Pro-apoptotic Effect of Soy Total Extract Incorporated in Lyotropic Liquid Crystals Formulation

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The paper aims to test the pro apoptotic capacity of soy total extract on the B164A5 mouse melanoma cells alone and after incorporation in the modern formulation lyotropic liquid crystals. DAPI dye and Annexin-FITC-7AAD double staining show that soy extract present both early and late apoptotic properties against B164A5 murine melanoma cell line. Furthermore , incorporation of soy extract in the modern formulation does not affect in a negative manner this property. As explained in the paper the benefits of such an approach, this preliminary study represent an obvious proof that incorporation of soy extract in this new formulation is a good option for furtherer in vivo studies on animal model of melanoma.

Keywords: soy extract, lyotropic liquid crystals, murine melanoma, apoptosis

Among the modern formulations used in the pharmaceutical field for local administration of different active agents, lyotropic liquid crystals (LLC) have gained increased attention due to their special physico-chemical properties [1,2] . As a brief description of the formulation-LLC are formed by surfactants having two different parts: a non polar, seldom hydrocarbon tail and a polar, seldom ionic, head [3]. LLC have the properties somewhere in between a conventional liquid and a solid crystal [4]. They have the ability to exhibit phase transitions depending on the temperature and on the concentration of the liquid crystals in the employed solvent which is frequently water [5]. The thermodynamic stability of these formulations and the intense similarity of these systems to those in living organism makes LLC a great excipient for the local administration of different active agents [6,7].

Pure natural compounds or plant total extract represent a wellspring of remedies for a variety of pathologies. During the past decades a spectacular number of remedies from nature turned in classical therapy, well accepted by an increasing number of patients. An intensively and controversy studied extract is represented by soy extract, respectively its activate isoflavones genistein and daidzein. Recent studies show that soy total extract induce apoptosis in prostate cancer cells [8]. Furthermore it shows anti menopause effects[9] . It has been show that soy extract is more potent than genistein on an animal model of breast cancer on tumor growth inhibition [10]. It has been reported its antiproliferative activity in a mouse model of skin cancer [11].Soy isoflavones have been studied for their ant diabetic and hypolipidemic effects [12]. The group of Catania *et al*., have published that in case of ovariectomized rats orally administered soy extract improves the endothelial dysfunction [13] . Soy germ extract have been proofed pro apoptotic properties *in vitro* in case of colon cancer [14] .

The paper represents a preliminary study of the soy based formulation for furtherer *in vivo* investigations on animal models of melanoma . It aims to test the pro apoptotic capacity of soy extract employing the B164A5 mouse melanoma cells alone and after incorporation in the modern lyotropic liquid crystals formulation.

Experimental part

Materials and methods

Extraction

Soy seeds were kindly provided from University of Agricultural Sciences and Veterinary Medicine, Timisoara, Romania, Department of Plant Culture. As previously described hydroalcoholic extracts were performed. Dry soybeans were put into powder. A hydroalcoholic solvent containing 70% ethanol was prepared. 10% vegetal product extracts were ultrasonicated for 10 min in the ultrasonic bath . Afterwards the solvent was evaporated and the powder was used for furtherer experiments [15].

Lamellar lyotropic liquid crystalline soy extract based formulation

Soy extract, the active agent was incorporated in the lamellar lyotropic liquid crystal formulation in a concentration of 10%. The carrier was the mixture of a non-ionic surfactant, Cremophor RH40 (Polyoxyl 40 Hydrogenated Castor Oil USP/NF). It was obtained from BASF (67056, Ludwigshafen, Germany). The water phase of the systems was purified water (Ph.Eur.6.), while the oil phase was isopropyl myristate (Merck Kft. 1121, Budapest, Hungary). The oil-surfactant mixture (oil:surfactant ratio = 2:1) was homogenized with a magnetic stirrer at room temperature. Then 10% of water was added to this mixture in small amounts while stirring. The 10 % soy extract was incorporated in the oil-surfactant mixture with a magnetic stirrer.

DAPI (4, 6-Diamidino-2-phenylindole) staining

B16 cells were seeded at a concentration of $5x10^4$ in a chamber slide system formed of 8 well glass slides in culture medium. After 24h cells were incubated in medium containing 10%FCS and 200μg/mL of soy extract, respectively 200μg/mL of soy extract corresponding to

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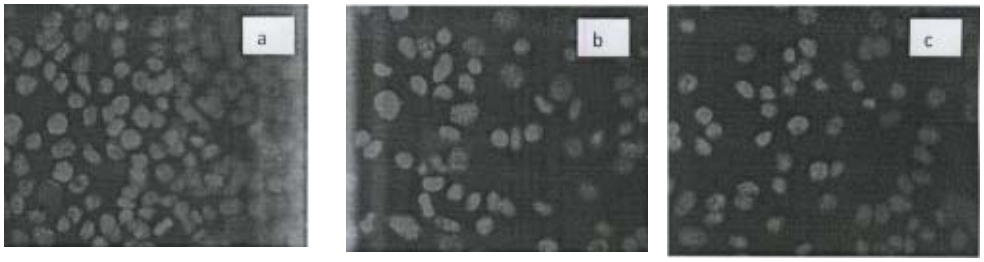


Fig. 1. DAPI staining after 72h incubaton of B164A5 cells with a) Medium; b) 200 μg/mL of soy extract c) 200 μg/mL of soy extract corresponding to the 10% LLC formulation

the 10% lamellar lyotropic liquid crystalline formulation. The total volume added in chamber was 400 μL. Cells were incubated for 72 h and afterwards the medium was removed. Cells were washed with PBS and afterwards 400μL of staining solution were added in each well. The staining solution consisted of a mixture of methanol and DAPI (Roche) as follows: 1 mL methanol: 2 μL DAPI (from a stock solution of 1mg/mL). Cells were incubated for 5 minutes with this staining solution and afterwards washed with PBS and analyzed by fluorescence microscopy.

Annexin-FITC-7AAD double staining

Cells were cultivated in a 6 well plates at a density of 80% using normal medium. After 24h medium containing 10% FCS and and 200 μg/mL of soy extract, respectively 200 μg/mL of soy extract corresponding to the 10% lamellar lyotropic liquid crystalline formulation were added. After 48h cells were detached using trypsin, washed with ice cold PBS and resuspended in 500 μLAnnexin binding buffer (1.19 g HEPES NaOH *p*H =7.4; 4.09 g NaCl; 0.138 g $CaCl_2$ in 50 mL distillated water and diluted 1:10) at a concentration of $1x10^6$ cells/mL. Cells were centrifugated 5 min at 1200 rpm, the supernatant was discarded and the cells were resuspended in 70 μL of Annexin binding buffer. 5 μL Annexin V-FITC (ImmunoTools) and 5 μL 7AAD (ImmunoTools) were added and cells were incubated 15 min on ice and in the dark. Samples were measured by FACS on FL1 and FL3 fluorescence channels using a BD Canto II FACS DIVA device. Flow Jo soft (7.6.3) was used for data analysis.

Statistics

One Way ANOVA followed by Bonferroni post test were used to determine the statistical difference between various experimental and control groups.*, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ compared to control group.

Results and discussions

The balance between proliferation and apoptosis has a vital role in many human diseases including cancer [16]. Among years different approaches have been conducted in order to fight cancer and one important strategy points towards finding pro-apoptotic agents [17]. DAPI staining is frequently used as a preliminary assay to test the pro-apoptotic capacity of different active agents [18] . After a period of incubation of 72h with 200μg/mL of soy extract, respectively 200μg/mL of soy extract corresponding to the 10% lamellar lyotropic liquid crystalline formulation, cells stained with DAPI displayed the typical characteristics of cells undergoing apoptosis (fig. 1). The blue stained nucleus allows to notice the morphological hallmarks of apoptosis: nuclear fragmentation, chromatin condensation, nuclear condensation as a sign of loss of cell membrane integrity, reduction of cellular volume, rounding up of the cell. It can not be detected a significant difference regarding the morphological signs of apoptosis between soy extract respectively the soy extract corresponding to the 10% lamellar lyotropic liquid crystalline based formulation. This aspect represent a good news for furtherer *in vivo* investigations, meaning that the formulation does not affect the biological activity of soy extract, but it helps, as explained in the introduction to its delivery. In addition it can be observed that after the 72 h of incubation both the extract respectively the extract incorporated in the formulation present also anti-proliferative effect, as it can be noticed by the decreased number of cells presented in a specific field. DAPI staining was previously used in order to test the pro-apoptotic activity of genistein, the most active isoflavone in soy extract, on human uterine leiomyoma, human prostate carcinoma PC-3 cells, Ewing's sarcoma CHP-100 cells, breast cancer MCF-7 cells [19-21].a

In order to be able to get more data about the apoptotic process, the double staining Annexin-FITC-7AAD was performed. This assay allows to get information about early apoptotic, late apoptotic and necrotic cells after incubation to different active agents [22] . Results show (fig. 2), that after a period of incubation of 72 h both the soy extract alone, respectively incorporated in the LLC formulation present early and late apoptotic properties. Average value corresponding to three different experiment show 3.38±2.89 early apoptotic cells in case of incubation with 200 μg/mL of soy extract and 2.60±0.84 early apoptotic cells in case of incubation with 200 μg/mL of soy extract incorporated in the LLC formulation. The tested active agents succeeded to induce more intense the phenomena of late apoptosis, as it can be depicted from figure 2, namely

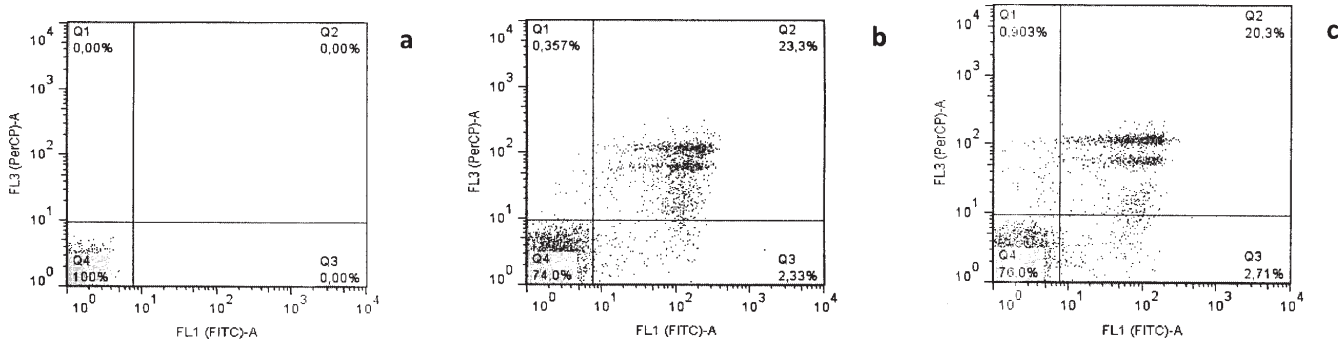
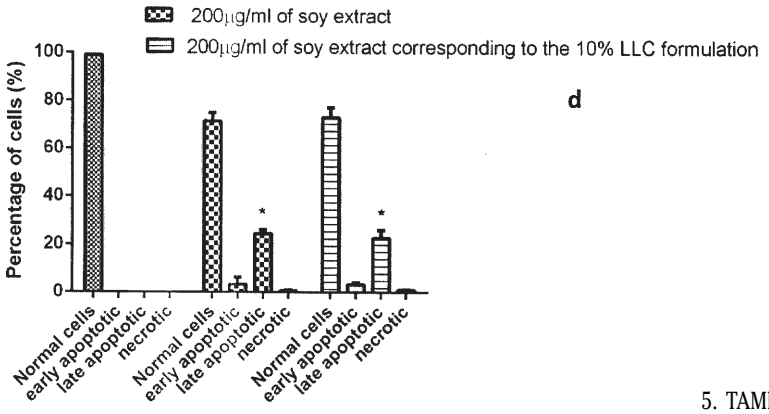


Fig. 2. Annexin-FITC-7AAD double staining after 72h incubation of B164A5 cells with: a) Medium; b) 200μg/mL of soy extract; c) 200μg/mL of soy extract corresponding to the 10% LLC soy extract based formulation



d

Fig. 2d cont. Average value corresponding to three different experiments

24.52±1.69 late apoptotic cells in case of incubation with 200µg/mL of soy extract and 22.66±3,34 late apoptotic cells in case of incubation with 200µg/mL of soy extract incorporated in the LLC formulation. A decreased number of necrotic cells could be also detected, namely 0.613 ±0.36 necrotic cells in case of incubation with 200 µg/mL of soy extract and 0.987±0.11 late apoptotic cells in case of incubation with 200µg/mL of soy extract incorporated in the LLC formulation. The pro-apoptotic capacity of soy extract was also described in case of prostate cancer cells, human AGS gastric cancer cells [8,23]. Soy isoflavones, the most active phytocompounds, as we previously discussed showed pro-apoptotic effect in case of breast cancer cells, murine and human bladder cancer cells, HT-29 colon cancer cells, human prostatic cancer cell line LNCaP, human hepatoma cell lines HepG2, Hep3B, Huh7, PLC, and HA22T [14.24.27].Furthermore, a recent study shows that the biotransformed soy extract fermented by *Aspergillus awamor* induces apoptosis in case of A375 human melanoma cell line [28]. Correlating to the data existing in the literature this study brings in to light the pro-apoptotic activity of soy extract on the murine melanoma B164A5 cell line.

Conclusions

Results show that soy extract present apoptotic properties against B164A5 murine melanoma cell line. Furthermore , incorporation of soy extract in the modern formulation lamellar lyotropic liquid crystals does not affect in a negative manner this property. As explained in the introduction the benefits of such an approach, this preliminary study represents an obvious proof that incorporation of soy extract in this new formulation is a good option for furtherer *in vivo* studies on animal model of melanoma

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