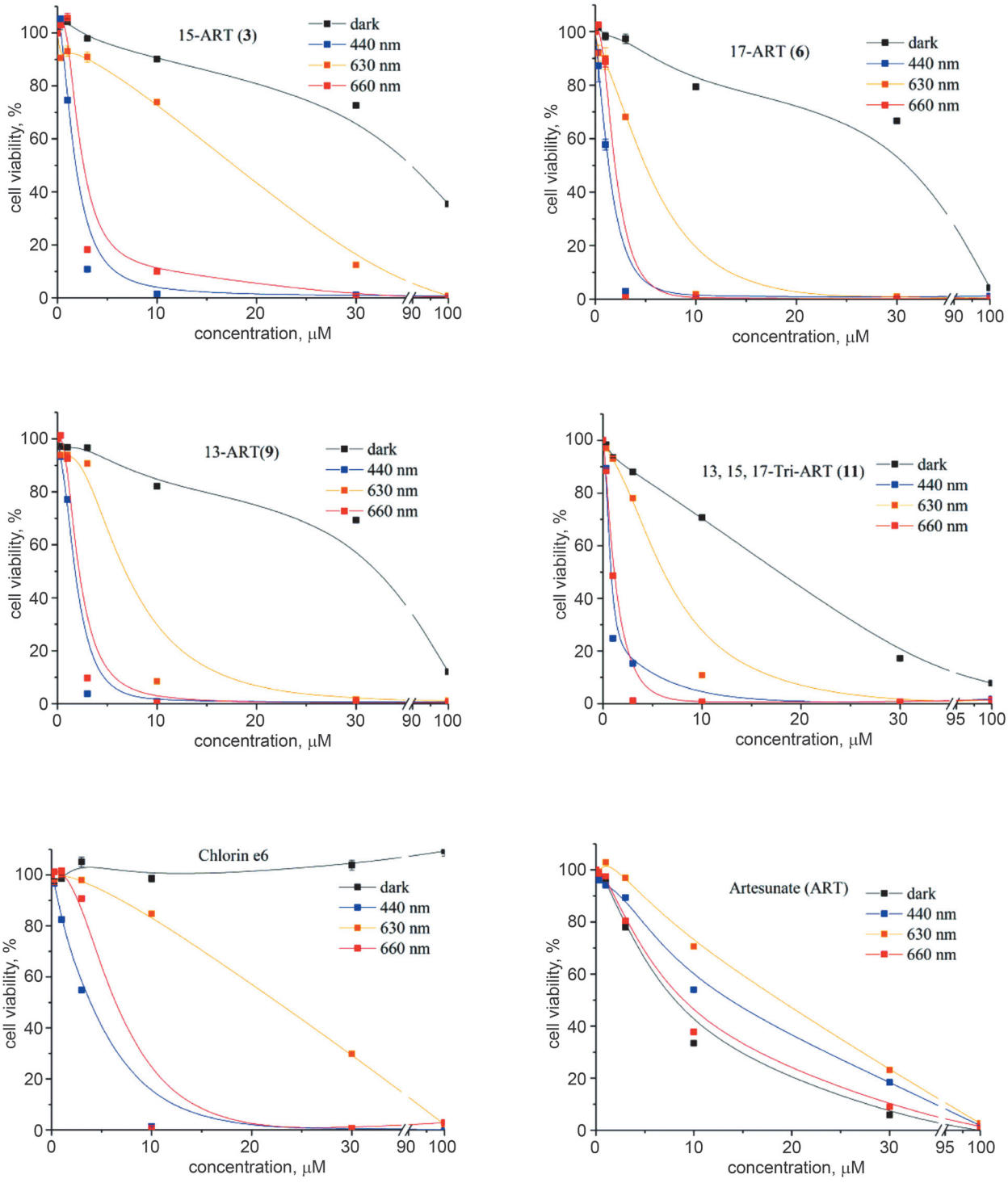


Figure 3. Cell viability results of phototoxicity and dark toxicity cells of chlorin photosensitizers in the light of different wavelengths.

intracellular ROS level at different wavelength. Although irradiation with 440 nm of light source produced the highest ROS level and the strongest phototoxicity in HepG2 cells, PDT with 440 nm of light was unpractical in clinical application because of the poor ability of short wavelength light to penetrate deep tissues. The four synthesized photosensitizers (**3**, **6**, **9**, **11**) possessed rather high phototoxicity with 660 nm of light, which locates in the long wavelength range and has the good penetration to deep tumor tissues. In conclusion, the conjugates of chlorin e_6-artesunate combined with irradiation at 660 nm exhibited significant photodynamic effect and will offer a promising approach in antitumor therapy.

Acknowledgements. This work was financially supported by the Natural Science Foundation of China (Grant No. 21506024), Doctoral Program Foundation of Liaoning Province (Grant No. 201501181), Scientific Research

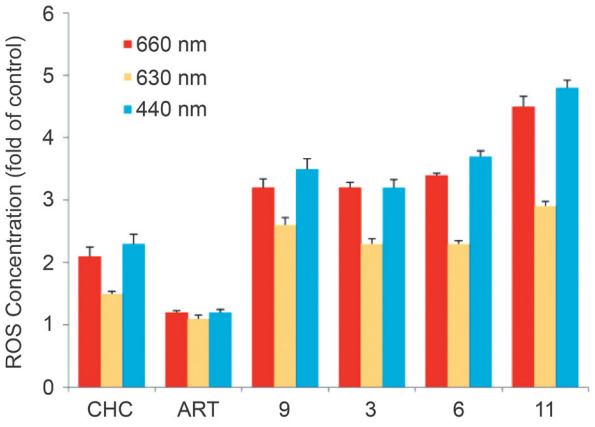


Figure 4. Concentration of chlorins photosensitizers-induced intracellular ROS in HepG2 cells after irradiation (using light of different wavelengths).

Foundation for the Returned Overseas Chinese Scholars of State Education Ministry of China (Grant No. 49), Fundamental Research Funds for the Central Universities (DUT13RC(3)45, DUT17LK10).

References

1. Phillips D. *Pure Appl. Chem*. **2011**, *83,* 733–748.
2. Cheng J., Li W., Tan G., Wang Z., Li S., Jin Y. *Biomedicine & Pharmacotherapy* **2017**, *87*, 263–273.
3. Zhou Z., Song J., Nie L., Chen X. *Chem. Soc. Rev.* **2016**, *45*, 6597–6626.
4. Avercheva O., Berkovich Y.A., Smolyanina S., Bassarskaya E., Pogosyan S., Ptushenko V., Erokhin A., Zhigalova T. *Adv. Space Res.* **2014**, *53*, 1574–1581.
5. Hogewoning S.W., Trouwborst G., Maljaars H., Poorter H., Van Ieperen W., Harbinson J. *J. Exp. Bot.* **2010**, *61*, 3107–3117.
6. Ma G., Zhang L., Setiawan C.K., Yamawaki K., Asai T., Nishikawa F., Maezawa S., Sato H., Kanemitsu N., Kato M. *Postharvest Biol. Tec.* **2014**, *94*, 97–103.
7. Ooi A., Wong A., Ng T.K., Marondedze C., Gehring C., Ooi B.S. *Sci. Rep.* **2016**, *6*, 33885.
8. Atif M., Fakhar-e-Alam M., Sabino L.G., Ikram M., De Araujo M.T., Kurachi C., Bagnato V.S., AlSalhi M.S. *Laser Phys. Lett.* **2011**, *8*, 386–392.
9. Chan Y., Lai C.H. *Lasers Med. Sci.* **2003**, *18*, 51–55.
10. He H., Ji S., He Y., Zhu A., Zou Y., Deng Y., Ke H., Yang H., Zhao Y., Guo Z., Chen H. *Adv. Mater.* **2017**, *29*, https://doi.org/10.1002/adma.201606690.
11. Hino H., Murayama Y., Nakanishi M., Inoue K., Nakajima M., Otsuji E. *J. Surg. Res.* **2013**, *185*, 119–126.
12. Menezes P.F.C., Bagnato V.S., Johnke R.M., Bonnerup C., Sibata C.H., Allison R.R., Perussi J.R. *Laser Phys. Lett*. **2007**, *4*, 546–551.
13. Merigo E., Conti S., Ciociola T., Fornaini C., Polonelli L., Lagori G., Manfredi M., Vescovi P. *Photodiagnosis Photodyn. Ther.* **2017**, *18*, 34–38.
14. Guo X., Wang L., Wang S., Li Y., Zhang F., Song B., Zhao W. *Bioorg. Med. Chem. Lett*. **2015**, *25*, 4078–4081.
15. Jinadasa R.G.W., Hu X.K., Vicente M.G.H., Smith K.M. *J. Med. Chem.* **2011**, *54*, 7464–7476.
16. Jinadasa R.G.W., Zhou Z.H., Vicente M.G.H., Smith K.M. *Org. Biomol. Chem.* **2016**, *14*, 1049–1064.
17. Nikolaeva I.A., Misharin A.Y., Ponomarev G.V., Timofeev V.P., Tkachev Y.V. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2872–2875.
18. Moon Y.-H., Kwon S.-M., Kim H.-J., Jung K.-Y., Park J.-H., Kim S.-A., Kim Y.-M., Ahn S.-G., Yoon J.-H. *Oncol. Rep.* **2009**, *22*, 1085–1091.
19. Efferth T. *Semin. Cancer Biol*. **2017**, *46*, 65–83.
20. Gaur R., Pathania A.S., Malik F.A., Bhakuni R.S., Verma R.K. *Eur. J. Med. Chem.* **2016**, *122*, 232–246.
21. Letis A.S., Seo E.J., Nikolaropoulos S.S., Efferth T., Giannis A., Fousteris M.A. *Bioorg. Med. Chem.* **2017**, *25*, 3357–3367.
22. Ooko E., Saeed M.E., Kadioglu O., Sarvi S., Colak M., Elmasaoudi K., Janah R., Greten H.J., Efferth T. *Phytomedicine* **2015**, *22*, 1045–1054.
23. Pang Y., Qin G., Wu L., Wang X., Chen T. *Exp. Cell Res.* **2016**, *347*, 251–260.
24. Guo X.H., Wang L., Wang S.S., Li Y.Q., Cao L., Cai R., Zhao W. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 4548–4551.

Received 29.11.2018
Accepted 05.12.2018