Hematopoiesis toxicity induced by 4-methylumbelliferon determined in an invertebrate model organism

Yan Fang1,2*, Yue He1,2*, Hua Wang1,2, Siqi Yan3, Rui Xing1,2, Weimin Yin4, Yanghu Sima1,2, and Shiqing Xu1,2

1Department of Applied Biology, School of Biology and Basic Medical Sciences, Medical College, Soochow University, Suzhou, China, 2National Engineering Laboratory for Modern Silk, Institute of Agricultural Biotechnology & Ecology, Soochow University, Suzhou, China, 3School of Architecture and Urban Environment, Soochow University, Suzhou, China, 4Gladstone Institute of Cardiovascular Disease, Medical College, Soochow University, Suzhou, China

Abstract

Umbelliferone has potential value as it has an inhibitory effect on tumor cells; however, its impact on an animal’s circulatory system and hematopoietic function has not been reported. In this study, 4-methylumbelliferon (4-MU), an umbelliferone derivative, was used as a model drug, and its potential toxicity on hemocytes and hematopoietic organs (HOs) was investigated using an invertebrate animal model, the silkworm, Bombyx mori. The results showed that the level of reactive oxygen species in HOs increased when larvae (third day of the fifth instar) were orally exposed to 4 mM 4-MU for 8 min, followed by the induction of improved antioxidative metabolism of coenzymes in hemolymph. Exposure to 4-MU also significantly upregulated the expression levels of several genes in the hemolymph and fat body (a detoxification tissue similar to the liver in mammals) including antimicrobial peptide gene cercopinA and moricin, and a phagocytosis-related gene, tetrspanin E, suggesting an increased antioxidant level and antimicrobial ability of the circulatory system. However, the percentage of dead hemocytes increased and hematopoiesis significantly decreased in HOs, indicating the toxic effect of 4-MU on hemocytes and hematopoiesis, despite it inducing enhanced antioxidant and antimicrobial activity in the circulatory system.

Introduction

4-methylumbelliferon (4-MU) is an umbelliferone derivative and is used as a model drug to study the pharmacodynamics of umbelliferones. 4-MU is rapidly metabolized into harmless substances by glycosylation and sulfation in mice, but produces different metabolites in different tissues (Chen & Pang, 1997). In the invertebrate model, Bombyx mori (the silkworm), 4-MU is mainly glycosylated (Hamamoto et al., 2009), which may be related to the extremely strong activity of glycosylase in the digestive tract and hemolymph of silkworms (Xu, 2013). In recent years, a large number of in vitro studies using mouse and human cells have reported the antitumor effect of 4-MU (Bhattacharyya et al., 2009; Oemardien et al., 2011; Twarock et al., 2011; Urakawa et al., 2012). One possible mechanism is that 4-MU may inhibit the synthesis of hyaluronic acid, an important marker for tumor development (Nakazawa et al., 2006). The double bonds which exist between carbon atoms and oxygen atoms in derivatives of the coumarin family produce reactive oxygen species (ROS) in vivo (Kuki et al., 2008). We previously observed that oral exposure to 4-MU induced a considerable increase in ROS (mainly H2O2) in gastrointestinal cells, increased antioxidant levels and the antibacterial ability of the digestive tract in B. mori, protected the tissues from injury (Fang et al., 2014a), and prevented cell damage (Fang et al., 2014b) in the digestive tract, indicating a new in vivo pathway for 4-MU.

Hemocytes are a suitable model for the study of innate immune response and DNA damage in vivo (Irving et al., 2005; Cherry & Silverman, 2006; Carmona et al., 2011). However, there have been no reports on the effect of umbelliferones on hematopoietic function. Bombyx mori is an ideal animal species for in vivo and in vitro research on the effects of toxins on hematopoiesis. The generation and release of hemocytes in hematopoietic organs (HOs) can be reliably repeated in vitro (Liu et al., 2014). Nearly all organs and tissues in insects such as flesh fly (Kurata et al., 1989) and B. mori (Liu et al., 2014) are immersed in hemolymph, and the results from in vitro cell culture experiments are close to the results from in vivo animal experiments. Thus, the limitations arising from tissue barriers in mammals can be avoided (Tsoi et al., 2013). This study assessed how 4-MU differentially acted on hemocytes and generated cytotoxicity.

Keywords

Antioxidant level, antimicrobial peptide, 4-Methylumbelliferon, hemolymph, hematopoiesis damage

Received 21 December 2014
Revised 15 June 2015
Accepted 2 August 2015
Published online 31 August 2015

*Yan Fang and Yue He are co-first authors, who contributed equally to this work.

Address for correspondence: Shiqing Xu, Department of Applied Biology, School of Biology and Basic Medical Sciences, Medical College, Soochow University, Suzhou, 215123, China. Tel: +86 0512 65880185. E-mail: szsqxu@suda.edu.cn
In particular, we determined whether 4-MU had a positive impact on the development of hematopoietic tissue and hematopoiesis function.

**Materials and methods**

**Preparation of animals**

The Dazao strain of *B. mori* was reared at 25°C with a photoperiod of 12 h light and 12 h dark. Similar-sized larvae (body weight 1.0 ± 0.1 g) were orally exposed to 4-MU (Sigma, St. Louis, MO, USA) on day 3 of the fifth instar. Following the method of Fang et al. (2014b), 4-MU was dissolved in 10% dimethyl sulfoxide (DMSO) to a final concentration of 4 mM. The instillation dose was 50 μL per individual. In order to minimize food residue, the animals were starved for 3 h before exposure to 4-MU; animals in the control group were also starved.

**Assessment of hemocytes and hematopoiesis**

Hemolymph was collected after the pereiopods were punctured using needles and placed in a centrifuge tube at 8, 30, 60, 120 and 180 min after 4-MU injection. A volume of 100 μL hemolymph was used for hemocyte acridine orange–propidium iodide (AO/PI) staining, and the remainder was used for biochemical assays and gene expression profile assays.

Following the method of Liu et al. (2014), the HOs were carefully removed using surgical forceps. The HOs were washed with precooled physiological saline and Grace’s insect tissue culture medium. A hanging drop culture was performed for each HO. The culture medium used was Grace’s insect tissue culture medium supplemented with 10% *B. mori* hemolymph and an appropriate amount of antibiotics. The volume of the culture medium was 10 μL, and the culture temperature was 25°C.

**AO/PI staining**

The hemocytes were double stained with AO/PI in order to assess cell apoptosis. AO (Item No. A742007) and PI (Item No. PB1112) were purchased from Sangon Biotec Co., Ltd (Shanghai, China).

**Biochemical assays**

Nicotinamide adenine dinucleotide oxidase (NOX-1), NAD(H) and NADP(H) were measured, respectively, using NOX assay kit (Item No. KY6), NAD(H) assay kit (Item No. KY1-2) and NADP(H) assay kit (Item No. BY1-2) from Comin Biotechnology Co., Ltd (Suzhou, China), according to the manufacturer’s instructions.

**Analysis of gene expression profiles**

Total RNA was isolated from five larvae hemolymph in each group using TRizol reagent (Life Technologies, Carlsbad, CA, USA) and purified using an Oligotex® mRNA Midi kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. The real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to investigate the expression level of genes using the primers listed in Supplementary Table S1. An SYBR® Premix Ex Taq™ kit (Perfect Real Time) was used for qRT-PCR and Ribosomal protein 49 (Rp49) and Elongation2 (Elo2) of *B. mori* was set as the reference genes. The reaction program was as follows: 95°C degeneration for 1 min, followed by 40 cycles at 95°C for 5 s, 58°C for 10 s, and 72°C for 10 s. The process was set automatically by the detection program and three replicates were used for each.

**Superoxide anion and reactive oxygen species (ROS) staining**

The levels of superoxide anion and ROS were measured using a dihydroethidium (DHE) kit (Beyotime, Shanghai, China) and ROS kit (Beyotime, Shanghai, China), respectively. The HOs were collected in diethylpyrocarbonate (DEPC) (containing 0.7% NaCl) to avoid air exposure and was then vortexed for 20 s in normal saline which included 0.1% DMSO. The HOs were then quickly placed into a staining solution for 7 min (for DHE) or 15 min (for 2’,7’-dichlorofluorescin diacetate, DCFH-DA), and then washed with saline for 5 min in the dark room. The red fluorescence (for superoxide anions) or green fluorescence (for ROS) was observed using a fluorescence microscope (Olympus Medical Systems Corp., Tokyo, Japan).

**Replication of experiments and statistical analyses**

All experiments were performed at least three times, and are presented throughout as mean ± SD. The significant differences between control group and treatment group were determined using the Student’s *t*-test. Data from multiple time points were analyzed by one-way ANOVA in combination with Origin7.5 Software.

**Results**

**4-MU exposure increased ROS levels in HOs and hemolymph**

We previously demonstrated that 4-MU passed through the membrane barrier of the digestive tract and entered the circulatory system of silkworms 8 min after oral exposure to 4-MU (Fang et al., 2014a). In this study, HOs were collected 8–60 min after oral 4-MU exposure and subjected to ROS and superoxide anion staining. The results showed that the level of ROS in HOs increased 8–30 min after 4-MU injection (Figure 1A) and the level of superoxide anion, a specific ROS, significantly increased 30 min after exposure (Figure 1B), suggesting that 4-MU induced ROS other than superoxide anion in HOs.

It was impossible to collect a sufficient amount of hydrogen peroxide (H$_2$O$_2$) for accurate analysis due to the small size of HOs. Furthermore, the hemolymph of silkworms was easily oxidized, which made it even harder to accurately measure H$_2$O$_2$ levels. Therefore, we examined the activities of NADH peroxidase (NOX-1), a key enzyme in the synthesis of H$_2$O$_2$ in hemolymph. As shown in Figure 1(C), NOX-1 activity was increased by 93% in hemolymph 16 min after 4-MU exposure, indicating that the level of H$_2$O$_2$, another important ROS, may have increased.
4-MU-induced metabolic changes in coenzymes in the hemolymph

As the activity of NOX-1 is related to coenzyme I and the metabolism of H$_2$O$_2$ is indirectly affected by coenzyme II, the substrates and products associated with the coenzyme I and II system were measured (Figure 2). The results showed that the NADH/NAD$^+$ ratio increased by 3.56–3.78 times within 32 min after 4-MU injection (Figure 2A), returned to the control level after 48 min, and further declined to 21% of the control level at 120 min. The NADPH/NADP$^+$ ratio increased threefold with a lower frequency and smaller amplitude within 120 min after 4-MU exposure, and then quickly decreased to the control level (Figure 2D). It is worth noting that NADPH and NADP$^+$ levels in the early stage (within 16 min) after 4-MU exposure were significantly lower than those in the control group, returned to the control levels at 32 min, and increased to much higher levels at 60 and 120 min compared with the control group (Figure 2E and F).

The NADH/NAD$^+$ ratio is an indicator of the level of glycolysis and metabolism in the tricarboxylic acid (TCA) cycle. A higher ratio indicates that cells are in an over-oxidized state with a higher level of oxygen consumption (Houtkooper et al., 2010). As shown in Figure 2(A, B and C), 4-MU-induced ROS quickly resulted in the hemolymph being in the over-oxidized state; however, the NADH/NAD$^+$ ratio was restored 48 min after exposure. As a reducing agent for...
biosynthesis, NADPH is involved in the activation of enzymes such as catalase and glutathione peroxidase in the body, and indirectly affects the metabolism of H$_2$O$_2$. Therefore, the NADPH/NADP$^+$ ratio is commonly used to evaluate the redox state in vivo (Jeon et al., 2012). Figure 2(D, E and F) shows that NADPH was rapidly used against 4-MU-induced ROS in hemolymph, and thus a large amount of NADPH was consumed. Oscillation of the NADPH/NADP$^+$ ratio also suggested that NADPH oscillated due to its synthesis and consumption in hemolymph.

4-MU-induced apoptosis of hemocytes and hematopoiesis in HOs

As shown in Figures 1 and 2, exposure to 4-MU increased ROS levels in hemolymph and tissues in HOs, suggesting that oxidative damage in hemocytes and HOs may have occurred. Therefore, AO/PI staining of hemocytes was performed and the results demonstrated that the percentage of dead hemocytes significantly increased 30–60 min after 4-MU exposure (Figure 3).

In order to clarify the reason for the increased percentage of dead hemocytes in hemolymph, we further investigated the expression levels of apoptosis-related genes in hemolymph. It was found that the mRNA levels of major genes involved in the apoptosis signaling pathway including, MAPK-ERK kinase (Mek) and extracellular regulated MAP kinase (Erk), slightly decreased within 3 h after 4-MU exposure. The mRNA level of p38 map kinase (p38) slightly increased for a short period of time followed by a slight decrease at 30 min, and a slight increase at 3 h after 4-MU injection (Figure 3D). It has been reported that cell apoptosis occurs only when the expression of Mek and Erk simultaneously decrease and the expression of p38 is significantly upregulated in an organism (Iyoda et al., 2003). As shown in Figure 3(C), the increase in the percentage of dead cells in hemolymph after 4-MU exposure might not be entirely, or even mainly, induced by apoptosis of hemocytes. Alternative reasons may exist.

The hematopoietic capacity of HOs was further investigated after HOs were exposed in vivo to 4-MU and cultured in vitro (Figure 4). The results showed that although 4-MU-treated HOs exhibited no obvious atrophy and

![Figure 3. The impact of 4-MU on the percentage of dead hemocytes in hemolymph. (A) The percentage of dead hemocytes stained by PI. (B) The concentration of hemocytes. The ordinate represents the numbers of hemocytes per milliliter hemolymph. (C) Dead hemocytes were stained red by PI. Live or dead hemocytes of prohemocytes, granulocytes and spherulocytes were stained green by AO and all hemocytes in a bright field. (D) Expression profile of apoptosis-related genes Mek, Erk and p38. In Figure 3(A, B and D), treatment was repeated 3 times, the animal number (sample size) of each hemolymph sample is 5 larvae, and each bar chart ± standard deviation represents mean value in triplicates. *p < 0.05 and **p < 0.01 indicate significant differences. X-axis shows the time after 4-MU exposure.](image-url)
apoptosis, these HOs had a much slower rate of hematopoiesis and produced significantly fewer hemocytes compared with the positive control (DMSO-treated HOs). HOs cultured in vitro beginning 30 min after 4-MU exposure had significantly lower hematopoietic capacity compared with those cultured earlier or later, indicating that the influence of 4-MU exposure on HOs continued to rise within 30 min, followed by self-repair of HOs. These observations were consistent with the changes in the percentage of dead hemocytes over time (Figure 3).

**4-MU affected the expression of phagocytosis-related and antimicrobial peptide genes**

It has been reported that umbelliferones have strong antibacterial effects on plant pathogenic bacteria in vitro (Zhang & Jiang, 2010). Our previous study showed that 4-MU can improve the antibacterial ability of the digestive tract once it enters the tract of *B. mori* (Fang et al., 2014b). The changes in the expression of antimicrobial peptide genes in the fat body (Figure 5A and B) and hemolymph (Figure 5C and D) were measured, and the results showed that both the hemolymph and fat body responded quickly to 4-MU exposure by upregulating the expression of antimicrobial peptide genes including cecropin A and moricin within 2 h after exposure. The expression levels of both genes were subsequently downregulated to normal levels. The expressed antimicrobial peptides (AMPs) in the fat body may be further secreted into hemolymph for their function. In other insect models (e.g. *Drosophila*), starvation is relative with immunity systemically (Brown et al., 2009). In this case, the animals were starved for 3 h before exposure to 4-MU. Compared to control group, the immune-related genes cecropin A and moricin were upexpressed in fat body and hemolymph within 3 h (Figure 5), it is mainly induced by the 4-MU. AMPs have been shown to be highly toxic to bacteria and tumor cells with almost no toxicity in normal tissue cells (Shin et al., 2000; Sergio et al., 2003). The results shown in Figure 5 indicate that 4-MU had potential pharmacological effects in improving antibacterial and antitumor abilities by inducing the expression of AMPs in *B. mori*.

As 4-MU upregulated the expression level of antimicrobial peptide genes in hemolymph (Figure 5), we speculated that phagocytosis of hemocytes may also be affected. The expression levels of phagocytosis-related genes were further examined. The results demonstrated that 4-MU exposure increased the expression level of tetraspanin E (TspE) gene without affecting the expression of Ced-6 (Ced-6) gene and ActinA1 (ActA1) gene (Figure 6), which suggested that 4-MU did not increase antibacterial ability by enhancing phagocytosis of hemocytes.

**Discussion**

Umbelliferones are widely found in plants, but have biological activities in many animal species and have been used for medicinal purposes (Morabito et al., 2010). These compounds also show strong antibacterial effects on plant pathogenic bacteria in vitro (Zhang & Jiang, 2010). In recent years, the

![Figure 4](image-url)

Figure 4. The impact of 4-MU on hematopoiesis in hematopoietic organs. (A) Hematopoiesis was affected by exposure duration in vivo and culture duration in vitro. (B) The form and function of HOs are displayed in Figure 4(B). +++ indicates normal hematopoiesis, ++ indicates only a small amount of hematopoiesis and + indicates very little or no hematopoiesis. Fifth-instar larvae were orally injected with 50 μL 4 mM 4-MU per individual for 48 h and control organisms were injected with the same volume of 10% DMSO. Exposure duration in vivo was the time to remove the HOs after exposure to 4-MU. Culture duration in vitro was the incubation time free from 4-MU. A single HO was cultured using the hanging drop method in 10 μL Grace’s insect medium at 25 °C with continuous illumination.
The ability of umbelliferones to inhibit the growth of cancer cells as well as the occurrence and development of tumors has been observed in many in vivo and in vitro experiments using mammalian cells including mouse and human cells (Bhattacharyya et al., 2009; Nakazawa et al., 2006; Oemardien et al., 2011; Twarock et al., 2011; Urakawa et al., 2012). Our previous research strongly suggested that 4-MU exposure induced a considerable increase in H2O2, and increased antioxidant levels and the antibacterial ability of digestive tract in B. mori (Fang et al., 2014b). Our experimental results also provide evidence to show that only trace amounts of 4-MU were observed in the fat body, a detoxification tissue similar to the liver in mammals, after exposure to 4-MU by oral injection, but strongly increased the ROS level and improved the antioxidant capacity in fat bodies, protected the tissues from injury and resulted in activation of the antioxidant enzyme system (Fang et al., 2014a). Similar antioxidant protection against cell damage was found in the digestive tract (Fang et al., 2014b).

In this study, oral 4-MU exposure induced changes in ROS levels and antioxidant capacity in hemolymph and HOs, which were similar to those observed in the digestive tract and fat body. It was also found that the expression levels of antimicrobial peptide gene, cecropinA and moricin, and phagocytosis-related gene, tetraspanin E, were significantly upregulated, indicating that antioxidant levels and the antibacterial ability of the circulatory system were simultaneously enhanced. However, the percentage of dead hemocytes in hemolymph significantly increased and hematopoietic capacity of HOs dramatically decreased within a short period of time.

**Conclusion**

4-MU has potential pharmacological effects in improving antibacterial and antitumor ability by inducing the expression of AMPs in B. mori. However, it has simultaneous potential toxicity on circulating hemocytes and their hematopoietic function.

**Declaration of interest**

The authors declare no conflict of interest.

This work was supported by the National Natural Science Foundation of China (Grant No. 31172264), National Natural Science Foundation of Jiangsu province (Grant No. 204 Y. Fang et al. Drug Chem Toxicol, 2016; 39(2): 199–205

![Figure 5](image5.png)

Figure 5. Expression level of antimicrobial peptide genes in the (A and B) fat body and in (C and D) hemolymph after 4-MU exposure. The X-axis shows the time after 4-MU exposure. Treatment was repeated 3 times, and each bar chart ± standard deviation represents mean value in triplicates. *p<0.05 and **p<0.01 indicate significant differences.

![Figure 6](image6.png)

Figure 6. Expression level of engulfment genes (A) TspE, (B) Ced-6 and (C) ActinA1 in hemolymph after 4-MU exposure. The X-axis shows the time after 4-MU exposure. Treatment was repeated 3 times, the animal number (sample size) of each hemolymph sample is 5 larvae, and each bar chart ± standard deviation represents mean value in triplicates. *p<0.05 and **p<0.01 indicate significant differences.
BK2011298), National High-Tech R&D Program of China (863 Program) (Grant No. 2011AA100306), Provincial Key Technology R&D Program of Jiangsu (Project No. BE2011327-1) and Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

Bhattacharyya SS, Paul S, Mandal SK, Banerjee A, et al. (2009). A synthetic coumarin (4-methyl-7 hydroxycoumarin) has anti-cancer potentials against DMBA-induced skin cancer in mice. Eur J Pharmacol 614:128–136.

Brown AE, Baumbach J, Cook PE, Ligoxygakis P. (2009). Short-term starvation of immune deficient Drosophila improves survival to gram-negative bacterial infections. Plos One 4:e4490.

Carmona ER, Creus A, Marcos R. (2011). Genotoxic effects of two nickel-compounds in somatic cells of Drosophila melanogaster. Mutat Res 718:33–37.

Chen J, Pang KS. (1997). Effect of flow on first-pass metabolism of drugs: single pass studies on 4-methylumbelliferone conjugation in the serially perfused rat intestine and liver preparations. J Pharmacol Exp Ther 280:24–31.

Cherry S, Silverman N. (2006). Host–pathogen interactions in drosophila: new tricks from an old friend. Nat Immunol 7:911–917.

Fang Y, Wang H, Zhu W, et al. (2014a). Antioxidative capacity in the fat body of Bombyx mori is increased following oral administration of 4-methylumbelliferone. Comp Biochem Physiol C Toxicol Pharmacol 159:31–37.

Fang Y, Wang H, Zhu W, et al. (2014b). Antioxidative properties of 4-methylumbelliferone are related to antibacterial activity in the silkworm (Bombyx mori) digestive tract. J Comp Physiol B 184: 699–708.

Hamamoto H, Tonoike A, Narushima K, et al. (2009). Silkworm as a model animal to evaluate drug candidate toxicity and metabolism. Comp Biochem Physiol 149:334–339.

Houthooper RH, Cantó C, Wanders RJ, Auwerx J. (2010). The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. Endocr Rev 31:194–223.

Irving P, Ubeda JM, Doucet D, et al. (2005). New insights into Drosophila larval haemocyte functions through genome-wide analysis. Cell Microbiol 7:335–350.

Iyoda K, Sasaki Y, Hortimoto M, et al. (2003). Involvement of the p38 mitogenactivated protein kinase cascade in hepatocellular carcinoma. Cancer 97:3017–3026.

Jeon SM, Chandel NS, Hay N. (2012). AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. Nature 485:661–665.

Kuki W, Hosotani K, Ohigashi H, Murakami A. (2008). Metabolism and absorption of auraptene (7-geranyloxyxocoumarin) in male SD rats: comparison with 7-ethoxyxocoumarin. Nutr Cancer 60:368–372.

Kurata S, Saito H, Natori S. (1989). Dissociation of Sarcophaga pergrina (flesh fly) fat body by pupal haemocytes in vitro. J Insect Physiol 35: 559–565.

Liu T, Xing R, Zhou YF, et al. (2014). Hematopoiesis toxicity induced by CdTe quantum dots determined in an invertebrate model organism. Biomaterials 9:2942–2951.

Morabito G, Trombetta D, Singh Brajendra K, et al. (2010). Antioxidant properties of 4-methylcoumarins in in vitro cell-free systems. Biochimie 92:1101–1107.

Nakazawa H, Yoshihara S, Kudo D, et al. (2006). 4-methylumbelliferone, a hyaluronan synthase inhibitor, enhances the anticancer activity of gemcitabine in human pancreatic cancer cells. Cancer Chemother Pharm 57:165–170.

Oemardien LF, Boer AM, Ruijter GJ, et al. (2011). Hemoglobin precipitation greatly improves 4-methylumbelliferone-based diagnostic assays for lysosomal storage diseases in dried blood spots. Mol Genet Metab 102:44–48.

Sergio HM, Gloria A. (2003). Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology. Electron J Biotechnol 6:271–284.

Tsoi KM, Dai Q, Alman BA, Chan WC. (2013). Are quantum dots toxic? Exploring the discrepancy between cell culture and animal studies. Acc Chem Res 46:662–671.

Twarock S, Freudenberger T, Poscher E, et al. (2011). Inhibition of oesophageal squamous cell carcinoma progression by in vivo targeting of hyaluronan synthesis. Mol Cancer 10:30. doi:10.1186/1476-4598-10-30.

Urakawa H, Nishida Y, Wasa J, et al. (2012). Inhibition of hyaluronan synthesis in breast cancer cells by 4-methylumbelliferone suppresses tumorigenicity in vitro and metastatic lesions of bone in vivo. Int J Cancer 130:454–466.

Xu X, Wang M, Wang Y, et al. (2013). Green cocoons in silkworm Bombyx mori resulting from the quercetin 5-O-glucosyltransferase of UGT86, is an evolved response to dietary toxics. Mol Biol Rep 40: 3631–3639.

Zhang WH, Jiang MG. (2010). Synthesis and antifungal activities of umbelliferone analogs. Chinese J Org Chem 30:254–259.

Supplementary material available online.

Supplementary Table S1