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ORIGINAL ARTICLE

Antimetastatic, antineoplastic, and toxic effects of 4-hydroxycoumarin in a preclinical mouse melanoma model

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Abstract
Purpose We have previously reported that in vitro treatment of B16-F10 melanoma cells with 4-hydroxycoumarin (4-HC) decreases their metastatic potential. However, the antimetastatic efficacy of 4-HC in vivo is unknown; therefore, we investigated the antimetastatic and antineoplastic effects of 4-HC in a mouse melanoma model. Based on the findings, the immunomodulatory and toxic effects of 4-HC were also studied.
Methods Experimental metastasis assay was performed in C57BL/6 mice that received 4-HC before intravenous injection of B16-F10 cells. Antitumor and antimetastatic efficacy of 4-HC was assessed in mice implanted subcutaneously with melanoma cells. Possible immunostimulant and toxic effects of 4-HC were studied in healthy mice.
Results 4-HC reduced the number of experimental lung metastases. Moreover, 4-HC diminished primary tumor growth and increased survival time in mice bearing melanoma tumors. Treatments also decrease spontaneous lung metastases in the same animals. Different to other coumarins, the antitumor effect of 4-HC seems to be unrelated to immunostimulation, since plasma concentrations of cytokines remained unchanged. In contrast, toxic histological changes in nephrons and bronchiolar epithelium and a pronounced anticoagulant effect were found in 4-HC treated animals.
Conclusions These results show that 4-HC not only exhibit antimetastatic effect in vivo, but also effectively reduces tumor growth and improves survival, even when it produce toxic effects. Although the molecular mechanism of 4-HC actions needs to be further defined, our data suggest that 4-HC may lead to the development of agents that could be used as adjuvants in the therapy of melanoma.

Keywords 4-Hydroxycoumarin · Coumarin · Melanoma · Metastasis

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Introduction

Melanoma incidence has been rising worldwide for the last four decades, especially in white populations. In that period, incidence rates have increased more than threefold in US and central Europe [1]. Even when melanoma mortality rates have remained relatively stable due to primary prevention, screening, and improved therapies, it is estimated that there were still 8,400 deaths in US during 2008 [2]. The leading cause of death in advanced melanoma patients is formation of metastases. Regardless of the numerous innovations on the treatment of the disease, the 5-year survival rate for patients with distant metastases is less than 10% [3]. Hence, the search for therapeutic agents that can inhibit metastasis is crucial for improving the management of the disease.

The production of metastasis is a highly complex process by which some cancer cells move away from the primary tumor and colonize other organs. This process requires phenotypical changes that allow cancer cells to migrate, survive in the blood circulation, extravasate, and proliferate in a tissue with a different microenvironment [4]. Adhesion of tumor cells to extracellular matrix (ECM) or other cells is essential to successfully complete the metastatic cascade [5]; therefore, adhesion receptors and their intracellular partners have been proposed as pharmacological targets for decreasing invasiveness of cancer cells [6, 7].

4-Hydroxycoumarin (4-HC) is a simple coumarin used as precursor for the synthesis of anticoagulant drugs and rodenticides that are 3-substituted-4-hydroxycoumarins. Previously, we have provided evidence that in vitro treatment of B16-F10 melanoma cells with 4-HC decreases paxillin expression and reduces its translocation to focal adhesions [8]. Inappropriate paxillin-mediated signaling reduces the activation of FAK and Rac-1, impairing the adequate generation of signals that promote malignancy [8]. All these effects produce a disorganization of the actin cytoskeleton, reducing the formation of stress fibers and lamellipodia, decreasing cell adhesion to ECM, and inhibiting cell motility [9]. In addition, 4-HC downregulates the expression of the adhesion receptor ARM-1 and inhibits adhesion of B16-F10 cells to lung slides [8]. Finally, we have shown that 4-HC decreases the capability of B16-F10 melanoma cells to originate experimental lung metastases without affecting cell proliferation or survival [8].

In the present study we analyzed the effect of oral administration of 4-HC to C57BL/6 mice on the formation of experimental metastases. Additionally, in mice implanted subcutaneously with B16-F10 cells, we studied the effects of 4-HC on primary tumor growth, survival time, and the generation of spontaneous metastases. Finally, possible immunostimulant and toxic effects of 4-HC were studied in healthy mice.

Materials and methods

Drugs

4-Hydroxycoumarin (4-Hydroxy-2H-1-benzopyran-2-one), its vehicle methylcellulose, and cyclophosphamide were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male C57BL/6 mice, 6–7 weeks of age, were purchased from Harlan (Mexico City, Mexico). Animals were acclimated to the animal facility of the Department of Pharmacology, School of Medicine, UNAM, for a minimum of 1 week. Mice were separately housed in ventilated cages under a controlled light cycle (12 h light/12 h dark) at a standard room temperature (22–24°C) and were allowed access to a conventional diet and tap water ad libitum. Tumors were induced to mice by injecting B16-F10 cells (American Type Culture Collection, Manassas, VA, USA) cultured in DMEM containing 10% fetal bovine serum at 37°C in a humid, 5% CO_2 atmosphere. All the experiments with mice comply with standards equivalent to the UKCCCR guidelines for the welfare of animals in experimental neoplasia [10].

Experimental metastasis assay

4-HC (10, 20 or 40 mg/kg/day) was administered via oral gavage to C57BL/6 mice during 7 days. Control mice received equivalent volumes of 0.2% methylcellulose. On day 7, each mouse was injected into the tail vein with a suspension of 8×10^5 untreated B16-F10 cells. The cells were collected from culture by detaching with a non-enzymatic cell dissociation buffer [4 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS] and had viability ≥98%. Mice were euthanized 2 weeks after melanoma cells injection, the lungs were excised, and the pulmonary metastatic tumors were counted in a blind manner under a dissecting microscope.

Survival/spontaneous metastasis assay

Mice were injected subcutaneously with 2×10^5 B16-F10 cells in order to induce a primary tumor. After 2 weeks, only the mice that showed a tumor between 3 and 6 mm of diameter were selected for the experiment. Those mice were distributed in five groups: (i) control (methylcellulose 0.2%); (ii) 4-HC 10 mg/kg/day; (iii) 4-HC 20 mg/kg/day; (iv) 4-HC 40 mg/kg/day; and (v) cyclophosphamide 200 mg/kg (unique dose i.p.) as positive control. Tumor size was estimated by measuring the tumor's axes and employing the formula: (major axis) × (minor axis)^2 × 0.52. Additionally, we registered the day of death for each mouse and these data were used to generate Kaplan-Meier graphs. When mice died, their lungs were excised and fixed in 3% neutral buffered paraformaldehyde solution. Posteriorly, the number of pulmonary spontaneous metastases was quantified with a dissecting microscope.

Multiplex cytometric bead array (CBA) assay for cytokines

Cytokine quantification was performed in plasma of healthy mice that received 4-HC (10, 20, or 40 mg/kg/day) for 7 or 30 days. Plasma samples were collected from blood obtained from the infraorbital plexus and stored at −70°C until the analysis day. FlowCytomix Multiplex kit for

mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, and TNF-α was purchased from Bender MedSystems (Vienna, Austria). The immunoassay was used according to the manufacturer's instructions. Briefly, 25 µl plasma samples were incubated with antibody coupled beads and biotinylated detection antibody. Complexes were washed, and then incubated with streptavidin–phycoerythrin prior to assessing cytokine concentration titers. Standard curves for each cytokine were generated in a range of 27–6,666 pg/ml by using the reference cytokines supplied by the manufacturer. Standard and test samples were analyzed with a CyAn ADP Analyzer (Beckman Coulter) equipped with Summit v4.3 software (Dako Cytomation) and the analyte concentration was calculated using the software FlowCytomix Pro (Bender MedSystems).

Toxicological evaluation

The renal, hepatic, and pulmonary sub-chronic toxic effects of 4-HC were evaluated in healthy mice that were oral gavage dosed (10, 20, or 40 mg/kg/day) for 60 days. Control mice received equivalent volumes of the vehicle (0.2% methylcellulose).

Serum and urine analysis

Urine of 24 h was collected using stainless steel metabolic cages. In urine samples we quantified: (i) total volume; (ii) creatinine concentration by using a commercial kit (Creatinine Assay Kit, BioAssay Systems); (iii) total protein concentration through Lowry's method (Dc Protein Assay, Biorad); and (iv) *N*-acetyl-*p*-D glucosaminidase (NAG) activity by using a commercial kit (*β*-*N*-Acetylglucosaminidase Assay kit, Sigma-Aldrich). In addition, serum samples were obtained from whole blood collected from the infraorbital plexus, and stored at −70°C until creatinine, urea nitrogen (BUN), and γ-glutamyltransferase (γ-GT) were determined. BUN and γ-GT concentrations were measured by using commercial kits (Urea Assay kit, BioAssay Systems, and γ-GT kit, Spinreact).

Histological studies

At 30 and 60 days, three mice of each treatment were euthanized and their kidneys, livers and lungs were excised. For photonic microscopy, organs sections were fixed in 10% neutral buffered formaldehyde solution, dehydrated in graded alcohol, and embedded in paraffin. Sections at 4 µm of thickness were obtained and stained with hematoxylin–eosin (HE). Histological damage was identified by analyzing 50 randomly selected fields with an Olympus BH2-RFCA microscope. For electron microscopy, intracardiac perfusion of mice was carried-out with Zamboni's fixer (0.3% picric acid in 6.2% neutral buffered formaldehyde solution). Fixation of dissected organs (kidneys, livers, and lungs) was completed by immersion in the same fixative during 24 h at 4°C. Then, samples were postfixed in 1% osmium tetroxide diluted with phosphate buffer 0.1 M (pH 7.2) during 2 h and dehydrated in graded ethanol until absolute propylene oxide. Finally, samples were infiltrated and embedded in Araldite 6005. Sections at 1 µm thickness were stained with toluidine blue for histological analysis. Ultrafine sections of 60 nm thickness were obtained with a diamond knife, contrasted with uranyl acetate–lead citrate, and analyzed with a transmission electron microscope Zeiss EM-10.

Coagulation tests

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured in citrated plasma of healthy mice that received 4-HC (10 mg/kg/day) for 30 days. PT determination was performed using Dade® thromboplastin C plus according to Quick's method [11]. aPTT was evaluated by addition of Dade® actin FS reagent; the mixture was incubated during 120 s at 37°C, and then clot formation was induced with 0.02 M $CaCl_2$ [12]. In both methods plasma samples were warmed at 37°C for 60 s before the addition of the indicated reagent and the time to clot formation was recorded by a Behring Fibrintimer II (Dade® Behring). Reference curves and accuracy controls were set up using the corresponding control plasma of the manufacturer.

Results

Oral administration of 4-HC impaired the formation of experimental metastases

To study if the antimetastatic effect produced by in vitro treatment of melanoma cells with 4-HC [8] could be reproduced in vivo, 4-HC was orally administered to mice during 7 days prior to the intravenous injection of B16-F10 cells. The evaluated doses of 4-HC (10, 20, or 40 mg/kg/day) reduced >85% the number of pulmonary tumors with respect to mice that received the vehicle methylcellulose (Table 1). As these doses effectively block metastasis, they were used in posterior assays.

Antineoplastic effect of 4-HC

4-HC was daily administered via oral gavage to C57BL/6 mice bearing a subcutaneous tumor generated by injection of B16-F10 melanoma cells. As positive control a group of

Table 1 Effect of 4-HC on experimental lung metastases

Treatment	Mice examined	Metastatic incidence	Lung metastases/mice (mean ± SD)
Control	7	100% (7/7)	51.5 ± 20.9
4-HC 10 mg/kg/day	5	100% (5/5)	3.2 ± 2.2**
4-HC 20 mg/kg/day	5	80% (4/5)	5.0 ± 3.6**
4-HC 40 mg/kg/day	5	100% (5/5)	6.8 ± 4.1**

4-HC was administered by oral gavage for 7 days prior to the intravenous injection of B16-F10 cells

** $P < 0.01$ versus control (Dunett's test)

mice received a single dose of the antineoplasic drug cyclophosphamide. Cyclophosphamide reduced the tumor size from day 16; however, it did not alter survival time. In contrast, 10 mg/kg/day of 4-HC, which diminished the tumor size from day 22, significantly increased survival time. Surprisingly, higher doses (20 or 40 mg/kg/day) of 4-HC did not affect tumor size nor survival (Fig. 1). Moreover, survival curve of mice that received 40 mg/kg/day of 4-HC showed a slight non-significant shift to the left, suggesting dose-dependent toxic effects.

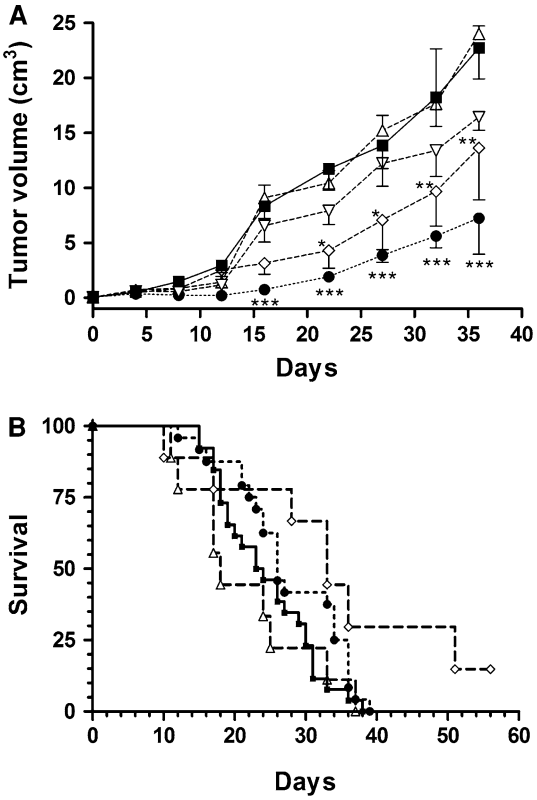

Fig. 1 Comparison of tumor growth and survival in mice bearing a subcutaneous tumor. **a** Mice were treated with either methylcellulose (*filled square*; $n = 25$), 4-HC 10 mg/kg/day (*open diamond*; $n = 10$), 4-HC 20 mg/kg/day (*inverted open triangle*; $n = 10$), 4-HC 40 mg/kg/day (*open triangle*; $n = 10$), or cyclophosphamide (*filled circle*; $n = 25$). Treatment of mice with 4-HC 10 mg/kg/day reduced tumor size from day 22, whereas the positive control cyclophosphamide did it from day 16 (*$P < 0.05$, ***$P < 0.001$ versus control; Bonferroni's test). Error bars represent standard deviation. **b** 4-HC 10 mg/kg/day (*open diamond*) increased mean survival time ($P = 0.02$; Mantel-Cox/log-rank test) with respect to control (*filled square*). In contrast, cyclophosphamide (*filled circle*) or the other evaluated doses of 4-HC [20 mg/kg/day (not graphed) and 40 mg/kg/day (*open triangle*)] did not modify survival time

4-HC reduced spontaneous metastases to lung

We analyzed the formation of secondary tumors in the same animals used for the survival assays. On the day of death, the lungs were excised and thereafter the macroscopic tumors were counted. Cyclophosphamide increased the number of secondary tumors in lung. In contrast, 20 or 40 mg/kg/day of 4-HC reduced spontaneous metastases number by 50% (Table 2). However, 10 mg/kg/day of 4-HC, which is effective reducing tumor size and increasing survival time, did not significantly decrease the number of lung tumors.

4-HC did not change plasma cytokine concentration

To analyze the possible immunomodulatory effect of 4-HC, we quantified the levels of 10 cytokines in plasma samples from mice that received 10, 20, or 40 mg/kg/day of 4-HC for 7 or 30 days. By multiplex CBA assay, we found no differences between 4-HC-treated and control mice in the plasma concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, or TNF-α (data not shown).

Toxic effects of 4-HC

Results from the survival assay suggest that 4-HC may have toxic effects in addition to their antineoplastic and antimetastatic effects. Thus, we evaluated the toxicity of 4-HC by administering 4-HC (10, 20, or 40 mg/kg/day) to healthy mice during 60 days.

Hepatic toxicity

The levels of serum γ-GT were not affected by 4-HC (Fig. 2a). Nevertheless, the histological study of liver showed that 20 or 40 mg/kg/day of 4-HC produced hepatic damage. The administration of 40 mg/kg/day induced cytoplasmic changes from day 30 in hepatocytes that are surrounding the portal vein (Fig. 2b). The same effect was found in mice that received 20 mg/kg/day for 60 days. In contrast, 10 mg/kg/day of 4-HC did not alter hepatic histology at the studied times. These results indicate that hepatotoxicity produced by 4-HC is dose and time-dependent.

Table 2 Effect of 4-HC on spontaneous lung metastases

Treatment	Mice examined	Metastatic incidence	Lung metastases/ mice (mean ± SD)
Control	25	100% (25/25)	11.9 ± 6.0
Cyclophosphamide	25	100% (25/25)	26.0 ± 20.8**
4-HC 10 mg/kg/day	10	90% (9/10)	7.0 ± 1.7
4-HC 20 mg/kg/day	10	100% (10/10)	5.5 ± 1.5 *
4-HC 40 mg/kg/day	10	90% (9/10)	5.8 ± 4.8 *

4-HC was orally administered to mice bearing subcutaneous tumors

* $P < 0.05$, ** $P < 0.01$ versus control (Dunett's test)

Renal toxicity

Creatinine clearance rate (CCR) was unaffected by treatments with 4-HC and remained within the normal range (1.14–17.8 µl/min/g [13]) all along the experiment (Fig. 3a). Additionally, 4-HC did not modify urinary concentrations neither of the tubular enzyme NAG nor protein (data not shown). These data indicated that 4-HC was not toxic to tubular cells and glomerular function was unaffected. However, 4-HC increased BUN levels above the normal limit (7–30 mg/dl [14]). The doses of 20 and 40 mg/kg/day raised BUN levels from third week, while 10 mg/kg/day do it from fourth week (Fig. 3b). Histological analysis of kidneys showed that 4-HC produced, in a dose-dependent fashion: (i) metaplasia of the parietal layer of the capsule of Bowman, and (ii) lost of microvilli in cuboidal epithelial cells of proximal convoluted tubule (Fig. 3c).

Pulmonary toxicity

Histological analysis was also performed in lungs; we observed that at all the evaluated doses 4-HC induced hyperplasia and lost in apical projections of Clara cells with leakage of its apical dome-shaped region, from day 30 (Fig. 4).

Electron transmission microscopy

Morphological alterations in kidneys and lungs were further studied in mice that received 10 mg/kg/day of 4-HC during 60 days, since only this dose showed therapeutic effects. We found that the cells from the parietal layer of the capsule of Bowman displayed cubic metaplasia with microvilli and corroborated the lost of microvilli in proximal convoluted tubular cells (Fig. 5). However, the endocytic system (apical tubulovesicles) of these cells showed no injury, which partially explains why morphological alterations are not reflected in functional damage. In lung, effects of 4-HC on Clara cells were confirmed. Additionally, lamellar bodies of most type II alveolar cells were partially empty or with vacuolization (Fig. 5).

Anticoagulant effect of 4-HC

PT and aPTT were evaluated in citrated plasma from healthy mice that had received 4-HC (10 mg/kg/day) for 30 days (Table 3). 4-HC increased aPTT and PT 2-fold and >15-fold, respectively, proving that 4-HC has anticoagulant effect. To study the reversibility of this effect, we analyzed PT in plasma obtained 48 h after the last administration of 4-HC (day 32). In those animals, the effect of 4-HC was totally lost.

Discussion

Previously, our group demonstrated that in vitro treatment of B16-F10 melanoma cells with 4-HC decreases their metastatic capability [8]. In the present study, in vivo intake of 4-HC is also effective in reducing experimental lung metastases at the evaluated doses. Since the pharmacokinetics of 4-HC is unknown, such doses were chosen by reviewing previous reports and by performing preliminary studies in our laboratory. 4-HC has been administered to rats at doses from 5 to 50 mg/kg/day [15] but there are not reported

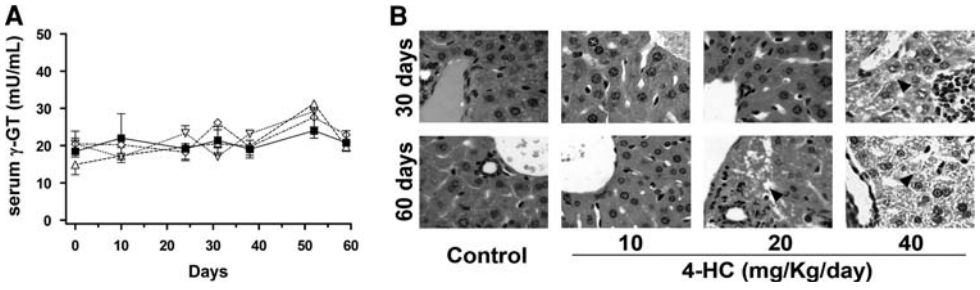

Fig. 2 Analysis of hepatic toxicity. **a** Quantification of γ-glutamyltransferase (γ-GT) in healthy mice that received either methylcellulose (*filled square*), 4-HC 10 mg/kg/day (*open diamond*), 4-HC 20 mg/kg/day (*inverted open triangle*), or 4-HC 40 mg/kg/day (*open triangle*). 4-HC treatments did not affect serum γ-GT levels. **b** HE-stained sections of livers (1000×). 4-HC produced a dose and time-dependent cytotoxicity (*arrowheads*) in hepatocytes close to the portal vein

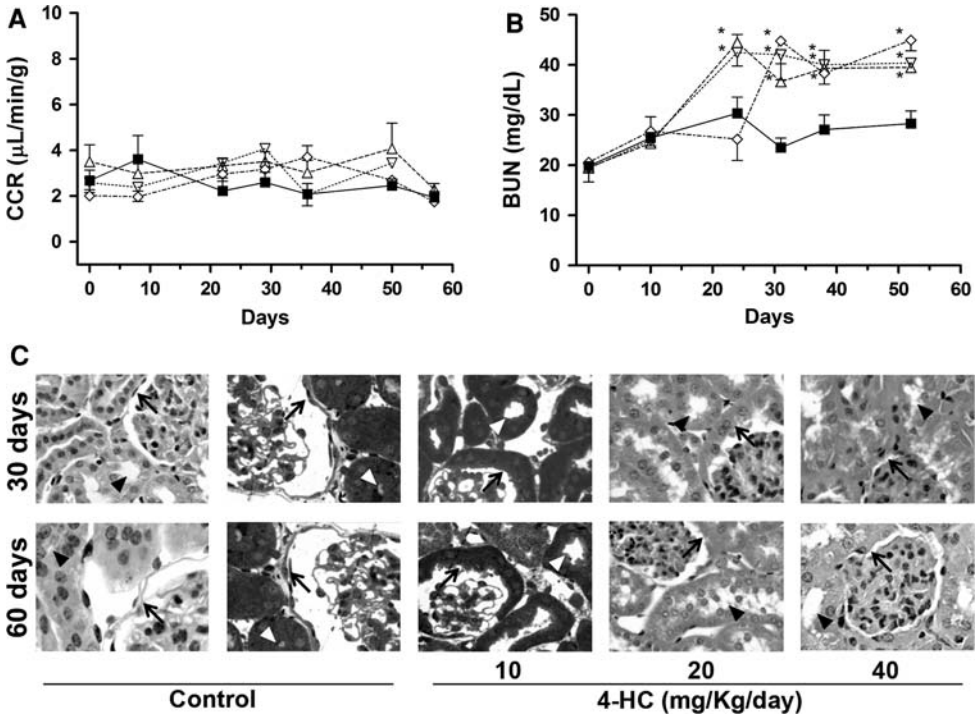

Fig. 3 Analysis of renal toxicity. Quantification of creatinine clearance rate (CCR) (**a**) and blood urea nitrogen (BUN) (**b**) in healthy mice that received either methylcellulose (*filled square*), 4-HC 10 mg/kg/day (*open diamond*), 4-HC 20 mg/kg/day (*inverted open triangle*), or 4-HC 40 mg/kg/day (*open triangle*). 4-HC did not altered CCR but increased BUN at all doses (*$P < 0.05$ versus control; Bonferroni's test). **c** Micrographs (1000×) of kidney sections stained with HE (first, fourth, and fifth columns) or toluidine blue (second and third columns). 4-HC produced metaplasia of the parietal layer of the capsule of Bowman (*arrows*) and lost of microvilli in proximal convoluted tubular cells (*arrowheads*), from day 30

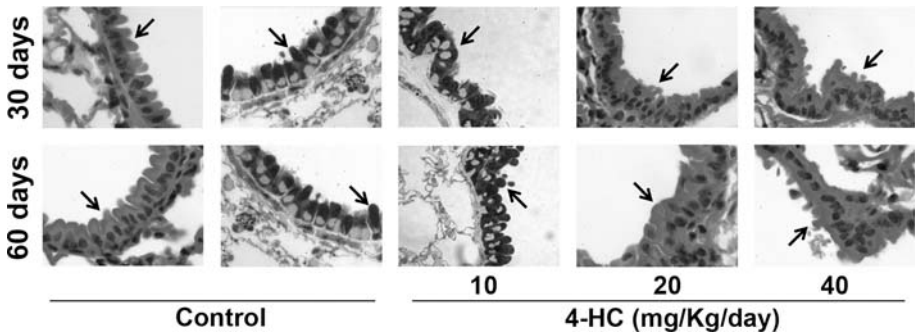

Fig. 4 Analysis of pulmonary toxicity. Micrographs (1000×) of lung sections stained with HE (first, fourth, and fifth columns) or toluidine blue (second and third columns). Administration of 4-HC caused hyperplasia of Clara cells (*arrows*) in the distal bronchiolar epithelium of healthy mice

studies in mice. In contrast, the closely structural related compound coumarin (1,2-benzopyrone) has been administered to several mice strains at doses ranging from 10 to 200 mg/kg/day [16–19]. Given that a unique dose of 50 mg/kg of ^{14}C-coumarin produces a plasmatic C_{max} of 202 µM [20] we hypothesized that if pharmacokinetics of 4-HC were similar to that of coumarin, doses from 10 to 200 mg/kg would produce plasmatic concentrations close to those needed for the in vitro reported effects of 4-HC (50–500 µM) [8, 9]. Nevertheless, in our preliminary studies mice that received doses higher than 80 mg/kg/day showed obvious toxicity; consequently, we set our higher dose at 40 mg/kg. Since all the doses employed in this study can efficiently reduce experimental metastases, concentrations of 4-HC in corporal fluids appear to be high enough to alter the metastatic capability of B16-F10 cells. Moreover, these findings indicate that 4-HC is orally active. However, further studies are needed to clarify the pharmacokinetics of 4-HC.

The precise mechanisms involved in the antimetastatic effect of 4-HC are not yet elucidated. Previous reports show that in vitro treatment of B16-F10 melanoma cells with 4-HC for 24 h disorganizes the actin cytoskeleton, reduces cell-substratum and cell-cell adhesion, and diminishes the generation of signals that promote migration, in a concentration-dependant fashion [8, 9]. In vitro treatment with 4-HC also decreases the metastatic capability of B16-F10 cells [8]. In contrast, 4-HC is neither cytostatic nor cytotoxic, and does not affect long-term survival of B16-F10 cells [8, 9, 21]. We demonstrated that prophylactic administration of 4-HC (prior to the intravenous injection of melanoma cells) reduces metastases number, which indicates that 4-HC affects early steps of experimental metastasis rather than metastatic growth. Strong adhesion to endothelial cells and transmigration are the first steps of the formation of experimental metastases and both require functional adhesion receptors as well as crosstalk of those receptors with the cytoskeleton [6, 7]. Therefore, the reported effects

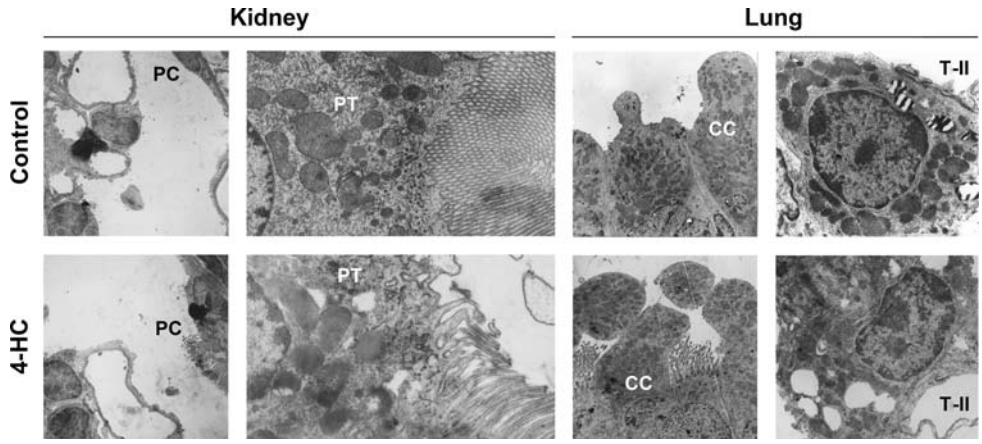

Fig. 5 Electron transmission microscopy of kidneys and lungs of mice treated 60 days with 4-HC (10 mg/kg/day). In the far left micrographs (2,500×), that display the glomerular urinary space, it is shown that 4-HC induced cubic metaplasia with microvilli on the parietal cells (PC) of the capsule of Bowman. Micrographs in the second column (8,000×) correspond to proximal convoluted tubular cells (PT); in 4-HC-treated mice microvilli decreased notoriously. In the bronchiolar epithelium (third column micrographs; 4,000×) 4-HC produced hyperplasia of Clara cells (CC). Micrographs in the far right (14,000×) show that 4-HC caused vacuolization in type II alveolar cells (T-II)

Table 3 Effect of 4-HC on activated partial thromblopastin time (aPTT) and prothrombin time (PT)

Treatment	*n*	Test (mean ± SD)	
		aPTT	PT
Control	4	62.8 ± 3.2 s	13.3 ± 1.0 s
4-HC 10 mg/kg/day	4	117.7 ± 21.2 s**	>200 s**
Control 48 h PA	2	n.d.	11.2 ± 0.7 s
4-HC 48 h PA	2	n.d.	11.8 ± 0.5 s

Measurements were performed on day 30 of 4-HC administration (10 mg/kg/day) or 48 h later (post-administration; PA)

** $P < 0.01$ versus control (unpaired *t* test)

of 4-HC on cell adhesion and signaling may be partially responsible of the antimetastatic effect shown here. In addition, recent studies have shown that integrin-mediated adhesion and the associated changes in the actin cytoskeleton are required to promote a transition from a quiescent to a proliferative state in vivo [22]; thus, the possibility that 4-HC can affect dormancy should not be discarded.

The antimetastatic effect of 4-HC was corroborated in a different metastasis model in which we treated mice implanted with subcutaneous tumors. 4-HC (20 or 40 mg/kg/day) reduce the spontaneous metastases to lung without affecting neither tumor growth nor survival time. In contrast, 10 mg/kg/day of 4-HC increases by 40% the median survival time, but does not decrease the number of metastases. Unexpectedly, this dose also reduces primary tumor growth, indicating that 4-HC has antineoplastic effect when it is administered for longer periods. This antineoplastic effect has been reported for other simple coumarins. Coumarin has shown antineoplastic effect in clinical protocols of renal [23] and prostatic carcinoma [24]. Similarly, coumarin and 7-hydroxycoumarin (7-HC) inhibit tumor growth of sarcoma-bearing mice [17] and both compounds inhibit the proliferation of several tumor cell lines in vitro [21, 25–28] by inducing cell cycle arrest and apoptosis [27, 28]. Our results, in which higher doses of 4-HC did not show antineoplastic effect, and the lack of cytostatic and cytotoxic effects in vitro [8, 9, 21] suggest that, differently to other coumarins, 4-HC does not elicit its antineoplastic effect by direct cytotoxicity.

On the other hand, it has been reported that coumarin [29, 30] and 7-HC [16] stimulate phagocytic activity of macrophages. Both compounds have synergic effect in vitro in the secretion of nitric oxide and IL-12 induced by LPS [17] as well as in the secretion of TNFα induced by heat-killed *Salmonella thyphimurium* [16]. In a mouse model of sarcoma, these immunomodulatory effects contribute to reduce tumor growth and increase survival time [17]. Therefore, we hypothesized that stimulation of immune system by 4-HC can be partially responsible of its antineoplastic effect. However, plasma concentrations of 10 cytokines were no different between 4-HC-treated and control mice, indicating that in the studied conditions 4-HC has not immunomodulatory effect and that other possible mechanisms by which 4-HC reduces tumor growth in vivo shall be analyzed. For example, other non-tumoral cell targets for 4-HC may include fibroblasts and endothelial cells; these cells, together with immune cells, soluble molecules, and the ECM, are present in the tumor microenvironment, which has been shown to be crucial in melanoma progression [31]. Currently there are no reports of the in vivo effects of simple coumarins in the tumor microenvironment.

Even when 4-HC showed antimetastatic and antineoplastic effects, the survival assay suggested a dose-dependent toxic effect. Analysis of the effects of 4-HC in mice that received 4-HC during 60 days, demonstrated the presence of hepatic centrolobular necrosis in a dose- and time-dependent fashion. Nevertheless, such effect was not sufficient to modify the levels of serum γ-GT, indicating that the toxic effect in liver is not transcendent. Accordingly, Ratanasavanh et. al. [32] have reported that 4-HC (500 μM) is not toxic to cultured hepatocytes. In contrast, coumarin produces hepatic hyperplasia and centrolobular necrosis in B6C3F1 mice [33, 34]. Hepatotoxic effects of coumarin are much more frequent in rats, where a single administration (200 mg/kg) produces hepatic centrolobular necrosis and increases the serum transaminase levels [34, 35]. This increased susceptibly in rats is associated with the biotransformation of coumarin to 3-hydroxycoumarin by CYP2A4; in turn, 3-hydroxycoumarin favors the generation of coumarin 3,4-epoxide, a highly reactive intermediate that produces toxicity by itself and by its conversion to *o*-hydroxyphenylacetaldehyde [36–39]. Contrary to rodents, coumarin-induced hepatotoxicity is rare in humans [40, 41] because the main biotransformation route is 7-hydroxylation, a reaction catalyzed by CYP2A6 [42]. Our results indicate that 4-HC can be biotransformed into a 3,4-epoxide in the liver of C67BL/6 mice. Importantly, 10 mg/kg/day of 4-HC show therapeutic effects without causing hepatic toxicity, suggesting that at low doses the generated reactive metabolites can be efficiently controlled by hepatic detoxification systems. Accordingly, Born et. al. [43] have reported that tolerance to coumarin is achieved by increasing detoxification, specially by conjugation with glutathione, rather than changes in 3,4-epoxide synthesis. This suggests that simultaneous stimulation of detoxification systems could inhibit some toxic effects of 4-HC, increasing its safety, and providing better models to study the mechanisms of its antimetastatic and antineoplastic effects.

Histological analysis demonstrated the presence of changes in lungs and kidneys of 4-HC-treated mice. 4-HC induced hyperplasia and lost in apical projections of Clara cells and vacuolization in type II alveolar cells. There are no previous reports of the effect of 4-HC on pulmonary cells, but several studies have described that coumarin produce necrosis in Clara cells and increases the incidence of lung adenomas and carcinomas in mice but not in rats [39, 44, 45]. Interestingly, the enzyme CYP2F2, which also generate 3,4-epoxide from coumarin [46], is expressed at higher levels in Clara cells from mice [39] and 3,4-epoxide formation occurs faster in microsomes from lungs of mouse than in those of rat [20]. These data indicate that the metabolic activity of lung, rather than the substrate concentration, is responsible of the lung toxicity caused by simple coumarins. Since CYP2F1 (orthologous to CYP2F2) is expressed at very small amounts in human lungs, it is unlikely that coumarins cause pulmonary toxic effects in humans [19], even when our results show that 4-HC toxic metabolites can be generated in lungs of C57BL/6 mice. Given the large interspecies differences in coumarins metabolism, further pharmacokinetical and toxicological studies, especially in non-rodent animals, are required to clarify the susceptibility to 4-HC-mediated toxicity.

In kidney, 4-HC induced cubic metaplasia in cells from the parietal layer of the capsule of Bowman and lost of microvilli in proximal convoluted tubular cells. The enzyme CYP2F2, which mediated coumarin toxicity in Clara cells, is expressed at low levels in mouse kidney [47–49]. In addition, 7-hydroxylation is also present in this organ [50], suggesting that other CYP isoforms can be participating in the renal metabolism of 4-HC. Thus, it is likely that the observed effects were caused by the mixture of locally generated metabolites. Importantly, morphological changes in proximal tubular cells are not related to cell death, since there are no changes in urinary levels of NAG. In agreement, 4-HC (500 μM) is not toxic to proximal tubular LLC-PK1 cells in vitro (unpublished results). Additionally, ultrastructural studies and determinations of CCR and urinary protein indicate that glomerule and the endocytic system of the proximal convoluted tubular cells are not seriously affected. The only functional abnormality we found in 4-HC-treated mice was an elevated BUN. Such BUN levels combined with no changes in CCR can be caused by an excessive formation of urea, as seen in cases of bleeding in the upper gastrointestinal tract. Increased risk of bleeding is clinically associated with intake of vitamin K antagonists [51], which are 3-substituted-4-hydroxycoumarins. Although it has been reported that 4-HC lacks anticoagulant activity [52], our results show that the employed administration schedules yield a pronounced anticoagulant effect, raising the risk of bleeding. Another possible explanation of elevated BUN is 4-HC accumulation in kidney, which may affect local osmolarity or organic ion transport. Accordingly, administration of ^{14}C-coumarin to B6C3F1 mice produces high concentrations of radioactivity in kidney, liver, contents of stomach, and small intestine [20].

The generation of toxic metabolites from 4-HC involves the participation of several CYP isoforms that differ in their localization, nature of metabolites formed, and rate of metabolism. Given its complex biotransformation, the possibility that 4-HC generates metabolites involved in the reported therapeutic effects exists. Coumarin has an accelerated metabolism that generates different compounds with biological activities [53], which has led to the proposal that coumarin acts as a prodrug [54]. The lack of anticoagulant effect 48 h after a long-term administration of 4-HC suggests that this compound is rapidly eliminated. The identification of the metabolic pathways activated by 4-HC would

therefore facilitate the elucidation of the mechanisms of its toxic and therapeutic effects.

Conclusions

This study shows that 4-HC inhibits the formation of melanoma metastasis when is orally administered. In addition, we found that 4-HC reduces primary tumor growth and increases survival time, even when it displays anticoagulant effect and its local biotransformation produce toxic effects in liver, lung, and kidney. The narrow therapeutic index of 4-HC limits its possible use as adjuvant in the therapy of melanoma; however, the identification of the molecular mechanisms of the antimetastatic and antineoplastic actions of 4-HC may lead to the development of structurally and/or mechanistically related agents useful in the prevention of metastasis.

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Conflict of interest statement None.

References

1. Garbe C, Leiter U (2009) Melanoma epidemiology and trends. Clin Dermatol 27:3–9
2. Ries LAG, Melbert D, Krapcho M et al (2008) SEER cancer statistics review, 1975–2005. National Cancer Institute, Bethesda, MD
3. Balch CM, Soong SJ, Atkins MB et al (2004) An evidence-based staging system for cutaneous melanoma. CA Cancer J Clin 54:131–149 quiz 182–134
4. Bogenrieder T, Herlyn M (2003) Axis of evil: molecular mechanisms of cancer metastasis. Oncogene 22:6524–6536
5. Hood JD, Cheresh DA (2002) Role of integrins in cell invasion and migration. Nat Rev Cancer 2:91–100
6. Hehlgans S, Haase M, Cordes N (2007) Signalling via integrins: implications for cell survival and anticancer strategies. Biochim Biophys Acta 1775:163–180
7. Velasco-Velazquez MA, Molina-Guarneros JA, Mendoza-Patino N et al (1999) Integrins and integrin-associated molecules: targets for the development of antimetastatic therapies. Rev Invest Clin 51:183–193
8. Velasco-Velazquez MA, Salinas-Jazmin N, Mendoza-Patino N, Mandoki JJ (2008) Reduced paxillin expression contributes to the antimetastatic effect of 4-hydroxycoumarin on B16–F10 melanoma cells. Cancer Cell Int 8:8
9. Velasco-Velazquez MA, Agramonte-Hevia J, Barrera D et al (2003) 4-Hydroxycoumarin disorganizes the actin cytoskeleton in B16–F10 melanoma cells but not in B82 fibroblasts, decreasing their adhesion to extracellular matrix proteins and motility. Cancer Lett 198:179–186
10. (1988) UKCCCR guidelines for the welfare of animals in experimental neoplasia. Br J Cancer 58:109–113
11. Quick AJ (1974) Letter: normal values for coagulation tests. N Engl J Med 290:751
12. Proctor RR, Rapaport SI (1961) The partial thromboplastin time with kaolin. A simple screening test for first stage plasma clotting factor deficiencies. Am J Clin Pathol 36:212–219
13. Qi Z, Whitt I, Mehta A et al (2004) Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. Am J Physiol Renal Physiol 286:F590–F596
14. Mohr U, Dungworth DL, Capen CC et al (1996) Pathobiology of the aging mouse. ILSI Press, Washington, DC
15. Luchini AC, Rodrigues-Orsi P, Cestari SH et al (2008) Intestinal anti-inflammatory activity of coumarin and 4-hydroxycoumarin in the trinitrobenzenesulphonic acid model of rat colitis. Biol Pharm Bull 31:1343–1350
16. Stefanova T, Nikolova N, Michailova A et al (2007) Enhanced resistance to *Salmonella enterica* serovar Typhimurium infection in mice after coumarin treatment. Microbes Infect 9:7–14
17. Stefanova TH, Nikolova NJ, Toshkova RA, Neychev HO (2007) Antitumor and immunomodulatory effect of coumarin and 7-hydroxycoumarin against Sarcoma 180 in mice. J Exp Ther Oncol 6:107–115
18. Orozco Arreaga A (2000) Estudio in vivo del efecto antineoplasico de la cumarina en un modelo de melanoma murino. Dissertation, Universidad Nacional Autónoma de México
19. Cruzan G, Bus J, Banton M et al (2009) Mouse specific lung tumors from CYP2F2-mediated cytotoxic metabolism: an endpoint/toxic response where data from multiple chemicals converge to support a mode of action. Regul Toxicol Pharmacol. doi:10.1016/j.yrtph.2009.07.002
20. Born SL, Api AM, Ford RA et al (2003) Comparative metabolism and kinetics of coumarin in mice and rats. Food Chem Toxicol 41:247–258
21. Kawaii S, Tomono Y, Ogawa K et al (2001) The antiproliferative effect of coumarins on several cancer cell lines. Anticancer Res 21:917–923
22. Barkan D, Kleinman H, Simmons JL et al (2008) Inhibition of metastatic outgrowth from single dormant tumor cells by targeting the cytoskeleton. Cancer Res 68:6241–6250
23. Dexeus FH, Logothetis CJ, Sella A et al (1990) Phase II study of coumarin and cimetidine in patients with metastatic renal cell carcinoma. J Clin Oncol 8:325–329
24. Mohler JL, Williams BT, Thompson IM, Marshall ME (1994) Coumarin (1, 2-benzopyrone) for the treatment of prostatic carcinoma. J Cancer Res Clin Oncol 120(Suppl):S35–S38
25. Marshall ME, Kervin K, Benefield C et al (1994) Growth-inhibitory effects of coumarin (1,2-benzopyrone) and 7-hydroxycoumarin on human malignant cell lines in vitro. J Cancer Res Clin Oncol 120(Suppl):S3–S10
26. Jimenez-Orozco FA, Molina-Guarneros JA, Mendoza-Patino N et al (1999) Cytostatic activity of coumarin metabolites and derivatives in the B16–F10 murine melanoma cell line. Melanoma Res 9:243–247
27. Lopez-Gonzalez JS, Prado-Garcia H, Aguilar-Cazares D et al (2004) Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines. Lung Cancer 43:275–283
28. Jimenez-Orozco FA, Lopez-Gonzalez JS, Nieto-Rodriguez A et al (2001) Decrease of cyclin D1 in the human lung adenocarcinoma cell line A-427 by 7-hydroxycoumarin. Lung Cancer 34:185–194
29. Piller NB (1978) A morphological assessment of the stimulatory effect of coumarin on macrophages. Br J Exp Pathol 59:93–96
30. Hardt TJ, Ritschel WA (1983) The effect of coumarin and 7-hydroxycoumarin on in vitro macrophage phagocytosis of latex particles. Methods Find Exp Clin Pharmacol 5:39–43
31. Villanueva J, Herlyn M (2008) Melanoma and the tumor microenvironment. Curr Oncol Rep 10:439–446

32. Ratanasavanh D, Lamiable D, Biour M et al (1996) Metabolism and toxicity of coumarin on cultured human, rat, mouse and rabbit hepatocytes. Fundam Clin Pharmacol 10:504–510
33. NTP (National Toxicology Program) (1993) NTP toxicology and carcinogenesis studies of coumarin (CAS No. 91–64-5) in F344/N rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep Ser 422:1–340
34. Cohen AJ (1979) Critical review of the toxicology of coumarin with special reference to interspecies differences in metabolism and hepatotoxic response and their significance to man. Food Cosmet Toxicol 17:277–289
35. Lake BG (1984) Investigations into the mechanism of coumarin-induced hepatotoxicity in the rat. Arch Toxicol Suppl 7:16–29
36. Vassallo JD, Hicks SM, Daston GP, Lehman-McKeeman LD (2004) Metabolic detoxification determines species differences in coumarin-induced hepatotoxicity. Toxicol Sci 80:249–257
37. Lake BG, Evans JG, Lewis DF, Price RJ (1994) Comparison of the hepatic effects of coumarin, 3,4-dimethylcoumarin, dihydrocoumarin and 6-methylcoumarin in the rat. Food Chem Toxicol 32:743–751
38. Lake BG, Gray TJ, Evans JG et al (1989) Studies on the mechanism of coumarin-induced toxicity in rat hepatocytes: comparison with dihydrocoumarin and other coumarin metabolites. Toxicol Appl Pharmacol 97:311–323
39. Vassallo JD, Hicks SM, Born SL, Daston GP (2004) Roles for epoxidation and detoxification of coumarin in determining species differences in clara cell toxicity. Toxicol Sci 82:26–33
40. Cox D, O'Kennedy R, Thornes RD (1989) The rarity of liver toxicity in patients treated with coumarin (1,2-benzopyrone). Hum Toxicol 8:501–506
41. Casley-Smith JR (1995) Frequency of coumarin hepatotoxicity. Med J Aust 162:391
42. Pelkonen O, Raunio H, Rautio A, Pasanen M (1997) The metabolism of coumarin. In: O'Kennedy R, Thornes RD (eds) Coumarins biology, applications and mode of action. Wiley, New York
43. Born SL, Fix AS, Caudill D, Lehman-McKeeman LD (1999) Development of tolerance to Clara cell necrosis with repeat administration of coumarin. Toxicol Sci 51:300–309
44. Born SL, Fix AS, Caudill D, Lehman-McKeeman LD (1998) Selective Clara cell injury in mouse lung following acute administration of coumarin. Toxicol Appl Pharmacol 151:45–56
45. Zhuo X, Gu J, Zhang QY et al (1999) Biotransformation of coumarin by rodent and human cytochromes P-450: metabolic basis of tissue-selective toxicity in olfactory mucosa of rats and mice. J Pharmacol Exp Ther 288:463–471
46. Born SL, Caudill D, Fliter KL, Purdon MP (2002) Identification of the cytochromes P450 that catalyze coumarin 3,4-epoxidation and 3-hydroxylation. Drug Metab Dispos 30:483–487
47. Nhamburo PT, Kimura S, McBride OW et al (1990) The human CYP2F gene subfamily: identification of a cDNA encoding a new cytochrome P450, cDNA-directed expression, and chromosome mapping. Biochemistry 29:5491–5499
48. Baldwin RM, Jewell WT, Fanucchi MV et al (2004) Comparison of pulmonary/nasal CYP2F expression levels in rodents and rhesus macaque. J Pharmacol Exp Ther 309:127–136
49. Ye X, Lu L, Gill SS (1997) Suppression of cytochrome P450 Cyp2f2 mRNA levels in mice by the peroxisome proliferator diethylhexylphthalate. Biochem Biophys Res Commun 239:660–665
50. Su T, He W, Gu J et al (1998) Differential xenobiotic induction of CYP2A5 in mouse liver, kidney, lung, and olfactory mucosa. Drug Metab Dispos 26:822–824
51. Dahri K, Loewen P (2007) The risk of bleeding with warfarin: a systematic review and performance analysis of clinical prediction rules. Thromb Haemost 98:980–987
52. Mueller RL, Scheidt S (1994) History of drugs for thrombotic disease. Discovery, development, and directions for the future. Circulation 89:432–449
53. von Weymarn LB, Murphy SE (2003) CYP2A13-catalysed coumarin metabolism: comparison with CYP2A5 and CYP2A6. Xenobiotica 33:73–81
54. Egan D, O'Kennedy R, Moran E et al (1990) The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds. Drug Metab Rev 22:503–529