［課程－２］

審査の結果の要旨

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This study is aimed to find out the molecular mechanisms and combination therapeutic effects of traditional Chinese medicine cinobufacini and its active compounds bufalin and cinobufagin. A series of experimental methods, such as HPLC, MTT assay, Real-time PCR, Mito-capture assay, western blot, and animal model, were employed to explore the molecular mechanisms and validate the combination therapeutic effect. The results are as follows:

1. Under the bioactivity-guided fractionation, effective anti-cancer components from cinobufacini were purified. Two purified fractions were isolated by preparative TLC and HPLC. The final detection of either of these fractions using HPLC reported that two active compounds. After analyzing and comparing these data online with reported compounds and references, the structures of two compounds were identified as bufalin and cinobufagin.
2. The anti-proliferation effect of cinobufacini and doxorubicin on hepatocellular carcinoma cells was estimated by MTT assay. The results suggested that cinobufacini had a significant growth inhibition effect on HLE and HepG2 cells
3. The anti-proliferation effect of the combination of cinobufacini and doxorubicin on hepatocellular carcinoma cells was assessed by MTT assay. The inhibitive level of cinobufacini combined with doxorubicin was higher than monotherapy in HLE and HepG2 respectively, and cinobufacini showed more obvious inhibitive ability than bufalin or cinobufagin. Through free Chou–Talalay method, it proved that combination of cinobufacini and doxorubicin produced synergy effects.
4. To investigate the effect of each experimental group on the morphology of apoptotic cells, Hoechst 33258 staining and mito-capture method was used. The combination group showed higher apoptotic ratio than monotherapy group, meanwhile cinobufacini group showed higher apoptotic level than bufalin group and cinobufagin group as well.
5. Through real-time PCR and western blot, apoptosis-related genes and proteins, such as bcl-2, bax, bid, cytochrome c, casase-3, caspases-9, caspases-8, and caspases-10, were detected. The results showed that combination group could induce strongest disruption of expression of genes and proteins significantly.
6. The *in vitro* findings were validated in vivo in a xenograft mouse model of human HepG2 liver cancer. Combination group led to a most significant suppression of tumor growth and decreased the final tumor weight. Meanwhile, the combination

group could induce strongest change of expression of genes and proteins significantly *in vivo*. Above all, these results suggested that cinobufacini and its active compounds, bufalin and cinobufagin, could induce apoptosis of HCC cells by activating mitochondrial- and Fas-related apoptotic signaling pathways and combination of cinobufacini and doxorubicin showed more obvious inhibitive activity on HCC cells and xenograft than monotherapies with cinobufacini or doxorubicin. This study contributes to illuminate the molecular mechanisms and combination therapeutic effect of cinobufacini, so it is considered worthy to award a doctoral degree.