NATURAL DRUGS

THE EFFECT OF ACETYLSHIKONIN ISOLATED FROM LITHOSPERMUM CANESCENS ROOTS ON TUMOR-INDUCED CUTANEOUS ANGIOGENESIS

AGNIESZKA PIETROSIUK, MIROSŁAWA FURMANOWA, EWA SKOPIŃSKA-RÓŻEWSKA', EWA SÖMMER', HENRYK SKURZAΚ and JANUSZ BANY

Department of Biology and Pharmaceutical Botany, Medical University of Warsaw, 1 Baracha Str., 02-097 Warsaw, Poland;
' Department of Laboratory Diagnostics and Immunology, National Institute of Tuberculosis and Lung Diseases, 26 Płocka Str., 01-138 Warsaw, Poland;
' Department of Immunology, Oncology Center, 5 Roentgena Str., 02-777 Warsaw, Poland;
' Department of Pharmacology and Toxicology, Military Institute of Hygiene and Epidemiology, 3 Koszelska Str., Warsaw, Poland

Abstract: This study has demonstrated that acetylsikronin (ACS), the isolated ingredient from Lithospermum canescens Lehlm, roots, in a daily dose of 200 μg for 3 days, inhibited cutaneous angiogenesis induced by L-1 sarcoma cells in Balb/c mice.

Keywords: Lithospermum canescens; acetylsikronin; L-1 sarcoma; angiogenesis; mice

The angiogenesis is the process of growth of new capillaries from pre-existing capillaries and post-capillary venules. It is a tightly controlled process that rarely occurs under normal conditions, except for instances of wound healing, embryonic development and development of the corpus luteum. It is generally accepted today that tumor growth is angiogenesis-dependent and that every increment of tumor growth requires an increment of vascular growth (1).

Many herbal extracts and secondary metabolites obtained from plants were examined as potential angiogenesis inhibitors and promoters (1, 2).

This study is focused on acetylsikronin (ACS), the compound isolated from Lithospermum canescens Lehlm. (Boraginaceae). It is a common prairie plant of Canadian origin. Although not so well known as Lithospermum erythrorrhizon Sieb. et Zucc., which is a component of the Chinese herbal preparation Zicao, Lithospermum canescens contains active compounds, sikronin and its derivatives which are of medicinal importance. Two naphthoquinone pigments, acetylsikronin and isopropylsikronin, were first isolated from intact plants of Lithospermum canescens and their chemical structures were determined (3).

Multiple pharmacological actions of sikronin and its derivatives are well known and described in the world literature. The detailed review of sikronin, elicamin and other naphthoquinones, including such aspect as biosynthesis, chemistry, synthesis, as well as propagation by cell cultures and medicinal properties were presented (4).

ACS was found to have the anticoagulant effect of heparin in rats. It inhibited an increased vascular permeability and acute edema induced by histamine, accelerated the proliferation of granulation tissue and promoted wound healing in rats. It also inhibited the respiratory burst in rat neutrophils (5, 6, 7).

More data concerned sikronin activity. Several therapeutic applications of sikronin, making use of its pleiotropic, anti-inflammatory and antitumor effects, were described (8, 9).

The investigations of Sankawa et al. (10, 11) suggest that sikronin and its derivatives may be potent antineoplastic agents.

The newly synthesized sikronin derivative 2-xyim-DMNQ-S33 was found to possess strong anticancer activity (12).

Sikronin inhibits angiogenesis in vivo and in vitro (13). Recently, many chemotherapeutic agents were reported to exert their antitumor effect by inducing apoptosis of cancer cells. Among them, shikonin and β-hydroxyisovalerylsikronin (β-HIVS), isolated from the root of Lithospermum erythrorhizon was observed to induce apoptosis in HL60 human promyelocytic leukemia cell line (14, 15, 16).

Shikonin also increased intracellular levels of phosphorylated apoptosis-related proteins, and de-
creased levels of proliferation-associated proteins in human epidermoid carcinoma cells (17).

Our research has been focused on investigating the biological activity of ACS – the shikonin derivative. An earlier paper (18) reports the immunomodulatory effect of ACS and isobutyrylshikonin (IBS) isolated from L. 
conescens on the cellular and humoral immunity in Balb/c mice and on F1 hybrids (Balb/c × C3H). The L. 
conescens roots extract was also found to possess antibacterial and antifungal activities. ACS and IBS showed strong antibiotic activity (19).

The aim of this study was to evaluate the effect of ACS on inhibition of cutaneous angiogenesis induced by cells isolated from the mouse L-1 sarcoma tumor. Although many studies concerning anti-tumor activities of shikonin derivatives isolated from L. erythrorhizon have been published, this report is the first to present evidence that Lithospernum 
conescens is a potential source of naphthoquinones for medicinal applications.

EXPERIMENTAL

Biological material and assays

The study was performed on L-1 sarcoma cells delivered by the Cell and Tissue Bank at the Oncology Center in Warsaw. The cells were passaged through four generations of locally bred Balb/c mice.

The mice (body weight ca. 20 g) were given orally, using an Eppendorf pipette, 40 μL per day of one of the following solutions: ACS in 10% alcohol, 40 μg or 200 μg; or 10% ethyl alcohol as control. The above mentioned doses correspond to 20 or 100 mg, respectively given to human weighing 70 kg, assuming the ratio of the body surface area to body mass in mice and men differs by the factor of 7. The doses used were determined on the basis of our preliminary investigations and literature data (12).

The evaluation of the effect of ACS on cutaneous angiogenesis induced by cells isolated from the mouse L-1 sarcoma tumor (3-days of treatment) was performed.

ACS isolation

ACS was isolated from roots of L. conescens. The plants were collected at Parkland Bot. Togo, Saskatchewan, Canada. A voucher specimen of L. conescens (Michx.) Lehnn. from South of Togo, Saskatchewan, found in sandy soils, is deposited at The W. F. Fraser Herbarium (SASK), University of Saskatchewan, Saskatoon, Saskatchewan, Canada, accession number 94815.

To obtain dyes fractions the crumbled roots (200.0 g) were extracted with n-hexane in Soxhlet’s apparatus for 72 h at 70°C. The solvent was evaporated from the extract solution under reduced pressure. The residue left was 5 g of deep red semisolid (yield 2.5%). The separation of the individual compounds was carried out using the flash chromatography method (stationary phase: Kieselgel 60, eluent: n-hexane–CHCl3 (90:10 to 5:95), monitored at λ = 212 nm, nitrogen pressure (1–1.5 bar). To check the purity of the isolated compounds, HPLC and UV spectra analysis were performed in DIONEX HPLC system equipped with automated sample injector (ASI-100) and UVD 340S detector under the following conditions: gradient elution – acetonitrile (40 – 0 cm)/0.04 M orthophosphoric acid (60 – 100 cm); flow rate 1.5 cm/min; column: EC 250/4.6 Nucleosil® 120 – 7 mm C18 (Macherey– Nagel); UV λmax (MeOH) for shikonin and its derivatives: 215, 278, 514 nm. Structures of the isolated compounds were determined with the 13C and 1H NMR method (3).

Influence of ACS on angiogenesis process

L-1 sarcoma cells were used for tumor-induced angiogenesis assay. The cutaneous angiogenesis assay was performed according to the method of Sidky and Auerbach (20) with some modifications (21). Cell suspensions were obtained from tumors removed on day 14 after subcutaneous implantation of 2 × 106 sarcoma cells into the mouse subscapular region. Then, each tumor was cut into smaller pieces, rubbed through the sieve and suspended in PBS (phosphate-buffered saline). The suspension was left for 10 min in room temperature, supernatant collected, centrifuged for 10 min at 300×g, and obtained cells washed twice and resuspended in Parker medium at density of 4 × 106 cells per 1 cm3. Multiple samples of 200 thousands sarcoma cells suspended in 0.05 cm3 of Parker medium were implanted intradermally into partly shaved, anaesthetised (chloral hydrate) Balb/c mice. The mice were fed 4 × 106 with the tested compounds, as previously described, for 3 days. After 72 h the mice were treated with a lethal dose of Morbital. All newly formed blood vessels were identified and counted in a dissection microscope on the inner skin surface (magnification × 6, central 1/3 of microscopic field) using the criteria proposed by the authors of the method (tortuosity, diversifications, small size, directed to the site of cells gathering). Statistical analysis of results was performed by Student’s t-test. Local Ethical Committee approved all experiments.
Table 1. Inhibitory effect of 3-days ACS administration on neovascular reaction induced in Balb/c mice after L-1 sarcoma cells grafting

<table>
<thead>
<tr>
<th>ACS daily dose (µg)</th>
<th>Number of tests</th>
<th>Mean number of newly-formed blood vessels ± SE</th>
<th>Statistical significance of difference from the control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>20.2 ± 0.23</td>
<td>n.i.</td>
</tr>
<tr>
<td>40</td>
<td>18</td>
<td>19.7 ± 0.28</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>200</td>
<td>12</td>
<td>16.9 ± 0.41</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

The results of the experiment are presented in Table 1. Administration of ACS in a daily dose of 200 µg, for 3 days after intradermal grafting tumor cells resulted in suppression of neovascular response. The lower dose used (40 µg) was ineffective.

The results showed a possible effect of ACS on angiogenesis followed by tumor growth reduction. To our best knowledge, the study described in this paper is the first ever investigation of the effect of ACS, isolated from Lithospermum canescens plants, on tumor-induced cutaneous angiogenesis.

Angiogenesis plays an important role in many developmental and repair processes but it also contributes to various pathological conditions such as diabetic retinopathy, rheumatoid arthritis, and cancer. It is critical for tumor growth and inflammation (13).

In the present study an inhibitory effect of ACS on cutaneous angiogenesis induced by L-sarcoma cells was observed. Angiogenesis was induced in the skin of Balb/c mice after grafting of L-1 sarcoma syngeneic cells. ACS was administrated to the mice in daily doses 40 and 200 µg, for 3 days. The lower dose of ACS was ineffective, but the higher dose (200 µg) markedly inhibited angiogenesis. The doses used in our study were high but did not exceed the toxic dose of shikonin − 30 mg kg⁻¹ per day (10). The dose 200 µg corresponded to the therapeutic dose of shikonin 10 mg kg⁻¹ per day used by Sankawa et al. (10) for inhibiting sarcoma 180 in mice.

Authors (l.c.) found also that shikonin caused acute toxicity at higher doses (> 30 mg kg⁻¹ per day), but was inactive at lower doses (< 5 mg kg⁻¹ per day).

The toxicity of ACS to mice by intraperitoneal administration is 41 mg kg⁻¹ or ca. 23 mg kg⁻¹ (LD₅₀) and is lower than toxicity of shikonin LD₅₀ = 20 mg kg⁻¹ (4).

In the literature, there are many papers on the antitumor action of shikonin isolated from a different species, namely Lithospermum erythrorhizon. Shikonin isolated from that plant inhibited TNF-α-induced (tumor necrosis factor – inflammatory cytokine) and B16 melanoma-induced angiogenesis in mice and normal developmental angiogenesis in the yolk-sac membranes of chick embryos (13). Authors (l.c.) demonstrated that shikonin had an inhibitory activity against three types of angiogenesis, i.e., inflammatory angiogenesis, tumor angiogenesis and normal programmed developmental angiogenesis.

Recent literature data demonstrate that shikonin could exert antitumor effect by different ways e.g., by inhibition of cancer proliferation (10, 11), inhibition of DNA topoisomerase (22), induction of apoptosis (14, 15, 16, 17), reduction of angiogenesis and carcinogenesis (13) or by chemoprotection (23).

The above mentioned literature data indicate that shikonin has been studied repeatedly for its antitumor effect, but very little is known about the activity of its derivative ACS and Lithospermum canescens, the plant, which contains several naphthoquinones.

Our findings indicate that Lithospermum canescens is a potential plant source of shikonin derivatives and its biological activity is comparable to that of Lithospermum officinale and Lithospermum erythrorhizon. It seems to be a promising source of angiogenesis inhibitors for applications in adjuvant cancer therapy, especially in immunocompromised patients, for example following chemotherapy.

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