SEMPERVIVUM MARMOREUM EXTRACT MODULATES THE CLASTOGENIC EFFECT OF CYTOSTATIC CCNU ON RAT NUCLEATED BLOOD CELLS

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Abstract: *Identification of chemical agents reducing the unwanted clastogenic side effects of cytostatics on normal cells is a major objective in the progress of the modern chemotherapy. Sempervivum marmoreum Griseb. is a monocarpic perennial Crassulaceaean, native in dry habitats in rocky zones. Previous experiments demonstrated antioxidant and anti-inflammatory properties of Sempervivum sp. extracts on various experimental models, suggesting a hematoprotector potential in clastogenic chemotherapy. Based on existing data regarding the pharmacological activity of Sempervivum spp and on metabolism-phylogeny correlation, we consider that Sempervivum marmoreum extract (SEMEX) should have anticlastogenic activity,-modulating the effect of the alkylant cytostatics. We hereby report the evaluation the modulatory effect of SEMEX on the DNA fragmentation in rat nucleated blood cells, with the scope of its use as adjuvant in the chemotherapy with CCNU (=N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea) – cytostatic finally producing DNA DSBs. Wistar rats were treated with CCNU and/or 3 concentrations (25%, 50%, 100%) of total extracts obtained from cultivated S. marmoreum; DNA fragmentation in nucleated blood cells was evaluated by PFGE-CHEF technique. Densitometric analysis of the DNA electrophoretic migration in the treatments clearly shows the following effects: 1-Consistent modification the DNA fragmentation profiles under the action of cytostatic, after the SEMEX administration; 2- Doze-dependence of the modulation intensity of (Deoxyribonucleic acid) DNA fragmentation. 3 - Evidence of a surprising intrinsic clastogenic effect of SEMEX administered alone. The densitometric profiles with different distribution of density maxima suggest a protective effect of SEMEX against the clastogenic activity of CCNU, decreasing DSB number and/or increasing of the postclastic DNA-repair efficiency.*

INTRODUCTION

Cancer was since immemorial times one of the greatest medical problems of the humankind and even now, it is far from being solved satisfactorily. Cancer therapy has advanced tremendously in the last decades, but despite its undeniable utility, it is still flawed by considerable side effects that sometimes render its application questionable. Antitumor chemotherapy is fundamentally based upon the premise that the clastogenic effect of the chemotherapeutic agent is considerably more prominent in the DNA of the tumour cells than in the healthy, normal cells, thus killing the abnormal cells at much lower drug concentrations and/or at a much higher rate. Unfortunately, the therapeutic window is often quite narrow for most clastogenic drugs, which translates in that the required doses to kill tumour cells are high enough to be highly toxic to normal, non-cancerous cells, fact that severely limits the clinical use of such antineoplastic drugs.

N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (CCNU) a nitrosoureic alkylating liposoluble cytostatic acting upon DNA and producing as a final point DNA-strand breaks of several types, amongst which also Double Strand Breaks (DSB).(1) DSB induction is followed by the DNA fragmentation, resulting in a mixture of DNA molecules of various sizes. DNA lesions immediately trigger enzymatic reactions attempting to repair the damaged DNA molecules. Extensive DNA breakage and/or inefficient DNA repair usually results in cell death. This action mechanism allows the use of CCNU as a chemotherapeutic antitumor agent. The clastogenic effect of CCNU is not restricted to the tumour cells, but it also kills normal cells of the affected organism; predominantly cells that undergo frequent mitoses. Unfortunately, this same action mechanism results in various side effects including severe, cumulative myelotoxicity, pulmonary fibrosis, impaired gonadal function, mutagenesis, and secondary cancers (frequently leukemia). Also, the incidence of secondary malignancies increases with dose and is more likely to follow CCNU association with radiotherapy.(2) Our experimental work on *Sempervivum marmoreum* Griseb. intended to identify protective agents against DNA fragmentation in normal cells and diminish some of the side effects of cytostatics goes along this growing trend in the progress of the modern chemotherapy. *Sempervivum marmoreum* Griseb. is a monocarpic perennial Crassulaceaean, with 12-16-merous flowers, with white-rose petals sometimes striated medially with pink-red or red-burgundy to dark red streaks. Leaves are ciliated, glandular-hairy on both sides to glabrescent. *S. marmoreum* Griseb is a carpato-balcanic species, with a range between 15°E-27°E meridians reaching towards East the western borders of Turkey and the Adriatic shores of Croatia towards West.(3,4) In Romania, it is found along with other medicinally-important Crassulacean species in the entire country, where it has a certain ecological amplitude, in varied biotopes from the shores of Danube to the highest peaks of the Romanian Carpathians, (at elevations varying between 50-2550m a.s.l.), with a climate and

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pedology also very diverse, this species occupying mostly dry habitats in rocky zones with a preference for higher altitudes.(5,6,7,8,9) Previous phytochemical research summarized in (10) demonstrated the presence in the leaf juice of *Sempervivum tectorum* L. (-a very closely related species to *S. marmoreum* Griseb.) of an entire array of compounds with known biological activity.(11,12,13). In this direction, Saša et al citing (10) older works states that "the benefits of juice and tea prepared from the leaves of *S. tectorum* can be attributed to the presence of many bioactive compounds, such as oligomeric and polymeric polyphenols, phenol carboxylic acids, ascorbic, citric malic acids, flavones- and flavonol-mono- and diglycosides with kaempferol and quercetin as aglycones, tannins, coumarines, oligo- and polysaccharides". Reviewing the mentioned compounds indeed points in the direction of antioxidant and protective effect of *S. tectorum* extracts against oxidative injuries, including the ones sustained by the normal, non-tumoral healthy cells during chemo- and radio-therapeutic procedures performed in cancer treatment. Previous experimental work demonstrated antioxidant, anti-inflammatory proprieties of *S. tectorum* extracts in several experimental models, fact which suggests a potential *in vivo* hemato-protective effect useful in the chemotherapy with clastogenic agents. Thus, Saša et al (10) cite the report of (14) stating that the extract of *S. tectorum* exhibited superoxide scavenger activity in a cell-free system, which suggested that this extract might also act as a direct scavenger of O_2^- in biological systems *in vivo*. Blázovics et al. (13) and Saša et al (10), -citing another article of Blazovics group (15), notice free radical scavenger and membrane stabilizing effects of *S. tectorum* extract in a rat system model

Sempervivum marmoreum Grieseb. is a species of the genus *Sempervivum* closely systematically related to *S. tectorum* L, with which shares a lot of common morphological characteristics in both reproductive organs (flower colour and shape, seed structure, pollen structure) and in vegetative parts (leaf succulent structure and shape, stem morphology, etc) to the point they were mistaken often in the later centuries in many botanical references.(5) Thus, the existence of Sempervivum tectorum as native plant in Romanian Carpathians was controversial (5) and more recent data (Barca et Niculae, unpublished) confirm that many of the early citations of *S. tectorum* L from Romania are in fact based on confusions with *Sempervivum marmoreum* Grieseb. specimens. The two species also exhibit in common a lot of ecological traits and environmental requirements, like their ability to live in arid dry environments, similar pollination and seed dispersal systems, etc. Moreover, they both share the same type of biochemistry and leaf morphological apparatus, both displaying a particular category of metabolism known as "Crassulacean Acid Metabolism" (CAM) that allows them to avoid oxygen toxicity during the light phase of CO_2 fixation of the photosynthesis, thus permitting them to open the stomata only during humid episodes at night and thus save water in water-deficient, dry seasons. Both species are also known to be winter hardy plants, capable of withstanding (after a period of proper acclimatization) long periods of very low temperatures, due to their high contend of mucopolysacharides that prevent frost-induced cell damage.

Romanian traditional herbal medicine extensively uses the juice extemporaneously squeezed from fresh leaves of members of the genus Sempervivum s.l. in the ear to alleviate pain and inflammations in the external and medial otitis, the plant being widely known as "urechelnita" or "iarba de ureche" (meaning "ear herb" or "herb for ear") (16), but also for other domestic uses.(17) Moreover, in recent years, the relative increased popularity of the species of the genus Sempervivum s.l. lead to conservation problems for some wild populations.(18) Based on the available pharmacological data regarding several species of *Sempervivum* and upon the above-mentioned correlations between the phylogeny and the metabolism and between the systematics and the metabolism of these *Sempervivum* species, we hypothesize that the extract obtained from *Sempervivum marmoreum* Griseb. (SEMEX) should have anticlastogenic modulating effect of the alkilant cytostatics. The anticlastogenic modulation effect should operate by modifying the efficiency with which the double strand breaks (DSBs) result in final fragmentation of DNA molecules in the cells. The cells also respond to alkilant injuries by increasing the efficiency of the DNA strand repair mechanisms, which tend to reduce the effect of DSB in living cells. At his experimental stage we propose to investigate the *overall* modifications induced by the treatment with SEMEX over the clastogenic effect of the alkilant CCNU, as evidenced by DNA fragmentation variations. This variation in DSB production and/or in repair mechanisms is expressed by modifications in the pattern of molecular fragmenting of DNA, with respect to size distribution or of frequency distribution pattern of various molecular weight fragments in the cells, evidenced by modifications in molecular frequency (MW) frequency distribution/size distribution in the purified DNA isolated form the treated cells. One method used in order to demonstrate the size distribution of DNA fragments from the treated cells is DNA Electrophoresis. Due to the phenomenon of reptation during electrophoretic migration of long filamentous molecules like DNA, conventional electrophoresis can only separate DNA fragments smaller than 20kbp. Larger DNA fragments migrate with lower but similar speed as they align themselves with the vector of the electrical field, ending at the top of the gel, in an unresolved, continuous long, intense, fuzzy band. Pulse Field Gel Electrophoresis (PFGE), invented in 1982 (19,20) resolves the long molecules of chromosome-sized DNA by alternating the direction of the vector of electric field forcing long DNA molecules to reorient themselves periodically and thus advance through the gel at speeds proportional to their molecular weight. This technique allows effective separation of much larger DNA molecules. Clamped Homogeneous Electrical Field (CHEF) – PFGE is a modern variation of the original pulsed field electrophoresis principle, which also uses pulsating but homogeneous electrical field (21,22) and allows good separation of DNA molecules up to 8-10MBp, in straight lanes.(23)

In this experimental study, we have evaluated the modulatory effect of the *Sempervivum marmoreum* Griseb. extract upon DNA fragmentation induced by the alkylant cytostatic CCNU in nucleated blood cells of Wistar rats. The DNA fragmentation in the treated cells was evaluated using the PFGE-CHEF technique to separate the damaged DNA by size classes. The fragmentation patterns were visualized and comparatively analysed by gel densitometry of the DNA bands stained with ethidium bromide, a conventional intercalating DNA fluorescent stain.

MATERIALS AND METHODS

Biological Plant Material and Extract Preparation

To obtain the plant extract we have used freshly harvested leaves from mature plants of *Sempervivum marmoreum* Griseb. maintained for more than 25 years in standard experimental culture from wild stock originating from Banat region, Southwest Romania. Mature, rosular peripheral leaves were first homogenized in a blender and the juice was immediately expressed, filtered, centrifuged. The pure extract thus obtained was immediately used directly as such, and/or the

50%, and 25% serial dilutions were prepared extemporaneously with isotonic saline solution.

Experimental Animals and Treatments followed a previously described protocol (Barca et al. in press) as follows: We have used young, healthy male Wistar rats, grown under standard laboratory conditions, weighing 150±10g. Two blood smears with acridine-orange supravital stain were made for each individual and then the individuals were randomly distributed in 8 treatment lots comprising 5 individuals each, as follows:

- Lot 1 - Positive control for SEMEX; - treated with 100% extract, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal.
- Lot 2 - Positive control for SEMEX; - treated with 50% extract, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal.
- Lot 3 - Positive control for SEMEX; - treated with 25% extract, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal.
- Lot 4 - Positive control for CCNU; - treated with CCNU, i.p. at day 4, at a dose of 10mg/Kg(b.W.)/animal.
- Lot 5 - treated with 100% extract, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal, followed by i.p. treatment with CCNU at day 4, at a dose of 10mg/Kg(b.W.)/animal.
- Lot 6 - treated with 50% extract, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal, followed by i.p. treatment with CCNU at day 4, at a dose of 10mg/Kg(b.W.)/animal.
- Lot 7 - treated with 25% extract, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal, followed by i.p. treatment with CCNU at day 4, at a dose of 10mg/Kg(b.W.)/animal.
- Lot 8 – Negative control, treated with isotonic saline solution, 7 days, by esophageal gavage at a dose of 20µL/g/day/animal.

The experimental work was performed before 2010, but experimental design and all procedures were in complete agreement with the recommendations of Directive 2010/63/EU. The number of animals used was the minimum required for statistical relevance of the experiments, and the 3R principle was observed. Special care was taken to avoid any stress or unnecessary pain to the animals and all procedures were performed gently and swiftly to minimize discomfort for the animals. All procedures involved are presently ranked as "superficial" or milder, and at the end of the treatments the animals were humanely euthanased as several organs and tissues were harvested.

DNA Preparation: Animals where CO_2 anesthetized, euthanased by cervical dislocation and then quickly decapitated, blood and tissues were immediately removed and processed. The nucleated blood cells separated from the harvested blood were immediately included in agarose blocks. The cells were treated osmotically and enzymatically within the agarose blocks to obtain purified intact DNA for CHEF-PFGE electrophoresis, according to standard protocols provided together with the CHEF-PFGE gear by Biorad.(23) The integrity of the cells and nuclei was controlled using the standard Tripan-Blue Exclusion Test with 0.2%Tripan Blue.

CHEF-PFGE Procedures: Obtaining the agarose miniblocks (plugs). Cells suspended in suspension buffer were included in gel agarose 1% in 1 x Tris-acetate-EDTA standard buffer (TAE); or in 0.5x TBE. The resulting miniblocks (plugs) were suspended in lysis buffer having the following composition: - ProteinaseK 15mg/ml, L-Sarcosine 0 .35mg/ml in TAE buffer with 0,5M Ethylenediaminetetraacetic acid (EDTA) and incubated 24h at 5^{o}C for cellular and nuclear lysis and for deproteination under continual agitation. After lysis, the miniblocks were stored until migration in standard TAE buffer with 0.5M EDTA. Immediately prior to the CHEF electrophoreses, the plugs were washed and suspended in 1x TAE.

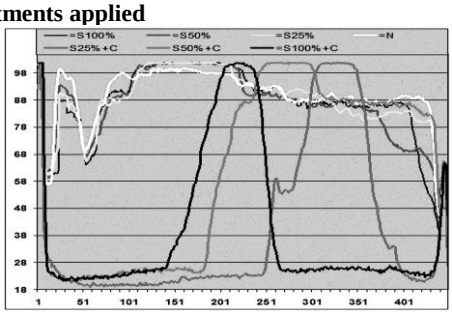
Preparation of the electrophoresis gels. The electrophoresis gels were standard 0.8% agarose gels prepared from migration agarose for CHEF (from Bio-Rad) in 1X TAE, stored for 1hour after setting in the fridge at +4C under 1xTAE for hardening.

MW markers:

- Pulse Marker 225-2.200KB (Sigma)
- DNA marker from Schizosaccharomyces pombe (3.5-5.7 mb) -CHEF DNA size Markers S. pombe DNA(Bio-Rad 170-3633)

Gel staining was done with the standard protocol with 0.5mg/mL ethydium-bromide, in distilled water for 40 minutes. Gel processing and analysis. *Image capture was performed using a Gel Doc imaging system produced by BioRad. The densitometric analysis was performed using the software Quantity One provided by the manufacturer BioRad. The migration lanes and bands were manually adjusted individually for each gel. The values of the optical density in 430 de points were read from the middle of the lanes and exported in tabular format for the comparative analysis of the migration patterns and the results presented in the form of graphical plots.*

Figure no. 1. Whole-gel (Rf 1-450) densitometric profiles for the treatments applied



RESULTS

The densitometric analysis of the DNA electrophoretic migration for the treatments shows several densitometric profiles with different distribution of density maxima (see figure no. 1) that indicate the following effects:

1. Consistent modification the DNA fragmentation profiles under the clastogenic action of the alkylant CCNU combined with the SEMEX administration, versus both negative control and controls positive for CCNU alone and/or for SEMEX alone;
2. Dose-dependent modification of the DNA fragmentation profiles indicating a dose dependent modulation of DNA fragmentation exerted by the 3 concentrations (25%, 50%, 100%) of SEMEX upon the clastogenic effect of CCNU.
3. Evidence of a surprising intrinsic clastogenic effect of SEMEX when administered alone as shown by the inversion of maximal densitometric values in the peak at Rf between 10-50; as illustrated in figure no. 1.

DISCUSSIONS

The consistent modification of the DNA fragmentation profiles suggests a protective effect of SEMEX against the clastogenic activity of CCNU, either by decreasing DSB number and/or increasing of the postclastic DNA-repair efficiency. The present findings do not allow a separation of

action mechanisms resulting in the observed modulation exerted by the SEMEX upon CCNU clastogenic effect, but several lines of thought come forward. The first mechanism potentially involved in the anticlastogenic modulation of SEMEX could be the potent antioxidant and radical-scavenger proprieties of the extracts from Sempervivum sp, as mentioned in (24) The somewhat surprising intrinsic mild clastogenic effect of SEMEX when administered alone could suggest as the most probable mechanism (among several others) a depression of DNA mismatch repair (MMR) pathway. This lack of MMR competence could explain the mildly increase in the observed DNA fragmentation due to unrepaired DSB caused by additional, non chemotherapy-related factors, while decreasing the DSB production efficacy under the action of CCNU which relies also on functional MMR pathway to eventually transform the O6-Me-guanine adducts into DNA-fragmenting, observable, DSBs. CCNU is a bi-functional alkylating agent and consequently, it produces inter-and intra-strand crosslinks (ICL) that are extremely efficient in arresting the replication forks. ICL recovery involves extremely complex repair mechanisms whose failure finally results in DSBs that, if un-repaired lead to cell death or cell cycle arrest. It is also conceivable that any sequence of this recovery mechanism could be influenced by compounds in SEMEX. Thus, SEMEX could modulate CCNU clastogenic effect either by enhancing the efficacy of ICLs recovery and with subsequent reduction of the DSB incidence, or by G2/M cell cycle arrest and succeeding evasion of further DNA damage and cell death. This observed aspect warrants the continuation of experimental work in this direction towards a better understanding of the mechanisms involved in the modulatory action of the extract. Finally, the findings of this study recommend the extract from *Sempervivum marmoreum* Griseb. as a viable candidate for further investigations of the possibility of its use as adjuvant in chemotherapy with nitrosoureic alkylating agents against brain tumours. This important potential use of this species also prompts for studies regarding the propagation, cultivation and general biology of this species in Romania, similar to other species of this family.(25)

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