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Auraptene and its Effects on the Re-emergence of Colon Cancer Stem Cells

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Abstract

Recent studies indicate that auraptene (7-geranyloxycoumarin, AUR), a geranyloxycoumarin extracted from fruits of edible plants belonging to the Rutaceae family, may represent a novel lead compound for dietary colon cancer chemoprevention in rodents. As a continuation of studies aimed to better depict the pharmacological effects and mechanism of action of the title natural compound, the current investigation was undertaken to determine whether AUR would be able to prevent the growth and sphere (surrogate tumors) formation of FOLFOX-resistant colon cancer cells that are highly enriched in cancer stem cells (CSCs). Our results demonstrate that AUR at a concentration of 10 μ M was able to inhibit the growth and formation of colonospheres of FOLFOX-resistant colon cancer HT-29 cells *in vitro*. The corresponding parental cells were also similarly affected by AUR at the same concentration level. The reduction in the growth and colonospheres formation in FOLFOX-resistant HT-29 was also associated with a concomitant decrease in phospho-epidermal growth factor receptor (pEGFR). These findings suggest that AUR could prevent the re-emergence of CSCs.

Keywords

auraptene; cancer chemoprevention; cancer stem cells; *Citrus*; colon cancer; geranyloxycoumarins

Introduction

Targeting cancer stem cells (CSCs) represent a novel frontier in oncology. To this aim several studies were recently reported in the literature suggesting new therapeutic approaches in cancer therapy (Pietras, 2011). CSCs are cancer cells that share with normal stem cells many features like the capacity to differentiate into all cell types found in a particular cancer. The first evidence of existence of CSCs was reported in 1997 by Bonnet and Dick in a subpopulation of leukemic cells. CSCs are hypothesized to exist in tumors as a distinct cell population and to be able to lead to relapse and/or metastasis by originating new tumors. In this context few studies on the effects of natural products on CSCs have been so far reported (Gunn *et al.*, 2011). Auraptene **1** (AUR) represents the most abundant geranyloxycoumarins found in nature (Genovese and Epifano, 2011).

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Conflict of Interest

The authors have declared that there is no conflict of interest.

The title compound has been isolated from plants belonging to many genus of the Rutaceae family, comprising several edible fruits and vegetables, like agrumes, carrot, celery, and several others. Although known for a long time, only in the last fifteen years AUR was seen to possess promising pharmacological properties as orally active cancer chemopreventive, anti-bacterial, anti-protozoal, anti-fungal, anti-inflammatory, and anti-oxidant agent, and to trigger key biological targets like metalloproteinases, glycoprotein P, and PPARs (Takahashi *et al.*, 2011). In particular our interest was focused on the ability of AUR to inhibit the growth of primary colon tumors in rodents (Tanaka *et al.*, 2010). Investigations on such a type of cancer is currently a field of great and growing interest due to the observation that nearly 50% of patients with colorectal cancer show recurrence of tumors that exhibit resistance to chemotherapy primarily due to the presence of CSCs (Botchkina G, 2012; Mollica *et al.*, 2012; Wu *et al.* 2012). The current investigation was undertaken to determine whether AUR would be able prevent the growth and sphere (surrogate tumors) formation of FOLFOX-resistant colon cancer cells that are highly enriched in CSCs.

Experimental

Chemistry

Auraptene was chemically synthesized as previously reported (Tanaka *et al.*, 2010). The recorded analytical data were in full agreement with those obtained with a commercial pure sample (Sigma Aldrich Chemical Co.). Its purity (> 98.6 %) was assessed by GC-MS analysis.

Pharmacology

MTT-test—HT-29 and HCT-116 wild type and FOLFOX resistant cells were plated in 96 well plates at a concentration of 1000 cells/well. The following day, medium was removed and replaced with Dulbecco's modified Eagle medium (DMEM) containing 2.5% fetal bovine serum (FBS) and AUR at various concentrations (2.5, 5, 10, 20 and 40 μ M). Controls without drug were measured in each group. The cells were incubated for 48 or 72 hours before the addition of MTT. Following the formation of formazan crystals, the medium was carefully removed and the crystals were dissolved in DMSO. Absorbance at 570 nm was measured and results were reported in percent absorbance of control group.

Western-Blot analysis—HT-29 and HCT-116 wild type and FOLFOX resistant cells were grown in DMEM containing 2.5% FBS and 10 μ M of AUR for 48 hours. Controls were grown in medium without drug. Western-Blot analysis were carried out as already reported (Nautyal *et al.*, 2012).

Colonsphere analysis—100 cells per well were plated in DMEM/F12 (1:1) medium with 1 $\times$ B27 supplement in 96 well plates. Each group consisted of 24 wells. Control groups did not receive treatment, while AUR group received 10 μ M of AUR. Cells were grown for 5 days before counting colonspheres 50 μ m in diameter. Control groups were set equal to 100%.

Statistics

Statistical analysis were carried out as already reported (Nautyal *et al.*, 2012).

Results and Discussion

Auraptene was chemically synthesized from commercially available umbelliferone (7-hydroxycoumarin) and geranyl bromide by means of environmentally friendly, easy to handle, and high yielding (98.4 %) route without the need of chromatographic method for

purification of the desired adduct (Tanaka *et al.*, 2010). Four cancer cell lines were used to perform pharmacological experiments, namely HT-29 (colorectal adenocarcinoma) wild type and FOLFOX-resistant, and HT-116 (colorectal carcinoma) wild type and FOLFOX-resistant. FOLFOX resistant cells have been grown in 2× FOLFOX (50µM 5-fluorouracil and 1.25 µM oxaliplatin). This type of cancer cells are known to be enriched in CSCs (Yu *et al.*, 2009). The MTT test revealed that after 72 h AUR was able to dose-dependently (concentration range 2.5 – 40 µM) inhibit the growth of parental and FOLFOX-resistant lines in both types of cells, the values of inhibition being around 40 % (Figure 2).

To further examine the biological action of AUR on colon CSCs we initially investigated the effect of AUR 10 µM on the expression of the phospho-epidermal growth factor receptor (pEGFR). It is in fact well known that elevated pEGFR is associated with poor prognosis in cancer patients (Magkou *et al.*, 2008). As reported in Figure 3 AUR was effective in decreasing (> 50%) the expression of pEGFR respect to untreated controls only in the case of HT-29 FOLFOX-resistant cells, while in the other three cell lines the presence of AUR 10 µM even stimulated the expression of the above cited receptor.

For this reason we decided to perform the subsequent experiments using only HT-29 cells. We carried out Western Blot analysis to examine the relative concentration of CD44 and CD166, two markers of colon CSCs (Horst *et al.*, 2009). As depicted in Figure 2 AUR at a concentration of 10 µM revealed strong differences in expressions of the above cited markers between FOLFOX-resistant and wild type cell lines. In the latter, values were equal to or even higher than untreated controls, while for FOLFOX-resistant cells the reduction in expression of both CD44 and CD166 was more than 50 %.

We finally examined the effect of AUR 10 µM on colonospheres (Ø 50 µm) formation, size, and integrity. As shown in Figure 4, in this case the title compound had virtually no effect on the wild type HT-29 cell line; the number of colon spheres were very similar to that of untreated control. In contrast AUR exhibited a fairly good action on FOLFOX-resistant cells, as evidenced by the 40 % reduction compared to the untreated control.

However, when comparing the average size of colonospheres (Ø 50 µm), AUR 10 µM recorded no significant differences with respect to untreated controls (data not shown).

In conclusion in the present work we confirmed that AUR possesses chemopreventive properties, with respect to recurrent colorectal cancers that are known to be resistant to conventional chemotherapy. Although the reasons for this resistance are not fully understood, one possibility could be the presence of cancer stem cells in recurrent tumors that are known to be resistant to chemotherapy. The observation that AUR inhibits the growth of both wild type and chemo resistant colon cancer cells as well as inhibits expression of CD44 and CD166 in chemo resistant HT-29 cells and suppresses formation of colonospheres that are thought to be surrogate tumors, supports this contention. EGFR appears to play a role in regulating this process. The latter is based on our recent observation that inhibition of EGFR by cetuximab (MoAb to EGFR) markedly attenuates the age-related and carcinogen-induced expression of several surface epitopes of colon cancer stem cells (Nautiyal *et al.*, 2012). However failure by AUR to disintegrate existing spheres suggests that while it may prevent the formation of cancer stem cells for drug resistant HT-29 cells, it does little to reduce their growth or force them to differentiate once they have already aggregated.

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Figure 1.
Structure of auraptene

Figure 2.
Growth inhibitory effects by AUR on HT-29 and HT-116 FOLFOX (R) and wild-type (WT) cell lines.

Figure 3.
Expression of tumoral markers relevant to CSCs in HT-29 cell lines.

Figure 4.
Effect of AUR 10 μM on colonosphere (Ø 50 μm) formation in HT-29 cell lines.