Herbacetin Is a Novel Allosteric Inhibitor of Ornithine Decarboxylase with Antitumor Activity

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Abstract

Ornithine decarboxylase (ODC) is a rate-limiting enzyme in the first step of polyamine biosynthesis that is associated with cell growth and tumor formation. Existing catalytic inhibitors of ODC have lacked efficacy in clinical testing or displayed unacceptable toxicity. In this study, we report the identification of an effective and nontoxic allosteric inhibitor of ODC. Using computer docking simulation and an in vitro ODC enzyme assay, we identified herbacetin, a natural compound found in flax and other plants, as a novel ODC inhibitor. Mechanistic investigations defined aspartate 44 in ODC as critical for binding. Herbacetin exhibited potent anticancer activity in colon cancer cell lines expressing high levels of ODC. Intrapерitoneal or oral administration of herbacetin effectively suppressed HCT116 xenograft tumor growth and also reduced the number and size of polyps in a mouse model of APC-driven colon cancer (ApcMin/−). Unlike the well-established ODC inhibitor DFMO, herbacetin treatment was not associated with hearing loss. Taken together, our findings define the natural product herbacetin as an allosteric inhibitor of ODC with chemopreventive and antitumor activity in preclinical models of colon cancer, prompting its further investigation in clinical trials. Cancer Res; 76(5); 1–12. ©2015 AACR.

Introduction

Polyamines play important roles in normal and cancer cell growth (1), proliferation (2), gene expression (3), and signal transduction from the cell membrane to the nucleus by activating mitogen activated protein (MAP) kinases, including Ras, MEKs, and ERKs (4–6). Ornithine decarboxylase (ODC) is a first rate-limiting enzyme in the polyamine biosynthesis pathway in mammals and it is highly expressed in the intestinal mucosa of individuals with familial adenomatous polyposis (FAP), a disease characterized by overexpression of c-myc caused by a deletion mutant adenomatous polyposis coli (APC) gene (7). Ras activation-mediated cell transformation induces ODC transcription, translation, and polyamine accumulation (8, 9). In addition, polyamines activate the phosphorylation of ERKs and induce the expression of oncogenes such as myc, jun, and fos (6, 10). Additionally, elevated ODC activity is observed in neoplastic tissues and is highly correlated with tumor growth (11). Furthermore, Myc-induced lymphomagenesis is suppressed by targeting ODC, suggesting that this enzyme is a potential target for cancer prevention or treatment (12).

Previous reports indicated that polyamine inhibitors, including the ODC inhibitor, S-adenosylmethionine decarboxylase (AMD), and N1, N13 or \(-\) N14-dihydrostpermine (DENSPM or DEHSPM; polyamine analogues), have been identified (13–16). However, in clinical trials, all of these polyamine inhibitors failed to be effective in treating various cancers (17–19). Difluoromethylornithine (DFMO), an FDA-approved drug, binds to the active site of ODC and acts as an irreversible and specific ODC inhibitor. DFMO significantly inhibited proliferation of adenocarcinoma, squamous, and leukemia cells (20) as well as cancers in numerous transgenic animal models (4, 21, 22). DFMO has been evaluated as a prevention agent against several cancers, including bladder, cervical, colorectal, breast, prostate, and nonmelanoma skin cancers (11). Intracellular putrescine and spermidine levels were strongly reduced by DFMO; however, in contrast, DFMO can promote the uptake rate of putrescine and spermidine (23). Thus, the initial colon cancer prevention trials with DFMO alone showed a dose-limiting cytotoxicity (24). Recently, a combination of low doses of DFMO and non-steroidal anti-inflammatory drugs (NSAID) has been studied and shown to have a considerable inhibitory effect on colon cancer (25, 26). Herbacetin is a novel flavonol compound found in natural sources such as herb of ramose scouring rush, flaxseed, and Roemeria hybrid (27).
Herbacetin is structurally close to quercetin and kaempferol and exerts various pharmacological activities, including antioxidant, anti-inflammatory, and anticancer effects (28). Previous studies have shown that herbacetin possesses a strong antioxidant capacity and can also induce oxygen species-mediated apoptosis in hepG2 liver cancer cells (29, 30). Additionally, phosphorylation of c-Met and AKT is strongly inhibited by herbacetin (31). However, this activity is not sufficient to explain herbacetin’s biologic activities. The aim of the present study was to identify a novel ODC inhibitor and to investigate the efficacy of the newly discovered ODC inhibitor, herbacetin, in the prevention or treatment of small bowel and colon tumors.

**Materials and Methods**

**ODC enzyme assay**

ODC activity was measured as the release of CO₂ from L-[1-C¹⁴] ornithine as previously described (32).

**Pulldown assay using CNBr–herbacetin–conjugated beads**

A recombinant human ODC protein (200 ng) or total cell lysates (500 μg) were incubated with herbacetin-Sepharose 4B (or Sepharose 4B only as a control) beads (50 μL, 50% slurry). The pulldown assay was performed as described previously (33).

**Measurement of polyamine content**

Intracellular polyamines were extracted with 0.6 N perchloric acid from herbacetin- or DFMO-treated cell pellets or mouse tissues, then dansylated or benzoylated, and content was measured by reverse-phase high-pressure liquid chromatography (HPLC) as described previously (34, 35). Polyamines were detected using a fluorescence detector with an excitation wavelength of 360 nm and an emission cutoff filter of 500 nm and analyzed using chromatography software.

**Computer docking and modeling**

The structure of ODC (PDB code 1NJJ) used as the receptor model in our docking program was an X-ray diffraction structure with a resolution of 2.45 Å. Before docking, the ODC protein was prepared for docking following the standard procedure outlined in the Protein Preparation Wizard (36) included in the Schrödinger Suite 2010 (37, 38). The binding pocket was selected by the G418 ligand, which was already bound to the crystal structure chosen. The Traditional Chinese Medicine Database, which contains more than 7,500 compound constituents from 352 different herbs, animal products and minerals, was chosen as the ligand database for docking against the ODC protein structure using the Schrödinger docking program Glide (39). One hundred compounds were chosen based on the docking score obtained by high-throughput virtual screening. This group was narrowed to 10 compounds based on standard precision and extra precision flexible docking.

**In vivo studies using the APC<sup>Min<sup>−/−</sup> mouse model**

Male C57BL/6/J<sup>Min<sup>−/−</sup></sup> mice were obtained from The Jackson Laboratory and maintained under “specific pathogen-free” conditions according to the guidelines established by the University of Minnesota Institutional Animal Care and Use Committee. APC<sup>Min<sup>−/−</sup></sup> male mice were bred with C57BL/6/J APC wild-type female mice. The progeny were genotyped by PCR assay to determine whether they were heterozygous for the min allele or were homozygous wild-type. APC<sup>Min<sup>−/−</sup></sup> male or female progeny were randomly assigned to groups after weaning at 3 weeks. Mice (5–6 weeks old) were divided into 3 groups: (i) untreated vehicle group (n = 8); (ii) mice treated with 0.4 mg herbacetin/kg of body weight (n = 8); and (iii) mice treated with 2 mg herbacetin/kg of body weight (n = 8). Herbacetin or vehicle was injected i.p. 3 times a week for 8 weeks.

**In vivo studies using the xenograft mouse model**

Athymic nude mice (6-week-old nu/nu female mice; Harlan Laboratories) were inoculated in the right flank with HCT116 cells (2 × 10⁶ cells/mouse). Mice were maintained under “specific pathogen-free” conditions based on the guidelines established by the University of Minnesota Institutional Animal Care and Use Committee. For treatment by i.p. injection, tumors were allowed to grow to an average of 74.1 ± 56 mm³ and then, based on tumor volume, mice were divided into groups to obtain a similar average tumor volume. Mice were divided into four groups as follows: (i) untreated vehicle group (n = 10); (ii) 0.4 mg herbacetin/kg of body weight (n = 10); (iii) 2 mg herbacetin/kg of body weight (n = 10); and (iv) 200 mg DFMO/kg of body weight (n = 10). Herbacetin, DFMO, or vehicle (5% DMSO in 10% tween 80) was injected 3 times per week for 14 days. For treatment by oral administration, tumors were allowed to grow to an average of 51.3 ± 51.6 mm³ and then mice were divided into 2 groups with a similar average tumor volume as follows: (i) untreated vehicle group (n = 15) and (ii) herbacetin at 100 mg/kg (n = 19). Treatment with herbacetin was initiated on day 17 after inoculation of cells and continued to day 35 (~3 weeks) and was administered by oral gavage 5 times a week. Tumor volume was measured 2 times a week and body weight was measured once a week. Herbacetin was prepared in 2.5% DMSO/5% PEG 400/5% Tween-80 in 1X PBS and sonicated for 20 minutes. Tumor volume was calculated from measurements of 2 diameters of the individual tumor base using the following formula: tumor volume (mm³) = (length × width × height × 0.52). Mice were monitored until tumors reached 1 cm³ total volume, at which time mice were euthanized and tumors extracted.

**Cell lines**

All cell lines were purchased from ATCC and were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials of each cell line were available to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less. HCT116 and HT29 human colon cancer cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 1% antibiotic–antimycotic. DLD1 human colon cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotic–antimycotic.

**Reagents and antibodies**

Hercacetin (purity: >90% by HPLC) was purchased from Indofine Chemical Company. CNBr-Sepharose 4B beads were purchased from GE Healthcare. The recombinant human ODC protein was obtained from Abnova. The screening to determine the effect of herbacetin on the activity of 13 kinases was performed by Millipore. The antibody to detect Xpress was from Invitrogen.
from Cell Signaling Technology. Antibodies against ODC and β-actin were purchased from Santa Cruz Biotechnology.

**Lentiviral infection**

The lentiviral expression vector, pLKO.1-shODC, and packaging vectors, pMD2.G and psPAX, were purchased from Addgene Inc. To prepare ODC viral particles, the viral vector and packaging vectors were transfected using JetPEI into HEK293T cells following the manufacturer’s suggested protocols. The transfection medium was changed at 4 hours after transfection and then cells were cultured for 36 hours. The viral particles were harvested by filtration using a 0.45-mm sodium acetate syringe filter and then combined with 8 µg/mL of polybrene (Millipore) and infected overnight into 60% confluent HCT116 cells. The cell culture medium was replaced with fresh complete growth medium and after 24 hours, cells were selected with 1.5 µg/mL of puromycin for 36 hours. The selected cells were used for experiments.

**Anchorag-indepedent cell growth**

Cells (8 × 10^3 per well) suspended in complete growth medium (McCoy’s 5A or RPMI-1640 supplemented with 10% FBS and 1% antibiotics) were added to 0.3% agar with different doses of each compound over a base layer of 0.6% agar. The cultures were maintained at 37°C for 2 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.4) program (Media Cybernetics).

**Luciferase assay for reporter activity**

Transient transfection was conducted using jetPEI (Qbiogene) and assays for the activity of firefly luciferase and Renilla activity were performed according to the manufacturer’s instructions (Promega). Cells (1 × 10^4 per well) were seeded the day before transfection into 12-well culture plates. Cells were cotransfected with reporter plasmid (250 ng) and internal control (CMV-Renilla, 50 ng) in 12-well plates and incubated for 24 hours. Colon cancer cells were treated with herbacetin for 48 hours and harvested in Promega Lysis Buffer. The luciferase and Renilla activities were measured using substrates in the reporter assay system (Promega). The luciferase activity was normalized to Renilla activity.

**Cell proliferation assay**

Cells were seeded (1 × 10^3 cells per well) in 96-well plates and incubated for 24 hours and then treated with different doses of each compound. After incubation for 1, 2, or 3 days, 20 µL of CellTiterGlo Aqueous One Solution (Promega) were added and then cells were incubated for 1 hour at 37°C in a 5% CO_2 incubator. Absorbance was measured at 492 nm.

**Statistical analysis**

All quantitative results are expressed as mean values ± SD or ± SE as indicated. Significant differences were compared using the Student t test or ANOVA. A P value of <0.05 was considered to be statistically significant.

**Results**

**Herbacetin is a specific and potent ODC inhibitor**

To identify potential active compounds targeting an allosteric site on ODC, we performed docking studies (Supplementary Table S1) using the Traditional Chinese Medicine Database. Results indicated that herbacetin was a potential allosteric inhibitory compound that targets ODC (Fig. 1A). To examine the interaction between herbacetin and ODC, we performed in vitro pulldown assays using herbacetin-conjugated Sepharose 4B beads (or Sepharose 4B as a negative control) and a recombinant ODC protein (Fig. 1B) or a HCT116 colon cancer cell lysate (Fig. 1C). Results confirmed that herbacetin directly binds to ODC. Furthermore, computer docking results indicated that Asp44, Asp243, and Glu384 on ODC might be involved in the binding. These sites were mutated to alanine (D44A, D243A, E384A) and ectopically expressed in HCT116 colon cancer cells. Pulldown assays using the wild-type or each mutant and herbacetin-conjugated Sepharose 4B beads revealed that the D44A mutant showed the most reduced binding affinity with herbacetin (Fig. 1D), suggesting that this site is important for binding. Next, we compared the effect of herbacetin and DFMO (Fig. 2A, left) and allosteric ODC inhibitors (Fig. 2A, right) on ODC activity using a recombinant ODC protein, HCT116 or DLD-1 colon cancer cell lysates (Fig. 2B), or intact HCT116 cells (Fig. 2C). Herbacetin showed inhibitory ODC activity similarly compared with DFMO in vitro (Fig. 2A and B). However, herbacetin was markedly more effective than DFMO in suppressing ODC activity in cell-based assays (Fig. 2C). Furthermore, ODC activity was similar in the wild-type and mutant ODC proteins because the mutated sites originated from the allosteric binding site of ODC rather than the active site. The ODC D44A mutant activity was less susceptible to the effects of herbacetin than the other mutants (D243A and E384A) or the wild-type ODC (Fig. 2D). Additionally, we docked herbacetin in silico to a selected pocket in the 1NJJ (ODC) protein structure, which not only allowed the ligand to be flexible but also allowed the amino acids forming the protein binding site to achieve a more realistic view of the possible protein–ligand interaction. Results indicated that herbacetin forms numerous favorable interactions and docked nicely within the ODC allosteric site, especially at residue Asp44 (1.64 Å). In contrast, a similar compound, kaempferol, could not form these interactions. In this model, important hydrogen bonds were formed between herbacetin and ODC’s backbone at Asp44, Asp243, and Glu384 (Supplementary Fig. S1A). In contrast, kaempferol formed hydrogen bonds at Glu384, Thr285, and Ser282 (Supplementary Fig. S1B). Kaempferol had little effect on ODC activity compared with herbacetin, suggesting that the binding of herbacetin with Asp44 might be more important for inhibiting ODC activity (Supplementary Fig. S1C). Next, to identify other direct molecular targets of herbacetin, we screened 13 kinases and S-adenosylmethionine decarboxylase (AdoMetDC) enzyme activity against herbacetin using in vitro kinase and enzyme assays. The results showed that none of these kinases were affected by herbacetin, whereas only high doses of herbacetin or DFMO significantly increased AdoMetDC enzyme activity (Supplementary Fig. S2A–S2N). Additionally, to determine the effect of herbacetin or DFMO on polyamine content, we measured the level of putrescine, spermidine, and spermine in HCT116 colon cancer cells (Fig. 2E–G). The findings indicated that putrescine and spermidine, but not spermine, levels are significantly inhibited by treatment with herbacetin or DFMO. Furthermore, to identify the effect on polyamine uptake by herbacetin or DFMO, cells were treated with herbacetin or DFMO for 24 hours and then 14C-conjugated putrescine or spermidine was added. Results showed that the intracellular 14C-putrescine and -spermidine levels were not changed by herbacetin (Fig. 2H and I). In contrast, DFMO significantly increased intracellular putrescine and...
spermidine uptake (Fig. 2H and I). Next, to determine the effect on intracellular herbacetin level by polyamines, cells were cotreated with herbacetin and putrescine, spermidine, or spermine. Results indicated that the intracellular herbacetin was not significantly changed by polyamine treatment (Supplementary Fig. S3).

**Anticancer effects of herbacetin**

We measured ODC activity in HCT116, DLD1, and HT29 colon cancer cells and determined that, of the three, HCT116 cells had the highest ODC activity (Fig. 3A). We compared the effect of herbacetin and DFMO on the doubling time and cell cycles of each colon cancer cell line (Fig. 3B; Supplementary Fig. S4). The findings indicated that herbacetin had the greatest inhibitory effect on colon cancer cells that expressed higher levels of ODC. We also examined the effect of herbacetin on anchorage-independent colon cancer cell growth. Herbacetin was at least 20-fold more effective than DFMO in suppressing anchorage-independent growth of colon cancer cells (Fig. 3C). Next, we determined whether herbacetin affected the reporter activity of the MAP kinase transcription factor, activator protein-1 (AP-1), in HCT116 cells. HCT116 cells were treated with herbacetin for 48 hours and then AP-1 reporter activity was measured (Fig. 3D). Treated cells were also subjected to Western blotting and herbacetin also suppressed phosphorylation of ERK1/2 as well as p90RSK (Supplementary Fig. S5). Overall, these results indicated that herbacetin decreases ODC activity, resulting in attenuated AP-1 activation.

The **inhibition of ODC by herbacetin is dependent on the expression of ODC**

To study the influence of ODC expression on cancer cell growth, we constructed HCT116 cells stably expressing mock (shMock) or knockdown of ODC (shODC; Supplementary Fig. S6A–S6E). We also constructed stable shODC HCT116 cells with rescued expression of ODC (shODC + ODC; Fig. 4A) and measured ODC activity (Fig. 4B). Results indicated that colon cancer cell growth is dependent on ODC expression (Fig. 4C and D). We then examined the effect of herbacetin or DFMO on growth of shMock, shODC, and shODC + ODC HCT116 cells. Data indicated that cells expressing shODC were resistant to herbacetin’s inhibitory effect on anchorage-dependent and -independent cell growth compared with cells expressing shMock (Fig. 5A and B, middle). The shODC cells expressing rescued ODC regained sensitivity to herbacetin (Fig. 5A and B, bottom). Furthermore, we investigated the effect of the polyamine, putrescine, plus herbacetin or DFMO on growth. Cells were treated with herbacetin or DFMO for 48 hours, and then putrescine was added and cell proliferation and polyamine pools measured after 48 hours. Results indicated that cancer cell growth inhibited by herbacetin or DFMO is rescued by putrescine treatment (Fig. 5C and D; Supplementary Fig. S7). Taken together, these findings indicated that the anticancer activity exerted by herbacetin is dependent on ODC and also its anticancer activity against ODC is reversed by putrescine. Next, to determine the influence of polyamine depletion with herbacetin treatment on cancer cell doubling time, we used HCT116 (fast growing) or HT29 (slow growing) cells stably expressing knockdown of ODC or SAT1 (spermidine/spermine N1-acetyltransferase 1). Results showed that cell doubling time was increased by shODC or by overexpressing SAT1 in both cell lines. In contrast, only HCT116 cells expressing SAT1 were more sensitive to herbacetin’s inhibitory effect on cell doubling time (Supplementary Fig. S8A–S8D).

**Herbacetin as a preventive agent against small bowel and colon tumorigenesis in vivo**

We examined the antitumor activity of herbacetin in colon tumorigenesis using two in vivo mouse models. ODC gene
expression is upregulated in the intestinal tissue of APC\(^{Min^+}\) mice, a model that mimics human FAP. APC\(^{Min^+}\) mice were administered herbacetin (0.4 or 2 mg/kg body weight) or vehicle 3 times/week for 8 weeks. At the end of 8 weeks, polyp number and size were determined and small intestine samples collected. Treatment of mice with 0.4 or 2 mg/kg of herbacetin

Figure 2.
Herbacetin inhibits ODC activity. The effect of herbacetin on ODC activity was assessed using a recombinant ODC protein (A) or colon cancer cell lysates (B) incubated for 15 minutes with reaction buffer and different doses of herbacetin or DFMO and then incubated at 37°C for an additional 1 hour. C, HCT116 cells were treated with herbacetin or DFMO for 48 hours and harvested. D, the effect of herbacetin on ectopically expressed wild-type or mutant ODC (WT, D44A, D243A, E384A) activity was measured as the release of CO\(_2\) from L-[1-\(^{14}\)C] ornithine. E-G, the effect of herbacetin or DFMO on polyamine (E, putrescine; F, spermidine; G, spermine) content was analyzed by HPLC in HCT116 colon cancer cells. H and I, the effect of herbacetin or DFMO on polyamine uptake was measured by using \(^{14}\)C-putrescine (H) or \(^{14}\)C-spermidine (I) in HCT116 colon cancer cells. Cells were treated with herbacetin or DFMO for 24 hours and the respective polyamine was or was not added for 30 minutes. After washing with PBS, the intracellular \(^{14}\)C-putrescine or \(^{14}\)C-spermidine levels were measured. For A-I, all data are represented as means ± SD of triplicate values from three independent experiments and the asterisk indicates a significant difference (*, \(P < 0.05\)) between herbacetin- or DFMO-treated samples compared with untreated controls.
Figure 3.
Anticancer effects of herbacetin. A, the effect of herbacetin on ODC activity in colon cancer cell lines was measured as the release of CO2 from L-[1-C14] ornithine. The asterisk indicates significantly decreased (*, P < 0.05) ODC activity in DLD1 or HT29 cells compared with HCT116 cells. B and C, the effect of herbacetin or DFMO on the doubling time of colon cancer cells (B) and on anchorage-independent colon cancer cell growth (C). For C, cells were incubated with respective compound for 3 weeks and then colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. D, the effect of herbacetin on AP-1 reporter activity in HCT116 colon cancer cells was analyzed using the substrates included in the reporter assay system. All data are represented as means ± SD of triplicate values from three independent experiments and the asterisk indicates a significant (*, P < 0.05) effect of herbacetin or DFMO compared with untreated control.
significantly suppressed polyp number, size, and ODC activity compared with the vehicle-treated group (Fig. 6A–C; \( P < 0.05 \)). Mice tolerated treatment with herbacetin without overt signs of toxicity or significant body weight loss (Fig. 6D). Furthermore, the effects of herbacetin on polyamine levels were evaluated by HPLC in the small intestine tissues after 8 weeks of treatment. Results indicated that putrescine and spermidine, but not spermine, content was markedly decreased by treatment with herbacetin (Fig. 6E–G). Additionally, we examined the effect of herbacetin or DFMO on HCT116 colon cancer cell xenograft tumor growth in mice. HCT116 colon cancer cells were injected into the flank of athymic nude mice, and mice were injected with herbacetin (i.e. 0.4 or 2 mg/kg body weight), DFMO (i.e. 200 mg/kg body weight) or vehicle 3 times/week for 2 weeks after the average tumor volume grew to about 74 mm\(^3\). Treatment of mice with herbacetin or DFMO strongly suppressed HCT116 tumor growth by over 70% relative to the vehicle-treated group (Fig. 6H; \( P < 0.05 \)). Furthermore, the effects of herbacetin on ODC expression and AP1 signaling were evaluated by Western blotting, immunohistochemistry, and H&E staining after 11 days of treatment. The expression of phosphorylated ERKs and RSK was markedly decreased by treatment with herbacetin or DFMO (Supplementary Fig. S9A). However, ODC expression in herbacetin or DFMO-treated tissues was similar to the vehicle-treated group (Supplementary Fig. S9A and S9C). Additionally, mice seemed to tolerate treatment with herbacetin or DFMO without overt signs of toxicity or significant loss of body weight similar to the vehicle-treated group (Fig. 6I). Additionally, we also examined the effect of oral administration of herbacetin (100 mg/kg body weight) on HCT116 colon cancer cell xenograft tumor growth in mice. HCT116 colon cancer cells were injected into the flank of athymic nude mice and mice were given herbacetin or vehicle by oral gavage 5 times/week for 18 days after the average tumor volume grew to about 51 mm\(^3\). Results showed that tumor growth and phosphorylated ERKs and RSK were significantly suppressed in mice fed herbacetin (Fig. 6I, Supplementary Fig. S9B) and body weight was not affected (Fig. 6K). These results indicated that herbacetin is a potent ODC inhibitor and an active anticancer agent against small bowel and colon tumor growth.

**Herbacetin is not involved in ototoxicity**

Although DFMO is an approved FDA drug as an irreversible inhibitor of ODC, high doses of DFMO in humans can cause hearing loss (19). Therefore, development of new potent, nontoxic ODC inhibitor is important. We demonstrated the anticancer effects of herbacetin as a novel ODC inhibitor against colon cancer and next determined whether herbacetin was associated with ototoxicity (i.e., toxicity to the ear) compared with DFMO. Prepulse inhibition (PPI) of the acoustic startle reflex is important to estimate hearing impairment in mice. PPI is the ratio of the startle with a prepulse to the baseline startle response and can be used to assess the behavioral salience of sound. A high percentage of PPI value indicates a good PPI. In other words, the subject shows a reduced startle response when a prepulse stimulus is presented compared with the response when the startle stimulus is presented alone and therefore is exhibiting normal hearing. Conversely, a low percentage of PPI value indicates a poor PPI or, in other words, the startle responses with and without the prepulse are similar and hearing is therefore defective. Our results indicated that on day 35, oral administration of DFMO (1 g/kg body weight) resulted in a 20% PPI compared with control group (Supplementary Fig. S10A; data are presented as a percentage of control with vehicle being 100%), suggesting that DFMO was associated with profound hearing loss in C57BL/6 mice compared with vehicle control. Two additional groups of mice were administered herbacetin intraperitoneally (2 mg/kg body weight) or orally (100 mg/kg body weight) to determine whether herbacetin had similar effects on hearing as a percentage of PPI. Our results indicated that the percentage of PPI (i.e., hearing) was unaffected by...
herbacetin administered orally (Supplementary Fig. S10B) or by intraperitoneal injection (Supplementary Fig. S10C). These results indicate that herbacetin is a potent ODC inhibitor without apparent ototoxicity.

**Discussion**

Many flavonol compounds have been reported to exert potent antiproliferative activities through inhibition of multiple targets.
Figure 6. Effectiveness of herbacetin as a preventive agent against small bowel and colon tumor growth in vivo. APC\(^{Min}\) mice were used as a small bowel tumorigenesis model treated or not treated with herbacetin (i.p. 0.4 or 2 mg/kg body weight) for 8 weeks. A and B, number (A) and size (B) of polyps from APC\(^{Min}\) mice treated or not treated with herbacetin were calculated following euthanization. C and D, ODC activity (C) and body weights (D) from vehicle- and herbacetin-treated groups of mice were measured. E–G, the effect of herbacetin on polyamine (E, putrescine; F, spermidine; G, spermine) content from APC\(^{Min}\) mice small intestine tissues was analyzed by HPLC. H, herbacetin or DFMO suppresses colon tumor growth. Mice were injected with HCT116 colon cancer cells and then treated with herbacetin (i.p. 0.4 or 2 mg/kg body weight), DFMO (i.p. 200 mg/kg body weight) or vehicle three times a week for 2 weeks and tumors harvested. I, herbacetin or DFMO has no effect on mouse body weight up to 22 days. J, oral administration of herbacetin significantly suppresses xenograft HCT116 colon cancer growth. When tumors reached \(\geq 51 \text{ mm}^3\), mice were administered herbacetin (100 mg/kg body weight) by oral gavage 5 times a week for 18 days. K, herbacetin has no effect on mouse body weight up to 35 days. All data are represented as mean values ± SE and the asterisk indicates a significant difference (*, \(p < 0.05\)) between herbacetin- or DFMO-treated groups compared to the vehicle-treated group. B.W., body weight.
such as MAPKs, MAPKPs, or COX enzymes and have been suggested as agents in the development of molecular target therapy for human cancers (40). Previous reports suggested that alanine mutagenesis in the dimer interface of ODC distant from active site inhibited catalytic activity (41) and G418 (geneticin) induced the disordering of residues in the active site of ODC and allosteric inhibition (42). Therefore, we screened for allosteric ODC inhibitors using computer docking modeling and identified herbacetin as a possible allosteric inhibitor.

Structural and computational technique-based drug discovery, especially the application of molecular modeling, molecular docking, virtual molecular high-throughput and targeted drug screening, has been utilized (43). Recent advances in the development of anticancer drugs involve an emphasis on molecular target-based preventive agents such as erlotinib, tamoxifen, and gefitinib (44). The present study suggests that predicting ODC inhibitors by computer modeling is a useful tool for identifying potential inhibitors. Additionally, computer modeling results of the predicted binding site between herbacetin and ODC showed that herbacetin interacts with Asp44, Asp243, and Glu384 on the ODC backbone and the Asp44 residue appears most important for the inhibitory effect (Figs. 1 and 2). Our in vitro results indicated that the herbacetin interaction with the Asp44 residue appears most important for the inhibition of ODC activity. Furthermore, we determined whether the hydroxyl (OH) residues of herbacetin were involved in its binding to ODC. We docked 4 different flavonoids, including herbacetin, luteolin, 7,3’,4’-trihydroxyisoflavone, and kaempferol, in silico to a selected pocket in the 1NJJ (ODC) protein structure and also performed an in vitro ODC activity assay. Herbacetin docked nicely to the ODC allosteric site at residue Asp44 (1.64 Å) as well as strongly inhibiting ODC activity. However, the other compounds could not bind to ODC at Asp44 and only had a weak inhibitory effect on ODC activity (data not shown). Interestingly, herbacetin has little effect on other kinases’ activity, suggesting that herbacetin is a specific ODC inhibitor (Supplementary Fig. S2A–S2M). Therefore, the present findings suggested that herbacetin appears to be relatively specific for ODC rather than other protein targets. Previous studies reported that DFMO inhibited the number of polyps in the middle and distal portions of the small intestine but did not affect polyp size (25). Additionally, oral administration of DFMO (500–2,000 mg/kg) was shown to strongly inhibit several types of tumor growth in vivo (47–49). However, high doses of DFMO induced cytotoxicity as evidenced by weight loss in several in vivo models (50–52). Therefore, to study potential anticancer effects and examine the possible toxicity of herbacetin, we performed an in vivo study in APCMin/+ mice treated with herbacetin (i.p. 0.4, 2, 10, or 20 mg/kg body weight). Results showed that the number and size of polyps was decreased by herbacetin (Fig. 6A–D) with no overt toxicity. The effects were associated with decreased polyamine content (Fig. 6E–G). In another in vivo study, xenograft tumor growth was also decreased by herbacetin (0.4 or 2 mg/kg body weight) or DFMO (200 mg/kg body weight) administered i.p., suggesting that herbacetin is more effective than DFMO. Notably, herbacetin administered orally (100 mg/kg body weight) was equally as effective as twice the dose of DFMO administered i.p. (Fig. 6H and I). Importantly, assessment of PI3K as an indicator of auditory function suggested that, unlike DFMO, herbacetin was not associated with ototoxicity.

A combination of DFMO and NSAIDs, sulindac and celecoxib, was shown to exhibit potent inhibitory effects (53, 54). Therefore, further studies are needed to investigate the effectiveness of combining herbacetin with NSAIDs in colon and skin cancers, and to further characterize herbacetin and perform pharmacokinetics and pharmacodynamics studies as well as to elucidate toxicological responses. Overall, the effectiveness of herbacetin as a preventive agent seems to suggest its use as a promising lead compound in the future. The results of this study might be highly significant in that herbacetin is a natural, nontoxic compound that could be combined with an NSAID, such as sulindac, for an immediate clinical trial to test its effectiveness against colon cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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