Design, synthesis and anti-cancer activity evaluation of podophyllotoxin-norcantharidin hybrid drugs

Hong-Wei Han, Han-Yue Qiu, Cui Hu, Wen-Xue Sun, Rong-Wu Yang, Jin-Liang Qi, Xiao-Ming Wang, Gui-Hua Lu, Yong-Hua Yang

PII: S0960-894X(16)30560-1
DOI: http://dx.doi.org/10.1016/j.bmcl.2016.05.063
Reference: BMCL 23922

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 29 March 2016
Revised Date: 14 May 2016
Accepted Date: 21 May 2016

Please cite this article as: Han, H-W., Qiu, H-Y., Hu, C., Sun, W-X., Yang, R-W., Qi, J-L., Wang, X-M., Lu, G-H., Yang, Y-H., Design, synthesis and anti-cancer activity evaluation of podophyllotoxin-norcantharidin hybrid drugs, *Bioorganic & Medicinal Chemistry Letters* (2016), doi: http://dx.doi.org/10.1016/j.bmcl.2016.05.063

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Design, synthesis and anti-cancer activity evaluation of podophyllotoxin-norcantharidin hybrid drugs

Hong-Wei Han,[a,b,†] Han-Yue Qiu,[a,b,†] Cui Hu,[a,b] Wen-Xue Sun,[a,b] Rong-Wu Yang,[a,b] Jin-Liang Qi,[a,b] Xiao-Ming Wang,[a,b,] Gui-Hua Lu[a,b,*] and Yong-Hua Yang[a,b,*]*

[a]*State Key Laboratory of Pharmaceutical Biotechnology, NJU-NJFU Joint Institute of Plant Molecular Biology, Nanjing University, Nanjing, 210023, China*

[b]*Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing, 210037, China*

† These Two authors equally contribute to this paper. E-mail: wangxm07@nju.edu.cn,Guihua.lu@nju.edu.cn,Yangyh@nju.edu.cn

ABSTRACT

In this study, we designed and synthesized eighteen podophyllotoxin-norcantharidin hybrid drugs which could exhibit more potent anti-cancer activity than the parent drugs. Through the anti-proliferation assay, the most potent anti-cancer agent was screened out, namely **Q9** (IC_{50}= 0.88±0.18 μM against MCF-7 cell line), and it showed lower cytotoxity against non-cancer cells, human embryonic kidney cells (293T) (IC_{50}=54.38±3.78 μM). Additionally, based on the flow cytometry analysis result, it can cause a remarkable cell cycle arrest at G2/M phase and induce apoptosis in MCF-7 cells more significantly than podophyllotoxin or norcantharidin per se. Moreover, the expression of cell cycle relative protein CDK1 was up regulated while a protein required for mitotic initiation, Cyclin B1 was down regulated. Furthermore, according to the confocal microscopy observation results, it was shown that **Q9** was a potent tubulin polymerization inhibitor and the effect is comparable to that of colchicine. For further investigation on the aforementioned mechanisms, we performed western blot experiments, thus finding the increase of the cleavage of PARP. Consistent with these new findings, molecular docking observations suggested that compound **Q9** could be developed as a potential anticancer agent.

Keywords:
Natural product
Podophyllotoxin
Norcantharidin
anti-tubulin

Natural products play a major role in the development of drugs, especially for the treatment of cancer [1-3] and infections [4], as well as immunosuppressive compounds [5]. However, there is limited number of organic products, whereas millions of hybrids as combinations of parts of different organic products can be prepared. This new approach appears to be promising for the development of new and more potent anti-cancer drugs, for that the biological activity of some new hybrids would exceed that of the parent compounds [6-8]. The advantage of this concept over a combinatorial chemistry approach is the extreme diversity, as well as the inherent biological activity of the hybrids.

Podophyllotoxin (1) **Fig. 1**, an active ingredient of pedophilia species, is a lignan useful in the semi-synthesis of commercially, which can be employed in chemotherapy for various cancer types [9], including cervical carcinoma [10], osteosarcoma, nasopharyngeal carcinoma [11], colon cancer [12], breast cancer, prostate cancer [13], testicular carcinoma, small cell lung cancer and lymphoma [14,15]. However, due to some unacceptable toxic side-effects, it is unable to be used in human clinical trials [16,17]. To overcome the limitations, numerous researchers have been conducted with the focus on the structural modification at the 4 position of cycloparaffin (C ring). It could thus generate some derivatives with superior pharmacological

profiles [18-20], such as NK611 (2), etoposide (3) teniposide (4) and 4-azapodophyllotoxin (5), which retains cytotoxicity as well as inhibition of tubulin polymerization comparable to that of podophyllotoxin [21]. In previous studies, we also synthesized some podophyllotoxin derivatives. On this basis, we found that the cytotoxic effects of the derivatives mentioned above in different types of tumors have been attributed to their ability to bind the tubulin during mitosis, which would thus inhibit microtubule assembly and induce apoptosis [22-24].

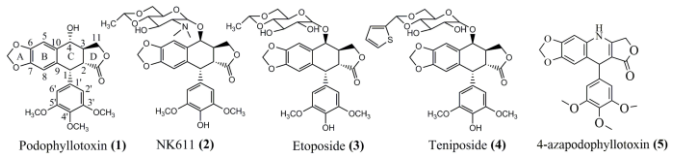
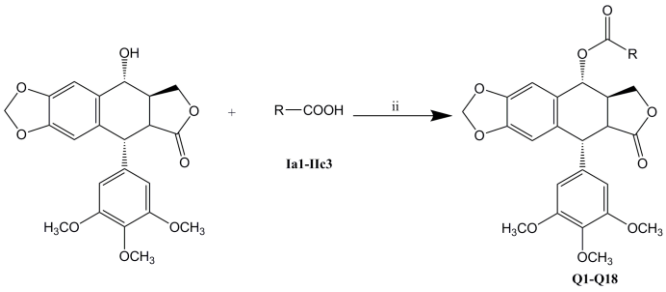


Fig. 1 Chemical structures of podophyllotoxin and some podophyllotoxin derivatives

Inspired by the hybrid concept, we would like to design a series of podophyllotoxin hybrid molecules, with the presence of the available natural product norcantharidin (NCTD) or its analogues, such as ring oxalic acid anhydride and noticeable. It is well known that as a premedicant for tumor treatment, NCTD is well used for cancer chemotherapy [25]. Previous studies reported that NCTD can interrupt cell mitosis and cause cell cycle arrest in M phase [26]. Given the same anti-cancer mechanism of podophyllotoxin and NCTD, we report the design and synthesis of three groups of hybrid molecules with NCTD or its two analogues based on podophyllotoxin scaffold, concerning which amino acid is used as the bridges. It is hoped that the new hybrids can achieve better effects than the original two parent drugs, namely podophyllotoxin and NCTD.

In the preliminary study, we synthesized the intermediate acid, as shown in **Scheme 1** and **2** [27]. (in the **Supplementary data**) In this study, we use the intermediate carboxylic acid to prepare a series of podophyllotoxin derivatives (**Scheme 3)**. These synthetic compounds were presented in **Table 1** and their ^{1}H NMR and ESI-MS results were consistent with the assigned structures, which were reported in the **Supplementary data**. In addition, these compounds were accounted for and characterized for the first time by ^{1}H NMR, elemental analysis, melting test, and mass spectroscopy, with the results in accordance with the depicted structures.

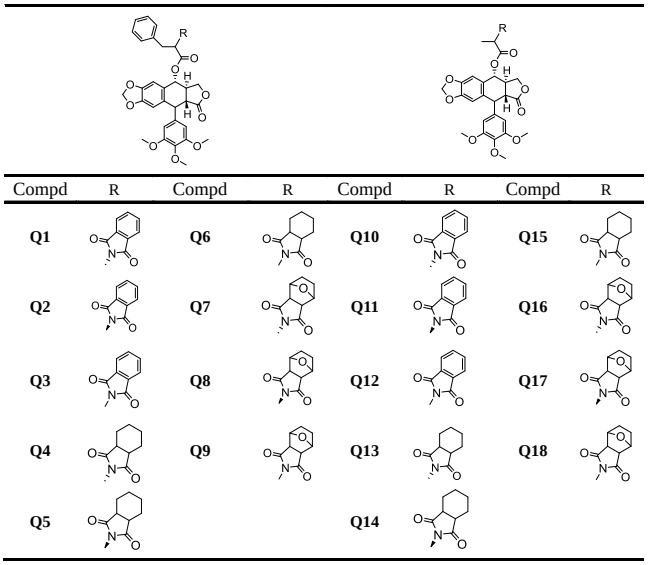
All the synthesized podophyllotoxin derivatives **Q1-Q18** were evaluated for their anti-proliferation activities against three human cancer lines, namely human breast cell line (MCF-7), human lung cancer cells (A549) and human cervical carcinoma cells (HeLa), as well as one non-cancer cell line, namely human embryonic kidney 293T cells (293T) by MTT (3-[4, 5-di-methylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay with etoposide as the positive control. It was observed that after modification not all the obtained compounds showed higher IC_{50} against the three cell lines compared with podophyllotoxin. As shown in **Table 2** (in the **Supplementary data**) the anti-proliferative activities of **Q6** (IC_{50}=1.17±1.26 μM), **Q9** (IC_{50}= 0.88±0.18 μM), **Q16** (IC_{50}=1.22±1.04 μM) and **Q17** (IC_{50}=1.17±0.76 μM) are better than podophyllotoxin (IC_{50}=4.21±1.11 μM) and the positive control etoposide (IC_{50}=1.74±0.35 μM). Interestingly, anti-proliferative effects of **Q9** (IC_{50}=7.82±3.60 μM) were much lower than that of podophyllotoxin (IC_{50}=5.32±1.36 μM) against A549 cells. In case of HeLa cells, the anti-proliferative properties of **Q9** (IC_{50}=3.21±0.99 μM) is superior to podophyllotoxin (IC_{50}= 6.56±1.45 μM) and etoposide (IC_{50}= 4.39±1.09 μM). Meanwhile, as shown from the anti-proliferation results, these compounds (**Q1-Q18**) exhibited low toxicity towards 293T cell line. According to the structure–activity relationship, we clearly found that cyclohexanol anhydride and NCTD substituted amino acids derivatives can significantly improve the anti-proliferation activity of podophyllotoxin and NCTD themselves. Previous experiments confirmed that podophyllotoxin and NCTD derivatives have better application prospects than the parent drugs in cancer chemotherapy. Moreover, it demonstrated that NCTD significantly suppressed the growth of highly-metastatic human breast cancer cell in vitro and ex vivo [28,29]. Taken together, we selected **Q9**, the best candidate for breast cancer cell lines MCF-7 in this study, for further investigation.



Scheme 3 The synthetic routes for **Q1-Q18**

To investigate whether **Q9** has anti-mitotic effects on MCF-7 cells, cell cycle analysis was performed by flow cytometry in which podophyllotoxin and NCTD are employed as positive controls. The results were shown in **Fig. 2**, 69.51% cells were arrested in G2/M phase while cells were treated with 8 μM **Q9** for 24 h. By contrast, the percentage of the cells that arrested in G2/M with NCTD or podophyllotocin is 24.89% or 15.77%, respectively. These indicating that **Q9** can potently arrest MCF-7 cells in G2/M phase while the effect was far better than NCTD and podophyllotoxin. Moreover, the expression of cell cycle related proteins CDK1 was at the same time up regulated, along with the down regulation of Cyclin B1. (**Fig. 3**)

Table 1 Chemical structures of **Q1-Q18**



Compd	R	Compd	R	Compd	R	Compd	R
Q1		**Q6**		**Q10**		**Q15**	
Q2		**Q7**		**Q11**		**Q16**	
Q3		**Q8**		**Q12**		**Q17**	
Q4		**Q9**		**Q13**		**Q18**	
Q5				**Q14**			

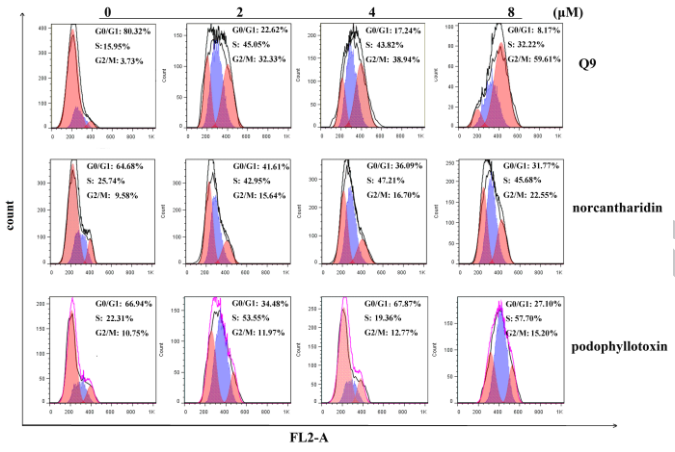


Fig. 2 Effects of **Q9**, norcantharidin and podophyllotoxin on the cell cycle distribution of MCF-7 cells. Cells treated with 0, 2, 4 and 8 μM **Q9**, norcantharidin and podophyllotoxin for 24 h were collected and processed for analysis.

In order to verify whether **Q9** can induce apoptosis in MCF-7 cells, we further performed apoptosis detection in a dose-dependent manner. (**Fig. 4**) We first treated MCF-7 cells with varying concentrations (0, 2, 4, and 8 μM) of **Q9** and also using NCTD and podophyllotoxin as the positive controls. After treatment, AnnexinV-FITC/PI assay was performed with cells, after which we found that MCF-7 cells showed considerable sensitivity to **Q9** in a dose-dependent manner. When the drug concentration rose to 2 μM, the proportion of the apoptotic cells was up to 64.50%. Additionally, we also found that NCTD could induce apoptosis in MCF-7 cells, but the effect was weaker than **Q9**. However, with the other positive control, podophyllotoxin showed no apoptosis inducing effect in MCF-7 cells and even at highest concentration, it only lead to 8.21% cells apoptosis. Thus, it can be concluded that **Q9** could significantly induce apoptosis of MCF-7 cells in a dose dependent manner.

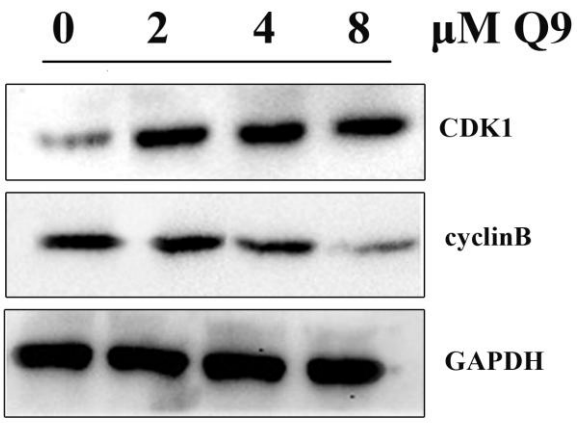


Fig. 3 Immuno-detection of cell cycle related proteins CDK1 and cycin B induced cell cycle arrest at G2/M phase in MCF-7 cells. Each band (left to right) represents the treatment using different concentrations (0, 2, 4, 8 μM) of **Q9**. GAPDH served as a loading control.

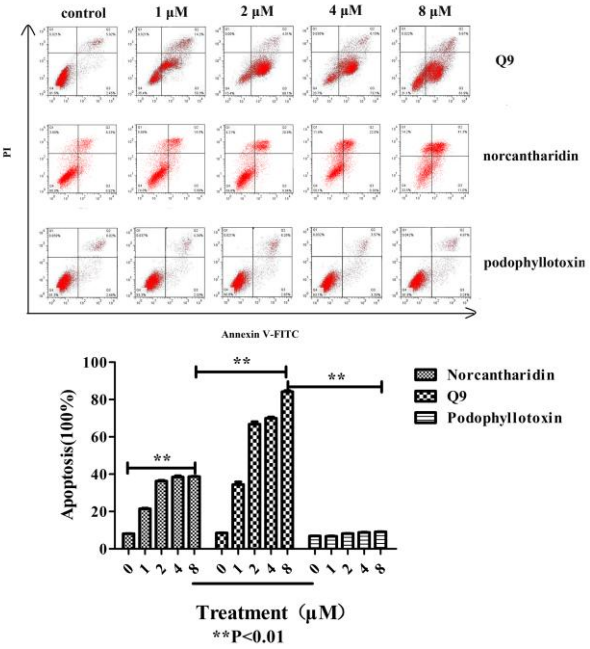


Fig. 4 AnnexinV/PI dual-immuno-fluorescence staining after treatment with different concentrations of **Q9**, norcantharidin and podophyllotoxin for 24 h revealed significantly increased number of apoptotic and necrotic cells (measured with Annexin V+/PI+ cells). Cells treated with 0, 1, 2, 4 and 8 μM **Q9**, norcantharidin and podophyllotoxin for 24 h were collected and processed for analysis. The percentage of early apoptotic cells in the lower right quadrant (annexin V-FITC positive/PI negative cells), as well as late apoptotic cells located in the upper right quadrant (annexin V-FITC positive/PI positive cells). Images are representative of three independent experiments. Data are mean±S.E.M. of three independent experiments. (**, $P < 0.01$).

To investigate the effect of **Q9** on cytoskeleton architecture, we performed confocal microscopy observation

on MCF-7 cells processed by **Q9** at indicated concentrations, as shown in **Fig. 5**. In the control group, we observed a typical array of radial organization and accumulation of microtubules at the microtubule-organizing center. However, in 4 μM **Q9** treated group, the architecture of the interphase microtubule network was deemed to be altered, accompanied by the destruction of tubulin cytoskeleton. Through the comparison of the two controls, colchicine and paclitaxel, we found that the effect of **Q9** on cytoskeleton architecture was analogous to that of colchicine and contrary to paclitaxel. The results confirmed that **Q9** can destroy the cytoskeleton structure and perturb mitosis in MCF-7 cells, and were in accordance to the cell cycle distribution consequences.

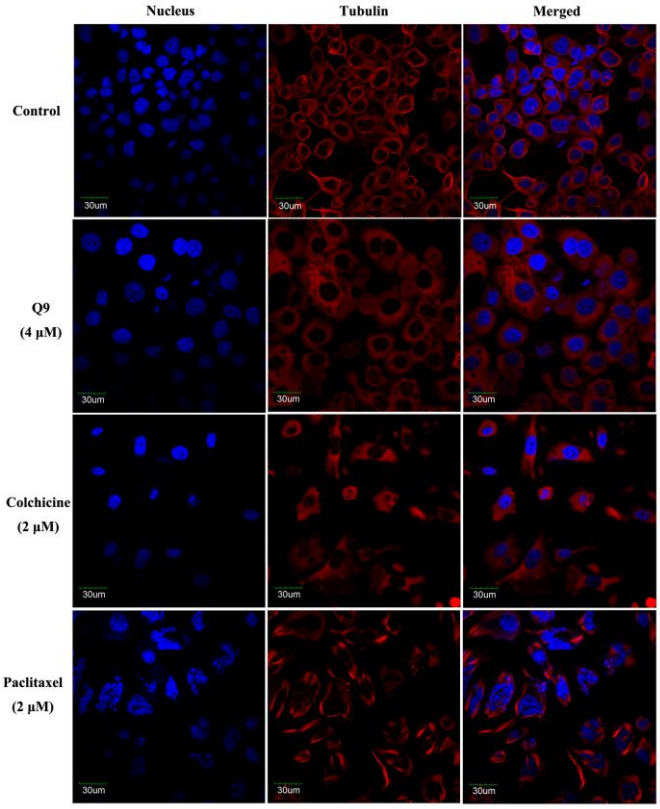


Fig. 5 Effect of **Q9** on interphase microtubules of MCF-7 cells using colchicine and paclitaxel as references. Microtubules tagged with rhodamine (red) and nuclei tagged with DAPI (blue) were observed under a confocal microscope.

Finally, on the basis of the above studies, we conducted a further detailed interpretation on the effect of inducing cells to apoptosis by **Q9**. We detected poly-ADP ribose polymerase (PARP), a family of proteins involved in DNA repair and programmed cell death. Compared with the control group, when the concentration of drug reached 8 μM, results showed that the expression of cleaved of poly-ADP ribose polymerase (PARP) were markedly increased (**Fig. 6**).

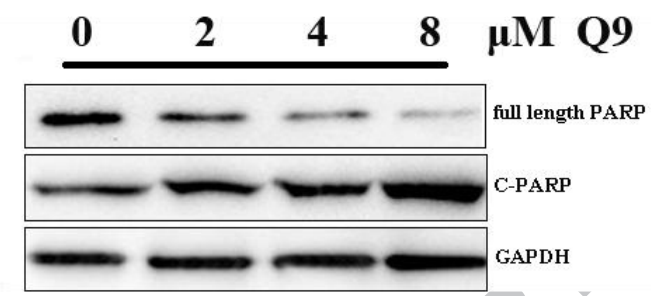


Fig. 6 Immuno-detection of apoptosis related proteins PARP and C-PARP induced apoptosis in MCF-7 cells. Each band (left to right) represents the treatment using different concentrations (0, 2, 4, 8 μM) of **Q9**. GAPDH served as a loading control.

In this study, we designed and synthesized a series of novel podophyllotoxin-norcantharidin hybrid drugs (**Q1-Q18**) in which phenylalanine and alanine are used as the bridges, and also evaluated their biological activities against three different cancer cell lines and one non-cancer cell line. Generally, introduction of NCTD or its analogues could really reduce the cytotoxicity side effect of podophyllotoxin toward non-cancer cell. Furthermore, **Q9** displayed the best anti-proliferation activities among all the compounds for breast cancer cell lines MCF-7. As indicated by the cell apoptosis and cell cycle analysis results, **Q9** could effectively prevent mitosis in cancer cells, thus resulting in cell cycle arrest and eventually inducing cell apoptosis. In order to confirm that **Q9** would exhibit better anti-cancer effect than the parent drugs, namely podophyllotoxin and NCTD, we used them as references in each assay and the results were in accordance with our prediction. Confocal microscopy results further confirmed that **Q9** can interrupt cancer cell mitosis, and cause cell cycle arrest in G2/M phase through inhibiting microtubules polymerization. Meanwhile, the expression of CDK1, G2/M phase check point regulatory protein which plays an important role on cell proliferation regulation, was also up regulated. In addition, Cyclin B1 is the regulatory subunit of the CDK1, while reduction of Cyclin B1 can stop cells in the G2 phase of the cell cycle and trigger cell death by preventing the chromosomes from condensing and aligning. The ability of **Q9** up- regulating intracellular Cyclin B1 levels suggests that the Cyclin B1-CDK1 plays a role in the events of mitosis. These data prove pro-apoptotic activities of **Q9** in MCF-7 cells and PARP is involved in the apoptosis process induced by **Q9**. In addition, the CDOCK interaction energy values of compounds bind to tubulin (PDB code: 1SA0) were performed. The results obtained are presented in the **Table 3**.(in the **Supplementary data**) In view of the interaction energy, **Q9** had a best estimated binding free energy of -74.3204 kcal/mol, which meant that the binding of **Q9** with tubulin was more stable than that of others and

this effect was more potent than that of colchicine. Thus, biological activity data along with molecular docking observations suggested that compound **Q9** could be developed as a potential anticancer agent.

Acknowledgements

The authors are grateful to the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R27), the fund for University Ph.D. Program from the Ministry of Education of China (20120091110037), the National Natural Science Foundation of China (NSFC) (Nos. 31470384, 31171161).

References and notes

1. Pull, L.; Bellettre, X.; Michel, J. F., Bouchaud, O.; Siriez, J. Y. *Archives de pediatrie: organe officiel de la Societe francaise de pediatrie*. **2013**, *20*, 1260.

2. Joel, T.; Frank, K.; Mehul D.; Vincent, J.; Francois, N.; Nicholas, J. W.; Philippe, J. G.; Patrice, P. *Antimicrob. Agents Chemother* **2013**, *57*, 5096.

3. Barbora, O.; Noemie, L.; Jana, P.; Mario, D.; Marc, D. *Cancer Treat Res*. **2014**, *159*, 123.

4. Mukne, A. P.; Viswanathan, V.; Phadatare, A. G. *Pharmacogn. Rev*. **2011**, *5*, 13.

5. Chen, J. C.; Li, W. L.; Yao, Q H.; Xu, J. Y. *Fitoterapia* **2015**, *103*, 231.

6. Prokopiou, E. M.; Ryder, Ryder, S. A.; Walsh, J.; *Angiogenesis*. **2013**, *16*, 503.7. Pal, C.; Sarkar, S.; Mazumder, S.; Adhikari, S; Bandyopadhyay, U. *Med. Chem. Commun*. **2013**, *4*, 731.

8. Gediya, L. K.; Khandelwal, A.; Patel, J.; Belosay, A.; Sabnis, G.; Mehta, J.; Purushottamachar, P.; Njar, V. C. O. *J. Med. Chem*. **2008**, *51*, 3895.

9. Zhou, J.; Qu, Z.; Yan, S.; Sun, F.; Whitsett, J. A.; Shapiro, S. D.; Xiao, G. *Oncogene*. **2014**, *33*, 1-11.

10. Williams, A. H. W.; Dai, X. D.; Blot, W.; Xu, Z. Y.; Sun, X. W.; Xiao, H. P.; Stone, B. J.; Yu, S. F.; Feng, Y. P.; Ershow, A. G.; Sun, J.; Fraumeni, J. F.; Henderson, B. E. *Br. J. Cancer*. **1990**, *62*, 982.

11. Barbora, O.; Noemie, L.; Jana, P.; Mario, D.; Marc, D. *Cancer treatment and research*. **2014**, *159*, 123.

12. Kaur, R.; Kapoor, K.; Kaur, H. *J. Nat. Prod. Plant Resour*. **2011**, *1*, 119.

13. Kamal, A.; Tamboli, J. R.; Nayak, V. L.; Adil, S. F.; Vishnuvardhan, M. V. P. S.; Ramakrishna, S. *Bioorg. Med. Chem*. **2014**, *22*, 2714.

14. Qi, Y. L.; Liao, F.; Zhao, C. Q., Lin, Y. D.; Zuo, M. X. *Acta Pharmacol. Sin*. **2005**, *26*, 1000.

15. Kamal, A.; Kuma, B. A.; Suresh, P.; Juvekar, A.; Zingde, S. *Bioorg. Med. Chem*. **2011**, *19*, 2975.

16. Shareef, M. A.; Duscharla, D.; Ramasatyaveni, G.; Dhoke, N. R.; Das, A.; Ummanni, R.; Srivastava, A. K.. *Eur. J. Med. Chem*. **2015**, *89*, 128.

17. Yang, L.; Nan, X.; Li, W. Q.; Wang, M. J.; Zhao, X. B.; Liu, Y. Q.; Zhang, Z. J.; Lee, K. H. *Med. Chem. Res*. **2014**, *23*, 4926.

18. Wang, H.; Tang, L. J.; Tang, Y. J.; Yuan, Z. P. *Bioorg. Med. Chem*. **2014**, *22*, 6183.

19. Cheng, W. H.; Cao, B.; Shang, H.; Niu, C.; Zhang, L. M.; Zhang, Z. H.; Tian, D. L.; Zhang, S.; Chen, H.; Zou, Z. M. *Eur. J. Med. Chem*. **2014**, *85*, 498.

20. Liu, Y. Q.; Tian, J.; Qian, K.; Zhao, X. B.; Natschke, S. L. M.; Yang, L.; Nan, X.; Tian, X.; Lee, K. H. *Med. Res. Rev*. **2015**, *35*, 1.

21. Kamal, Ahmed; Reddy, T. Srinivasa; Polepalli, S.; Paidakula, S.; Srinivasulu, V.; Reddy, V. G.; Jain, N, Shankaraiah, N. *Bioorg. Med. Chem*. **2014**, *24*, 3356.

22. Lin, H. Y.; Li, Z. K.; Han, H. W.; Qiu, H. Y.; Gu, H. W.; Yang, Y. H.; Wang X. M. *RSC Adv*. **2015**, *5*, 47511.

23. Lin, H. Y.; Bai, L. F.; Wang, F.; Wu, X.; Han, L. J.; Baloch, S. K.; Yang, Y. H.; Wang, X. M. *RSC Adv*. **2015**, *5*, 27775.

24. Kamal, A.; Reddy, T. S.; Polepalli, S.; Shalini N.; Reddy, V. G.; Rao, A. V. S.; Jain, N.; Shankaraiah, N. *Bioorg. Med. Chem*. **2014**, *22*, 5466.

25. Wang, G. S. *J. Ethnopharmacol*. **1989**, *26*, 147, 162.

26. Hong, C. Y.; Huang, S. C.; Lin, S. K.; Lee, J.; Chueh, L. L.; Lee, C. H. K.; Lin, J. H.; Hsiao, M. *Biochem. Bosch. Res. Co*. **2000**, *276*, 278.

27. Baloch, S. K.; Ma, L., Wang, X. L.; Shi, J.; Zhu, Y.; Wu, F. Y.; Pang, Y. J.; Lu, G. H.; Qi, J. L.; Wang, X. M.; Gu, H. W.; Yang, Y. H. *RSC Adv*. **2015**, *5*, 31759.

28. Lin, H.Y.; Li, Z. K.; Han, H. W.; Qiu, H. Y.; Gu, H. W.; Yang, Y. H.; Wang, X. M. *RSC Adv*. **2015**, *5*, 47511.

29. Huang, Y.; Liu, Q.; Liu, K.; Yagasaki, K.; Zhang, G. Y. *Cytotechnology* **2009**, *59*, 201.

Graphical Abstract

Design, synthesis and anti-cancer activity evaluation of podophyllotoxin-norcantharidin hybrid drugs

Hong-Wei Han, Han-Yue Qiu, Cui Hu, Wen-Xue Sun, Rong-Wu Yang, Jin-Liang Qi,[a,b] Xiao-Ming Wang, Gui-Hua Lu and Yong-Hua Yang

[a]*State Key Laboratory of Pharmaceutical Biotechnology, NJU-NJFU Joint Institute of Plant Molecular Biology, Nanjing University, Nanjing, 210023, China*

[b]*Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing, 210037, China*

It proves pro-apoptotic activities of **Q9** in MCF-7 cells and PARP is involved in the apoptosis process induced by **Q9**.

.