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ORIGINAL PAPER

IN VITRO activity of β-amirinic phytocompounds – a basic screening for breast cancer therapy

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Abstract

As a result of biotechnological research concerning the indigene phytotherapeutic potential follow-up, we obtained two β-amirinic compounds (GSO-1 and GSO-2) with antitumorous indications. In order to define the specific target action of these compounds, we developed an "in vitro" experimental model for antiproliferative status evaluation on adherent, epithelial type breast adenocarcinoma (MCF-7 cell line). The biocompounds were tested at different concentrations, at 24 and 48h cell culture treatment, as a screening of most important parameters for antitumorous activity (cell proliferation, DNA synthesis, apoptosis). The target parameters were obtained by cellular analysis methods as microscopy and flow cytometry, using competitive software. The results marked a significant and differentiated activity of GSO-1 and GSO-2. The first one is a relevant apoptosis inducer, but the second compound acts as an inhibitor for MCF-7 cells proliferation and DNA synthesis.

Keywords: β-amirinic compounds, MCF-7 cell line, cell proliferation, DNA synthesis, apoptosis.

Introduction

Cancer patients usually receive chemotherapeutic agents during the course of their illness, treatment based on cell destruction potential by growth interruption. Many plants could have potent anticancer effects with soft adverse reaction and could be a natural source of very active pharmaceutical compounds (ex. vinblastine, vincristine, podophyllotoxin, taxol). The latter was extracted from species of the genus *Taxus*, constituting the most used antineoplastic agent at the present time [1].

However, the precise pathways through which these molecules produce an antitumor effect has not been yet fully characterized, also because their mechanism of action appears to be dependent to the type of tumor cell under study [2]. It has been reported that antitumoral drugs can act through different cellular mechanisms, e.g., by inducing apoptosis, cell-cycle arrest, or cell growth inhibition, but also by targeting angiogenesis and cell migration [3].

Tumourigenesis is the result of cell cycle disorganisation, leading to an uncontrolled cellular proliferation [4]. Specific cellular processes-mechanisms that control cell cycle progression and checkpoint traversation through the intermitotic phases are deregulated. Normally, these events are highly conserved due to the existence of conservatory mechanisms and molecules such as cell cycle genes and their products: cyclins, cyclin dependent kinases (Cdks), Cdk inhibitors (CKI) and extra cellular factors (i.e. growth factors) [5]. Revolutionary techniques using flow cytometry and specific software are available to quantify and evaluate cell cycle processes and cellular growth. S-phase fraction measurements, including ploidy values, using histograms and estimation of indices such as the mitotic index and tumour-doubling time indices, provide adequate information to the clinician to evaluate tumour aggressiveness, prognosis and the strategies for therapy in experimental researches [6], [7].

Apoptosis is a morphological and biochemical distinct form of programmed cell death that occurs in many cell types, MCF-7 being widely utilized to study this process. In apoptotic cells, phosphatidilserine (PS) - normaly found on the internal part of the membrane - becomes

translocated to the external possition, this being considered an early event that precedes the DNA fragmentation. Annexine V is a 35-36 kDa proteine binding phospholipids in a Ca^{2+} dependent process, with high affinity for PS.

The number of cell replications (cell proliferation) was assessed with the aid of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA). CFSE (carboxy fluorescein diacetat succin imidil ester) is a cell permeant fluorescein-based dye which covalently attaches to cytoplasmic components of cells, resulting in uniform bright fluorescence. Upon cell division, the dye is distributed equally between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry [8]. We designed our studies in order to explore the effect of two amirinic compounds on apoptosis, DNA synthesis and proliferation of MCF-7 cell line. These compounds are triterpenic structures derivatives of the same β-amirinic nucleus, oleanan bi-glycosidate (GSO-1) and oleanan penta-glycosidate (GSO-2) and were obtained in Biotehnos laboratories as result of original technologies.

Materials and methods

Cell culture: MCF-7 - (Human Caucasian breast adenocarcinoma, originating from ECACC- 86012803). Cells were cultured in EMEM with 10% fetal bovine serum, 2mM Glutamine, under standard culture conditions (37°C, 95% humidified air and 5% CO_2), harvested 48h before treatment, and 24 h or 48 h with tested substances.

The optimal doses for GSO-1 and GSO-2 were established during viability tests on MCF-7cell line. We compared GSO-1 effects with culture control and GSO-2 with solvent control at appropriate dilution. As positive control, 5nM Methotrexate – a well known citostatic drug - was used.

Equipments: Flow cytometer FACS CANTO II with DIVA 6 software and MODFIT LT V2.0 for DNA analysis.

VIABILITY TESTS - TRYPAN BLUE EXCLUSION METHOD

Reagents: trypan blue (Sigma)

The dye exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented below, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm

DNA STAINING FOR FLOW CYTOMETRY CELL CYCLE ANALYSIS

Reagents: Cycle TEST PLUS DNA Reagent (BD PHARMINGEN).

After dissolving the cell membrane lipids with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA and stabilizing the nuclear chromatin with spermine, propidium iodide was stoichiometrically bound to the clean, isolated nuclei; stained nuclei emited fluorescent light primarily at wavelenghts between 580 and 650 nm. Flow cytometry detection was done with a detector equipped with a 585/42 bandpass filter, used to analyze light emitted by stained cells. The resulting fluorescence histograms was analized to detect S-phase modulation (DNA synthesis) [9].

CELL PROLIFERATION ASSAY – FLOW CYTOMETRIC ANALYSIS OF CELL DIVISION BY DYE DILUTION (CFSE STAINING)

Reagents: Cell Trace CFSE Cell Proliferation Kit (Invitrogen)

CFSE staining technique is based on 10μM CFSE incubation of cells for 10 min at 37°C, followed by quenching with culture medium with 10% fetal bovine serum [10]. After harvesting, cells are analysed through flow cytometry, in terms of quantifying the green fluorescence emission of CFSE labelled cell generations on FITC-area scale.
Results are presented by calculating the proliferative index - (PI) - the sum of the cells in all generations divided by the calculated number of original parent cells (*specific soft FCS Express*). It is useful for comparing quantity of cell division between cultures of the same cells undergoing different treatments. GSO-1 and GSO-2 effects were estimated as % of proliferation variation for each sample:

$$\% \text{ of proliferation variation} = (\text{IP sample} \times 100 / \text{IP control})$$

APOPTOSIS EVALUATION THROUGH PHOSPHATIDIL SERINE (PS) TRANSLOCATION

Reagents: ANNEXIN V - FITC apoptosis detection Kit (BD PHARMINGEN)

The procedure consists of the binding of Annexin V-FITC to PS in the membrane of cells which are begining the apoptotic process and the binding of propidium iodide to the cellular DNA in cells where the cell membrane has been totally compromised. Annexin V-FITC is detected through flow cytometry as green fluorescence, PI is detected as red fluorescence [11].

Results and discussion

Cell viability assay

We performed trypan blue staining in order to define optimum concentration for significant biological activity on MCF-7 cell line. Five concentrations were tested by successive dilutions between 162.5 μM and 8.125 μM for GSO-1 and for GSO-2 between 120.2 μM and 6.14 μM (Fig.1, Fig. 2).

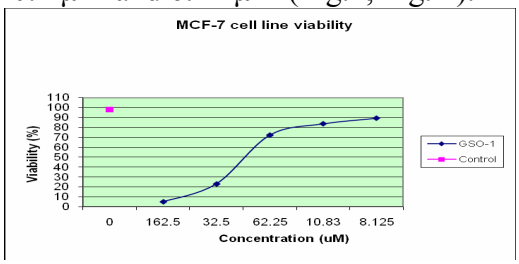


Fig. 1. MCF-7 cell line viability in presence of GSO-1

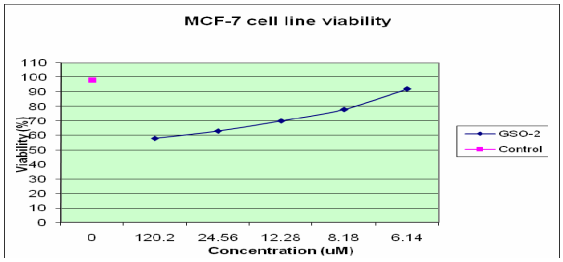
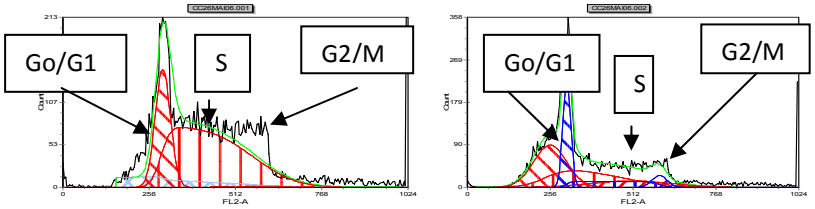


Fig. 2. MCF-7 cell line viability in presence of GSO-2

For further investigation only samples with viability over 60% were considered. MCF7 cell line was more resistant to GSO-2 action, thus the corresponding viabilities for efficacy testes were obtained beginning with 16.25 μM for GSO-1 and 24.56 μM for GSO-2.

DNA synthesis assay

We studied cell cycle using kinetic measurements of culture evolution at 24 and 48 h incubated with GSO-1 (two concentrations: 16.25 μM and 8.12 μM) and GSO-2 (three concentrations: 24.56 μM, 12.28 μM and 6.14 μM). Fig. 3 represents a selection of flow cytometry results: propidium iodide fluorescence histograms showing cell cycle Go/G1, S, G2/M phases modulation induced by GSO-2 12.28μM. The obtained results based on flow cytometry analysis are presented in table 1(average of 5 experiments) and graphics (Fig. 4-7).



A: control B: GSO-2: 12.28µM

Fig. 3. Selection of flow cytometry results: Cell cycle S-phase reduction in MCF-7 cell line treated with GSO-2 12.28 µM.

Table 1. MCF-7 cell cycle modulation induced by GSO-1 and GSO-2

Sample / µM	G0/G1		S		G_2/M	
	24 h	48 h	24 h	48 h	24h	48 h
Control	23.98	25.75	59.01	70.2	17.02	4.01
Solvent control c1	23.5	12.41	61.2	86.56	15.3	1.03
Solvent control c2	23.6	12.2	58.3	84.2	18.1	3.6
GSO-1/16.25	33.1	25.7	52.3	68.4	14.6	5.9
GSO-1 /8.12	40.2	42.75	42.5	56.37	17.3	0.88
GSO-2 /24.56	38.8	43.11	49.1	54.6	12.1	2.32
GSO-2 /12.28	39.3	46.24	46.41	51.91	14.27	2.47
GSO-2 /6.14	39.06	41.32	57.9	58.48	3	0.21
Methotrexate 5nM	52.6	60.3	30.2	32.6	17.2	7.1

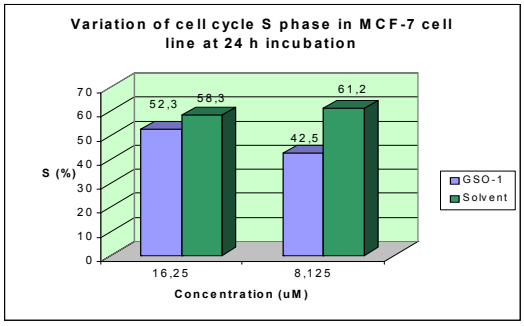


Fig. 4. Variation of cell cycle S phase in MCF-7 cell line at 24 h incubation with GSO-1

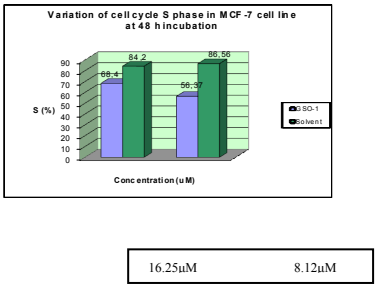


Fig. 5. Variation of cell cycle S phase in MCF-7 cell line at 48 h incubation with GSO-1

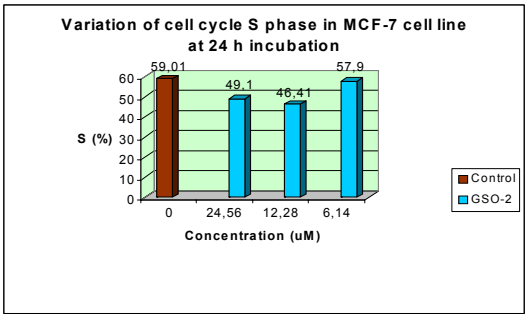


Fig. 6. Variation of cell cycle S phase in MCF-7 cell line at 24 h incubation with GSO-2

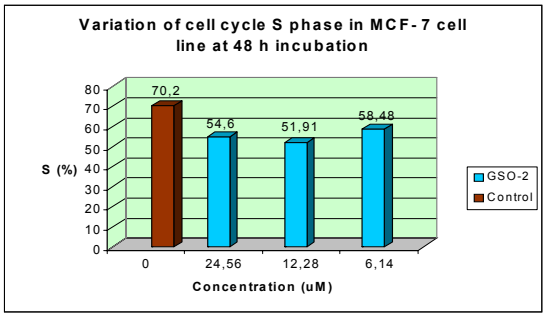


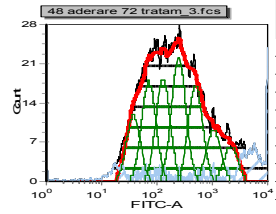
Fig. 7. Variation of cell cycle S phase in MCF-7 cell line at 48 h incubation with GSO-2

The optimal time of action regarding the S phase of cell cycle is 48 h. After this period of time the percent of cells being in S phase is diminished up to 30% compared with the solvent control, both for GSO-1 in concentration of 8.125 µM and the three progressive dilutions of GSO-2 (in a dose dependent manner).

There are no significant differences (< 5%) between tested concentrations.

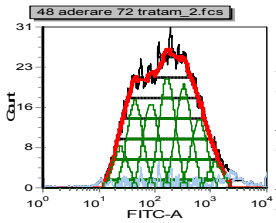
Cell proliferation

After cell cycle evolution previously discussed we selected 48 h as optimum incubation time of GSO-1 and GSO-2. A selection of flow cytometry results is presented in Fig. 8.



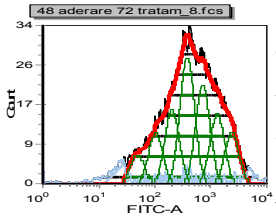
Generation	Generation #	Peak Channel	Peak Value	Log Std Dev	Log CV	% of Orig Cells
Undivided Cells	0.00	0.00	0.00	0.00	0.00	0.00
Generation 1	1.00	0.00	0.00	0.00	0.00	0.00
Generation 2	2.00	1860.08	6.00	47.94	5.61	5.75
Generation 3	3.00	938.98	9.00	47.94	6.20	8.64
Generation 4	4.00	461.38	17.00	47.94	6.95	16.41
Generation 5	5.00	235.01	22.00	47.94	7.83	21.18
Generation 6	6.00	118.64	18.00	47.94	8.88	17.49
Generation 7	7.00	69.16	18.00	47.94	10.11	16.94
Generation 8	8.00	36.19	12.00	47.94	11.91	10.95

A: control



Generation	Generation #	Peak Channel	Peak Value	Log Std Dev	Log CV	% of Orig Cells
Undivided Cells	0.00	1104.00	5.00	52.42	6.64	4.30
Generation 1	1.00	562.34	13.00	52.42	7.33	12.78
Generation 2	2.00	319.08	20.00	52.42	8.15	19.34
Generation 3	3.00	153.99	21.00	52.42	9.21	21.08
Generation 4	4.00	77.04	16.00	52.42	10.61	16.02
Generation 5	5.00	42.17	17.00	52.42	12.46	16.40
Generation 6	6.00	23.08	8.00	52.42	14.79	7.18

B: 16 µM GSO-1



Generation	Generation #	Peak Channel	Peak Value	Log Std Dev	Log CV	% of Orig Cells
Undivided Cells	0.00	2186.97	11.00	47.23	5.47	9.76
Generation 1	1.00	1207.90	15.00	47.23	5.92	13.58
Generation 2	2.00	667.14	21.00	47.23	6.46	19.33
Generation 3	3.00	352.27	27.00	47.23	7.16	25.22
Generation 4	4.00	192.82	16.00	47.23	7.99	14.32
Generation 5	5.00	97.34	11.00	47.23	9.14	9.68
Generation 6	6.00	42.55	6.00	47.23	10.91	5.37

C. 5 nM Methotrexate

Fig.8. Selection of flow cytometry results (FCS Express software): MCF-7 biding proliferation induced by GSO-2:**A:** basic proliferation of MCF-7 cell line (control);**B:** 16 µM GSO-1 action on MCF-7 proliferation. **C:** 5 nM Methotrexate – positive control

It was noticed the decrease of the proliferative number of generations and the rise of the % of originating cells in parent population induced by GSO-1, similar with the citostatic drug Methotrexate. Next table and graphics show the effects on cell proliferation as % of cells accumulated in parent generation and proliferation index (Table 2, Fig.9 and Fig.10) for all the studied samples. For a better understanding of GSO-1 and GSO-2 effects on MCF-7 cell line we represent % of proliferation variation compared with control cells considered 100% of proliferation.

Table 2: MCF-7 cell line proliferation induced by GSO-1 and GSO-2

Sample(µM)	**% cells in parent population (0 generation)**	**Proliferation index (IP)**	**% variation of proliferation (compared with appropriate control)**
Untreated control	0	2.9	100
Solvent control 1/1000	0	3.6	100
Solvent control 1/2000	0	3.4	100
GSO-1 /16.25	4.3	2.8	77.8
GSO-1 /8.12	10.5	2.7	79.4
GSO-2 /24.56	14.6	2.5	86.2
GSO-2 /12.28	17.5	1.9	65.5
GSO-2 /6.14	16.2	1.7	58.62
Methotrexate 5nM	9.76	1.8	62.06

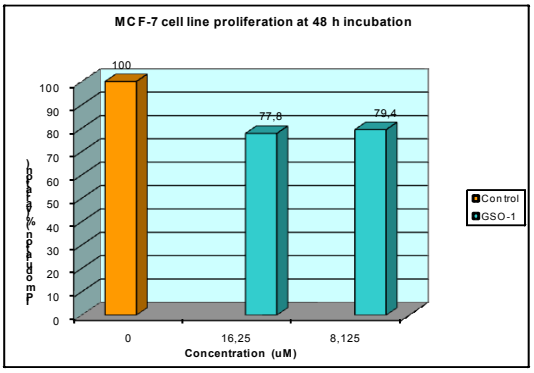


Fig. 9. MCF-7 cell line proliferation at 48 h incubation with GSO-1

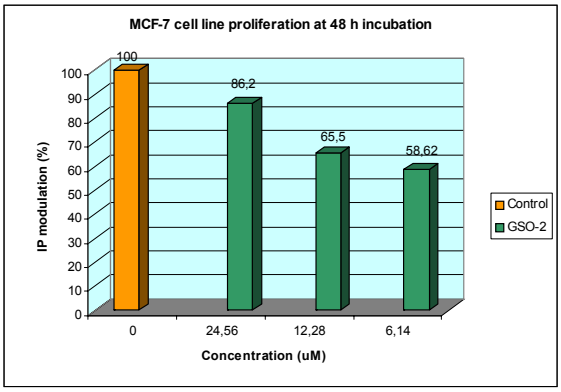


Fig.10. MCF-7 cell line proliferation at48 h incubation with GSO-2

It can be seen a biding of proliferation induced by GSO-1 and GSO-2, showed by increased number of cells in 0 generation and proliferative index. Both compounds stopped the DNA synthesis, arresting the cell cycle in G0/G1, and as a consequence, the number of proliferative generations decreased.

APOPTOSIS: Apoptosis induction was investigated after 48 h of cells cultivation with GSO-1 and GSO-2. Annexin-V FITC (apoptotic cells, expressing phosphatidylserine externalisation) and PI stained cells (late-apoptotic or necrotic cells, with disrupted membrane) are shown in Fig. 11 as "dot-plot" representation (FITC-A/PE-A) and fluorescence histograms (FITC-A- phosphatidyl serine positive). Percent of live/ death cells or early/late apoptosis where estimated as % of cells in Q1/Q2/Q3/Q4 quadrants with Diva 6 software statistics and presented in Table 3 and Fig. 12-18.

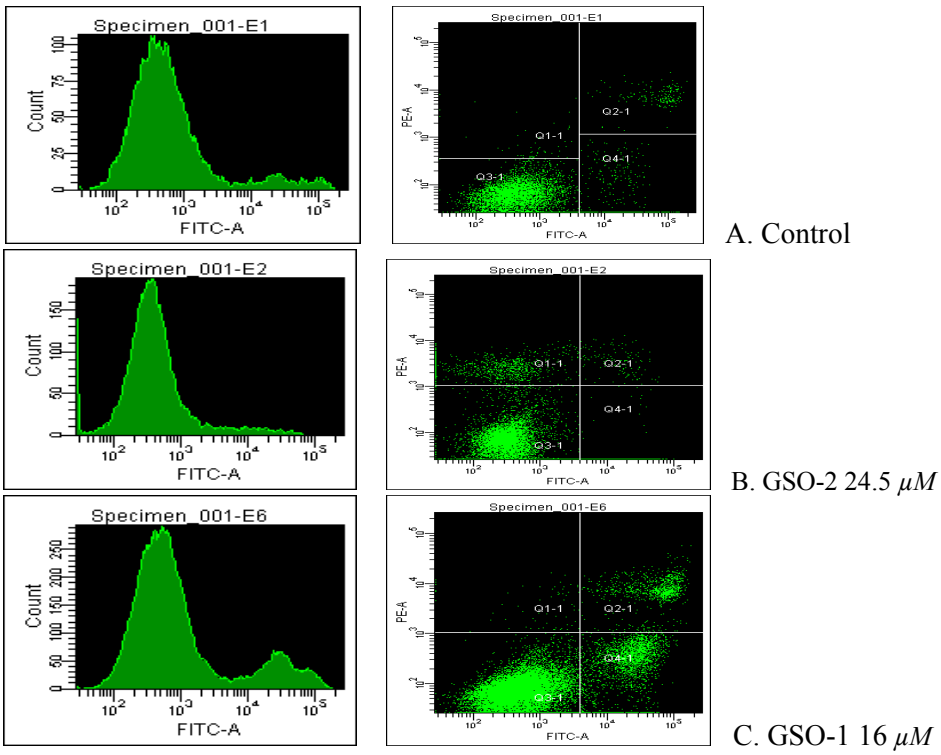


Fig. 11. Selection of flow cytometry results: Apoptosis induction of 16µM GSO-1, 24.5 µM GSO-2 on MCF-7 cell line

Table 3. MCF-7 apoptosis induction at 48 h of GSO-1 and GSO-2 action

Sample (µM)	% live cells	% death cells	% early apoptosis	% late apoptosis
Control	74.02	15.52	3.54	6.93
Solvent c1	69.08	23.14	4.32	3.48
Solvent c2	76.53	12.69	2.37	8.42
GSO-1 /16.25	61.58	15.13	18.32	4.98
GSO-1 /8.12	73.21	11.23	7.42	8.14
GSO-2 /24.56	57.03	38.7	1.12	3.15
GSO-2 /12.28	60.03	26.28	4.45	9.23
GSO-2 /6.14	61.08	27.07	3.24	8.59
Methotrexate 5nM	56.81	16.31	20.34	6.54

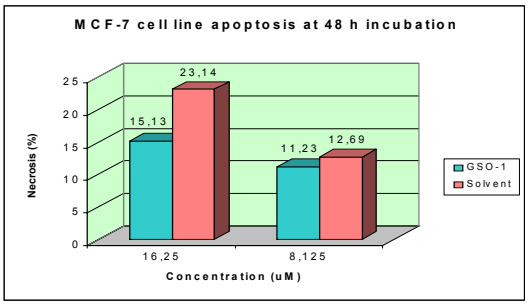


Fig.12. MCF-7 cell line necrosis at 48 h incubation with GSO-1

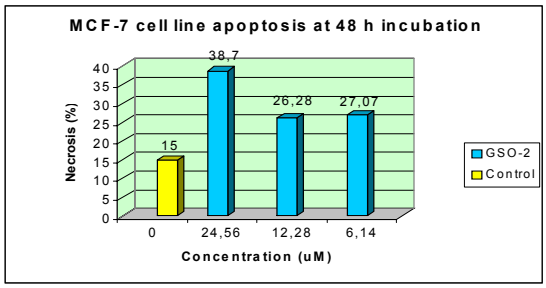


Fig.13. MCF-7 cell line necrosis at 48 h incubation with GSO-2

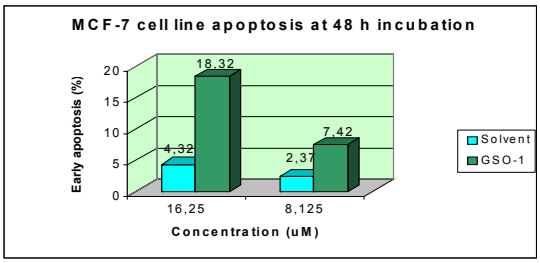


Fig.14. MCF-7 cell line apoptosis at 48 h incubation with GSO-1

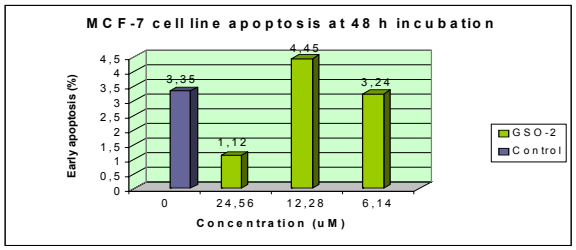


Fig.15. MCF-7 cell line apoptosis at 48 incubation with GSO-2

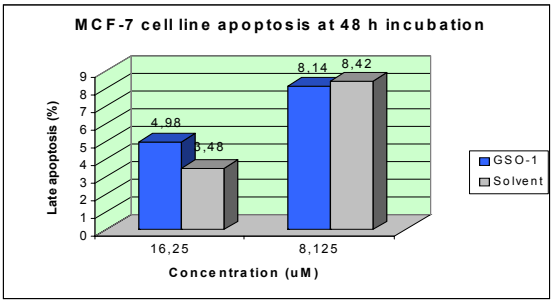


Fig.16. MCF-7 cell line apoptosis at 48 h incubation with GSO-1

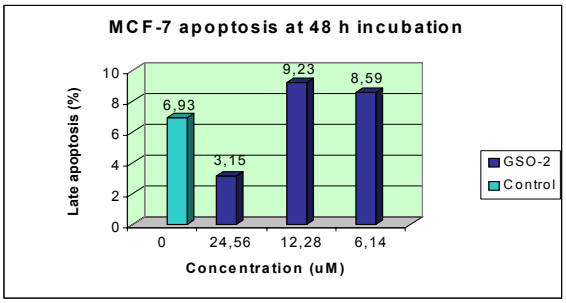


Fig17. MCF-7 cell line apoptosis at 48 h incubation with GSO-2

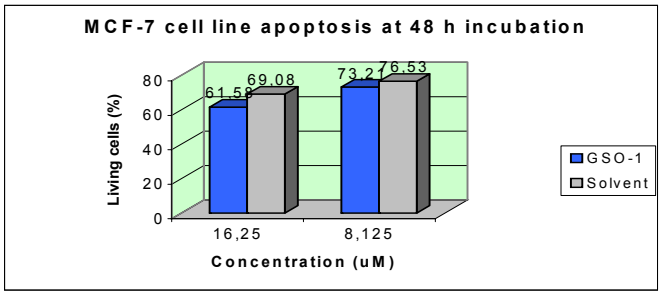


Fig. 18. MCF-7 living cells percent at 48 h incubation with GSO-1

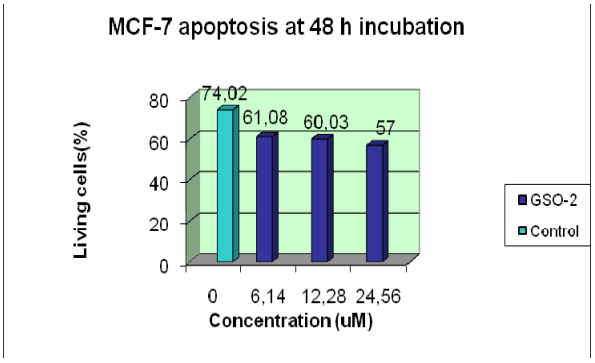


Fig. 19. MCF-7 living cells percent at 48 h incubation with GSO-2

The GSO-1 effect (16.25 μM) was remarkable in inducing phosphatidylserine externalization, raising with 16% the cells presenting this early event in apoptosis evolution. GSO-2 has rather a toxic cellular effect, rising the percent of necrotic cells.
It can be stated that on MCF-7 cell line the optimal time of action of these substances is 48 h. GSO-1 16.25 μM is the most efficient modulator for apoptosis induction, as well as for inhibition of cell proliferation. GSO-2 inhibits the MCF-7 cells proliferation and DNA synthesis but does not have a significant effect on apoptosis.

Conclusions

This study permitted a comprehensive evaluation of antitumor profile of GSO-1 and GSO-2, Biotehnos self-developed substances. The most effective solution for cellular resistance to tumor therapy is to specifically attack the target cell by different convergent mechanisms. Assessing proliferation, cell cycle arrest and apoptotic end-points in MCF-7 cell line could be first steps for anticancer drug development. We improve our "in vitro" screening by applying the fast, quantitative and reliable technique of flow cytometry.

G0/G1 cell cycle arrest induced by both GSO-1 and GSO-2 is reflected in the decrease number of proliferative generations. In addition, GSO-1 16.25 μM induce early apoptosis in breast cancer cells.

This manner of GSO-1 and GSO-2 synergic action (antiproliferative and pro-apoptotic effects) would be an important step to future research for therapeutic breast cancer improvement.

Acknowledgments

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