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**Effects of Bio-Normalizer Alone or in Combination
with Anticancer Drugs on Cancer Cells.
A Preliminary Study**

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INTRODUCTION

Since oxygen radicals have been implicated in the genotoxic events which can initiate and promote cancer development (for review: Ames *et al.*, 1995), numerous clinical trials have been carried out involving anticarcinogenic effects of natural or synthetic antioxidants (for review: German, 1995). Moreover, experimental and clinical studies have also indicated that some natural antioxidants enhance therapeutic treatment of established carcinomas. Mechanisms for antitumor activity of antioxidant compounds generally involve cell differentiation and stimulation of the immunological defense. Recent studies have also revealed some potentiality for direct inhibition of cell growth.

Numerous experimental results are consistent with the assumption that the cellular environment becomes more prooxidizing during differentiation (Allen, 1991; Nagy *et al.*, 1993; Nagy *et al.*, 1995). Generally, it is assumed that antioxidants are inhibitory to differentiation in many types of cells (Takenaga *et al.*, 1981; Allen, 1991; Allen & Venkatraj, 1992). However, natural retinoids and carotenoids have inhibited the growth of several tumors *in vivo* due to increasing the rate of differentiation of malignant cells (for review: Cornic *et al.*, 1994; Bertram & Bortkiewicz, 1995). Consequently, all-*trans* retinoic acid has been used with success in clinical trials, allowing complete remission in acute promyelocytic leukemia patients (Warell *et al.*, 1991). Differentiation is mediated by nuclear retinoid receptors which play an important role in the control of gene expression (Pfalz *et al.*, 1994).

Interestingly, natural antioxidants such as d-limonene, β -carotene, canthaxanthin, and α -tocopherol have caused regression of tumors, exhibiting similarities in anticancer responses with certain chemotherapeutic agents (Elegbede *et al.*, 1986; Shklar *et al.*, 1987; Schwartz & Shklar, 1988; Schwartz *et al.*, 1989; Shklar *et al.*, 1989; Schwartz & Shklar, 1992). For example, α -tocopherol inhibits tumor cell growth through a process that reduces free radical production (Schwartz *et al.*, 1993). α -Tocopherol bound to membrane associated proteins inhibits the development of peroxidation products. The reduction of peroxidation products directly alter the induction of transcription factors such as *c-fos* and *c-myc*, genetic products that promote the proliferation of tumor cells (Amstad *et al.*, 1990; Stoler, 1991). Available evidence also indicates that α -tocopherol inhibits the expression of mutant p53, while increasing the expression of the wild type form (Schwartz *et al.*, 1993). α -Tocopherol may regulate phospholipid products, ultimately modifying calcium flux and membrane kinase activity. A reduction in kinase activity may affect cyclin activity enhancing antioncogene expression such as p53.

Therefore, strong indications support that natural antioxidants can exhibit antiproliferative activity and would probably yield to new drugs in cancer therapy. Nevertheless, high doses of antioxidant compounds were generally required to obtain cytostatic activity *in vitro*, and cause toxic side effects *in vivo*. Thence, the search for natural antioxidants with low toxicity and high free radical quenching activity is of critical interest. In the present study, we have investigated the biological action and toxicity of the natural free radical scavenger Bio-Normalizer (Santiago *et al.*, 1991; Santiago *et al.*, 1992a).

Among the different analysis performed, cell proliferation assays have been carried out, on cisplatin-sensitive and -resistant human ovarian adenocarcinoma cells, in presence of Bio-Normalizer alone or in combination with two anticancer agents (cisplatin and melphalan). As a matter of fact, recent studies suggest that antioxidants may also find a role in conjunction with cytotoxic chemotherapy. Several natural antioxidant compounds have been used to limit the toxicity due to prooxidant effects of anticancer drugs (Sundstrom *et al.*, 1989; Bienvenu *et al.*, 1992; Stahelin, 1993; Vasavi *et al.*, 1994). Furthermore, other studies have demonstrated that antioxidants can also enhance the effectiveness of antitumor drugs by increasing the sensitivity of tumor cells to these drugs. For example β -carotene and α -tocopherol were able to potentiate the activity of anticancer drugs such as cisplatin and melphalan (Schwartz *et al.*, 1992; Teicher *et al.*, 1994). This ability could be of particular interest in order to overcome clinical resistance to platinum or alkylating agents.

I MATERIAL and METHODS

I.1. Reagents and Drugs. Owen's reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and phenazine methosulfate (PMS) were purchased from Promega, melphalan from Sigma Chemical Co., and *cis*-diamminedichloroplatinum(II) (cisplatin) from Laboratoires Roger Bellon.

I.2. Cell lines. Two experimental cellular models were used in this study. IGROV1 human ovarian adenocarcinoma cells grow as monolayers in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. This cell line exhibits an epithelial character, highly tumorigenic properties and a 24h doubling time (Bénard *et al.*, 1985). A cisplatin-resistant subpopulation, IGROV1-DDP, was established by stepwise escalation with increasing concentrations of cisplatin in the growth medium. Resistant cells were adapted to grow in presence of 1 μ g/ml cisplatin. The selection of cisplatin-resistant cells led to a cytologically heterogeneous cell population. Two morphological types coexist; small mononuclear cells, morphologically similar to parental IGROV1 cells, and enlarged polynuclear cells with heterogeneous karyotype characteristics. IGROV1-DDP cells, when exposed continuously to 1 μ g/ml cisplatin, have an average cell cycle time of 66 h. Growth conditions were at 37°C in an humidified atmosphere of 5% (v/v) CO₂ in air. Cells were passaged when they reached confluence by treatment with trypsin/EDTA solutions (0.05/0.02%), with a transfer factor of 1/4.

I.3. Cell proliferation assays. For these experiments, IGROV1 and IGROV1-DDP cells were grown in plastic 96-well microtiter plates. Cells in exponentially growth were exposed either to various concentrations of Bio-Normalizer, cisplatin or melphalan alone, or in combination. Incubation times were of 2h for cisplatin and melphalan, and 2 or 24h for Bio-Normalizer. After drug exposure, a recovery period of 48h for IGROV1 and 72h for IGROV1-DDP cells was performed, then cells were incubated during 2h with 333 μ g/ml MTS and 25 μ M PMS. Solutions of reagents and drugs were prepared just prior to addition to assay plates. MTS is bio-reduced by cells into a formazan by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan produced is directly proportional to the number of living cells in culture. After 4h of reaction, the absorbance of the formazan was measured at 490 nm with a Bio-Tek Instruments EL 307 microplate reader. 12 wells were analyzed for each condition of drug exposure in order to obtain a significant mean value of the absorbance. The statistical significance of differences between groups was assessed by the Student's t-test for paired samples ($P < 0.05$).

I.4. Chemical analysis. Retinoids, β -carotene and α -tocopherol, in 10 mg/l Bio-Normalizer solutions in K_2HPO_4 (5 mM, pH 8), were simultaneously quantified by liquid chromatography (HPLC). The HPLC method was a modification of that described previously (Simonoff *et al.*, 1992). Absorbances of α -tocopherol, retinoids, and β -carotene were measured respectively at 292, 325 and 436 nm. Selenium content of Bio-Normalizer was analyzed by particle induced X-ray emission (PIXE), using yttrium as internal standard. The methodology for PIXE analysis in food and biological samples has been described in (Simonoff *et al.*, 1988). Fatty acids in Bio-Normalizer lipid extracts were measured by gas chromatography. Methods of extraction and quantification of fatty acids were based on those described by (Lepage & Roy, 1986).

I.5. Cytological observations. IGROV1 and IGROV1-DDP cells were incubated during two weeks in presence of 4 mg/ml of Bio-Normalizer in complete culture medium. Cell morphology was examined during this high dose incubation by phase contrast light microscopy. Video images of the characteristic cell types of both cell lines were taken to be compared with controls. Growth characteristics (doubling times) were also controlled.

I.6. Nuclear Micro-Probe Analysis. NMPA was accomplished using the CENBG nuclear microprobe. The features of this device, and its capabilities for biological samples analysis, have been previously reported (Llabador *et al.*, 1990; Moretto *et al.*, 1993). The methodology for specimen preparation of IGROV1 cells has been described in (Ortega *et al.*, 1994). Platinum and trace metal were quantified in IGROV1 cells after 5h incubation with 10 μ g/ml cisplatin alone, or in combination with 1000 μ g/ml Bio-Normalizer. Large square areas ($500 \times 500 \mu m^2$) were irradiated to assess the average metal composition of each cell line. Samples were irradiated with a 1.0 nA beam of 2.5 MeV protons focused to a 5 μ m spot. Irradiation times of nearby 5 h were necessary for trace metal determination. High speed scans (1 ms per step) were performed to avoid thermal damage. Simultaneous measurements of particle induced X-rays and backscattered protons were achieved. PIXE spectra were treated with the GUPIX software (Maxwell *et al.*, 1989). Backscattered particles were collected with a 20 mm² silicon detector, at a scattering angle of 135°. The backscattering data were processed using an in house computer software, specifically designed for the treatment of spectra from biological samples (Moretto & Razafindrabe, 1995).

II RESULTS

II.1. Cell proliferation assays

II.1.a Bio-Normalizer alone. IGROV1 and IGROV1-DDP cells were incubated with various concentrations of Bio-Normalizer: 0, 0.1, 1.0, 10, 100 and 1000 μ g/ml. At these concentrations, no modification of IGROV1 cell proliferation could be noticed after 2h exposure (Fig.1), or after 24h exposure (Fig. 2). In the case of IGROV1-DDP cells, after 2h exposure, proliferation was virtually not modified except a slight enhancement at 1000 μ g/ml (Fig.3). After 24h incubation, IGROV1-DDP cell proliferation was slightly enhanced even at low concentrations of Bio-Normalizer (Fig. 4).

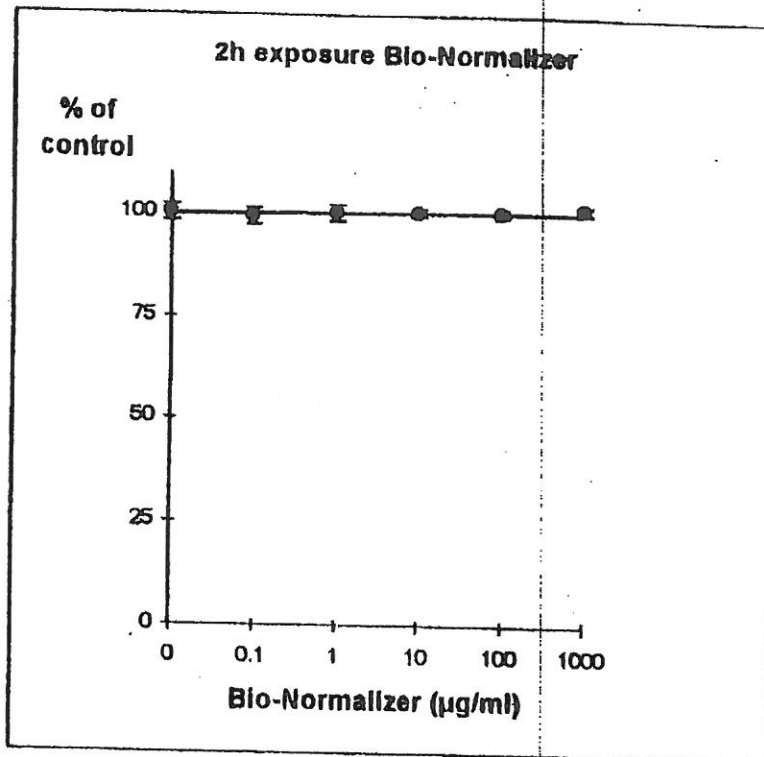


Figure 1. Effects of different concentrations of Bio-Normalizer on IGROV1 cell growth (expressed as % of control), after a short treatment (2h).

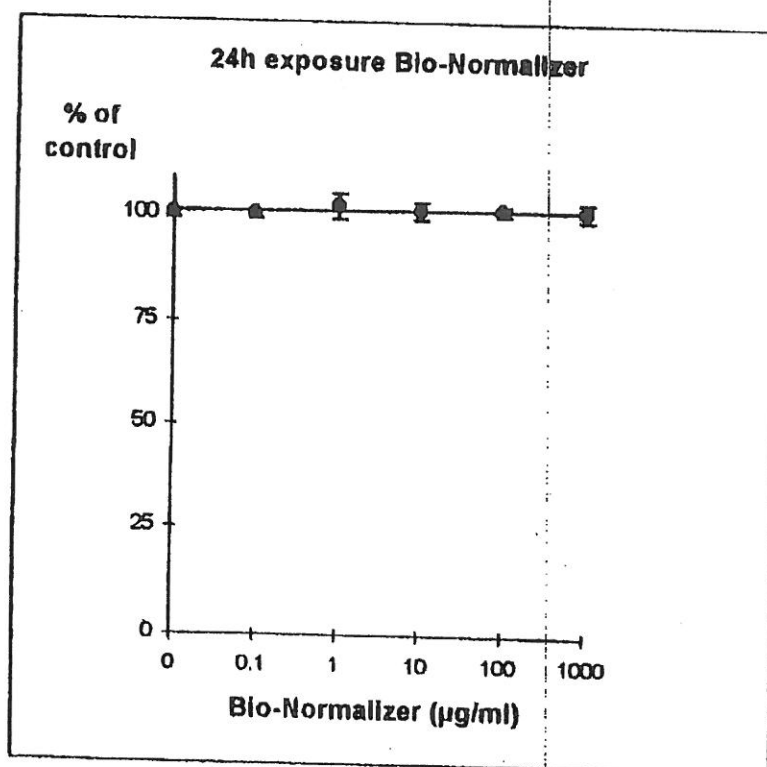


Figure 2. Effects of different concentrations of Bio-Normalizer on IGROV1 cell growth (expressed as % of control), after a long treatment (24h).

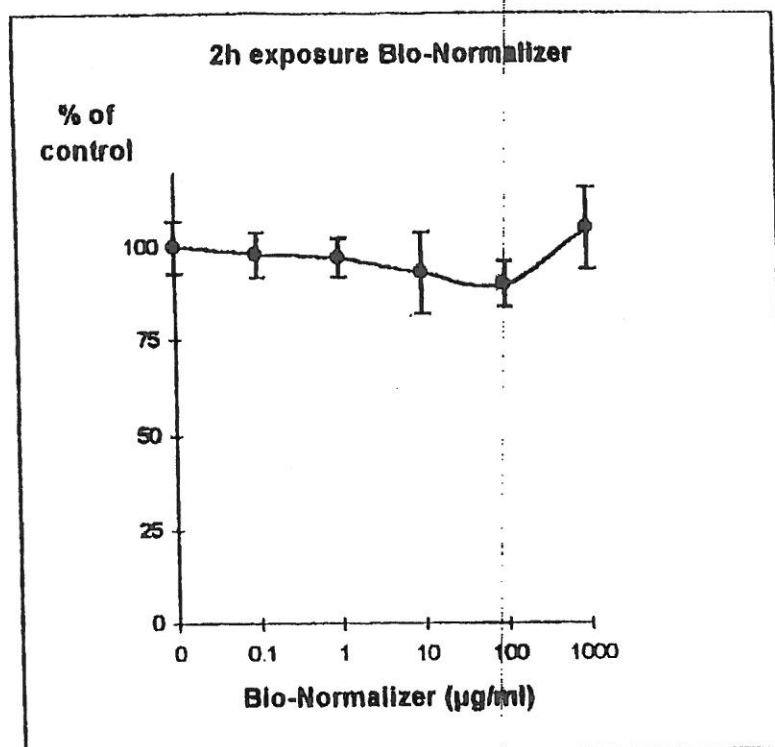


Figure 3. Effects of different concentrations of Bio-Normalizer on IGROV1-DDP cell growth (expressed as % of control), after a short treatment (2h).

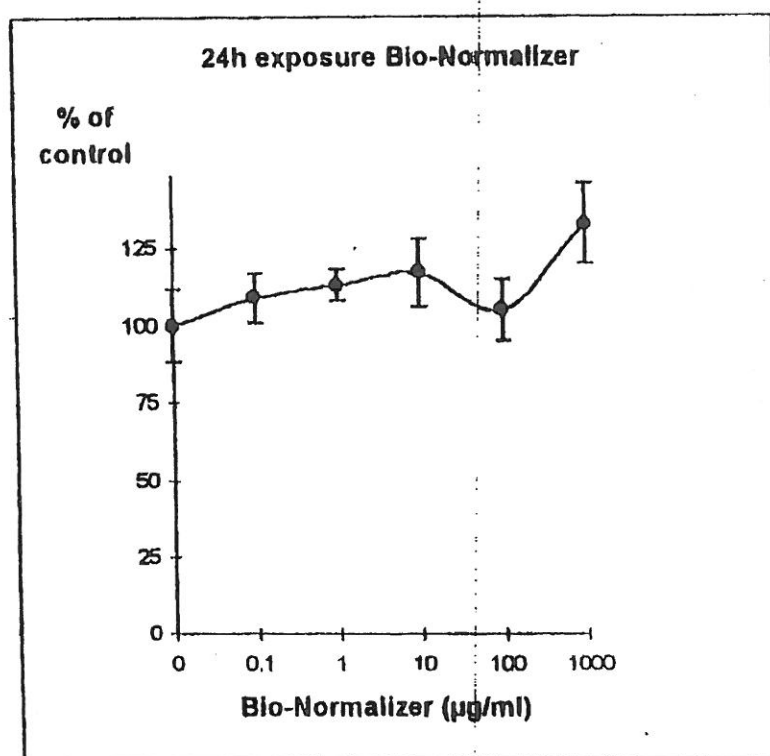


Figure 4. Effects of different concentrations of Bio-Normalizer on IGROV1-DDP cell growth (expressed as % of control), after a long treatment (24h).

II.1.b Determination of cisplatin IC₅₀. Cisplatin concentrations inhibiting 50% of cell proliferation (IC₅₀) were determined both on IGROV1 and IGROV1-DDP, after 2h of drug exposure (Fig. 5 and 6): for IGROV1, IC₅₀ = 1 µg/ml cisplatin; for IGROV1-DDP, IC₅₀ = 85 µg/ml cisplatin.

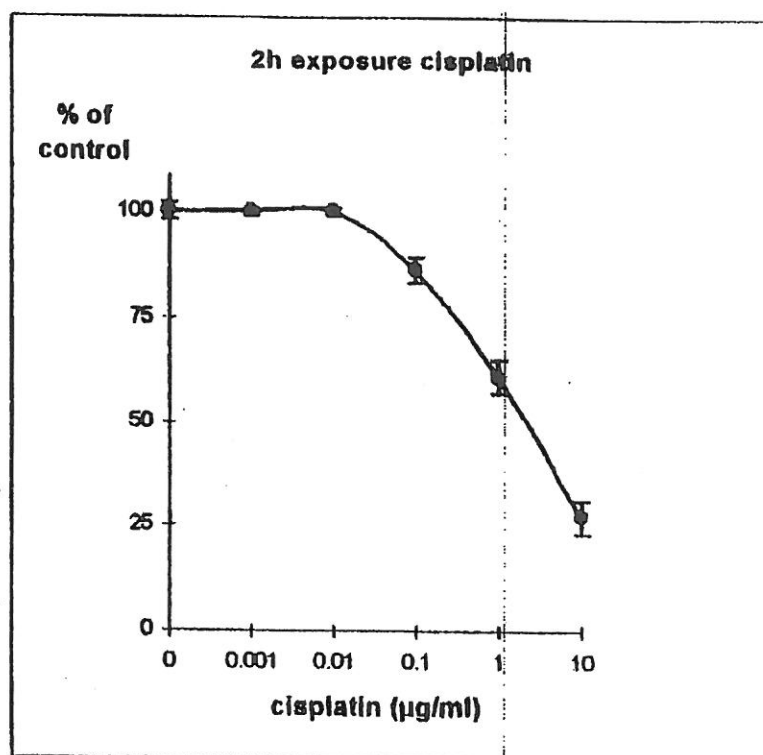


Figure 5. Effects of different concentrations of cisplatin on IGROV1 cell growth (expressed as % of control), after 2h exposure.

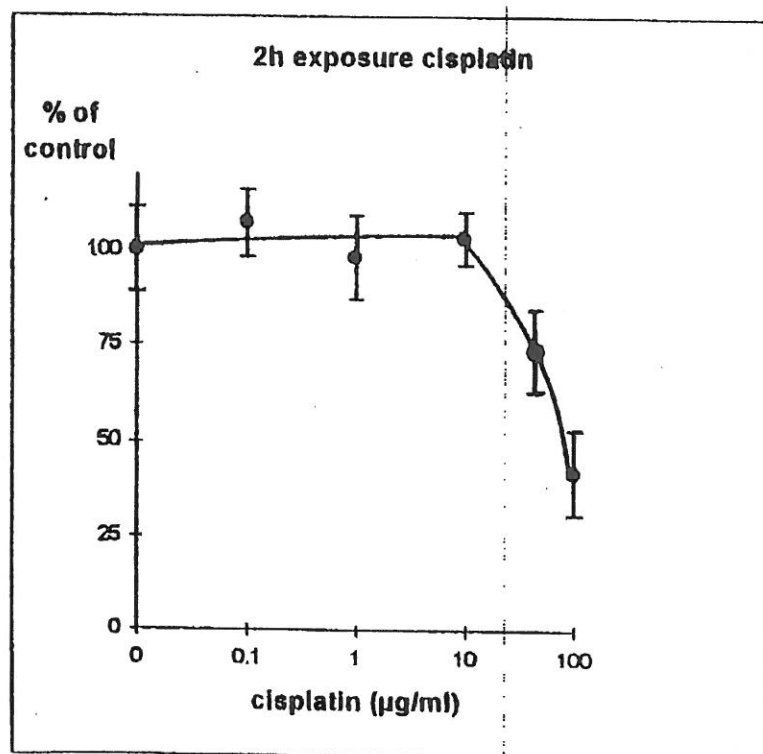


Figure 6. Effects of different concentrations of cisplatin on IGROV1-DDP cell growth (expressed as % of control), after 2h exposure.

II.1.c. Bio-Normalizer and cisplatin. IGROV1 and IGROV1-DDP cells were incubated during 2h with their respective IC50 concentrations of cisplatin, and simultaneously with various concentrations of Bio-Normalizer (0, 0.1, 1.0, 10, 100, 1000 $\mu\text{g/ml}$), or pre-incubated with these concentrations of Bio-Normalizer during 24h. For a short treatment on IGROV1 cells, cisplatin cytotoxicity was partially suppressed by Bio-Normalizer, even at low concentrations of Bio-Normalizer (0.1 $\mu\text{g/ml}$) (Fig. 7). Cell proliferation varied between 74% (at 1 $\mu\text{g/ml}$ Bio-Normalizer) and 80% (at 1000 $\mu\text{g/ml}$ Bio-Normalizer), instead of 50% normally observed for a 2h exposure with 1 $\mu\text{g/ml}$ cisplatin without Bio-Normalizer. The same phenomenon was observed, and amplified, when IGROV1 cells were pre-exposed during 24h to several concentrations of Bio-Normalizer (Fig. 8). In this case, cell proliferation reached 88% to 96% of control.

For IGROV1-DDP cells, after a short incubation with Bio-Normalizer, cisplatin cytotoxicity, measured after 2h exposure with 85 $\mu\text{g/ml}$ cisplatin, was totally suppressed. Cell proliferation compared to controls varied between 92 and 111%, instead of 50% without Bio-Normalizer (Fig. 9). The same was observed after 24h pre-exposure of IGROV1-DDP cells with Bio-Normalizer; cisplatin cytotoxicity was nearly totally suppressed with cell proliferation rates between 82 and 100% instead of 50% (Fig. 10).

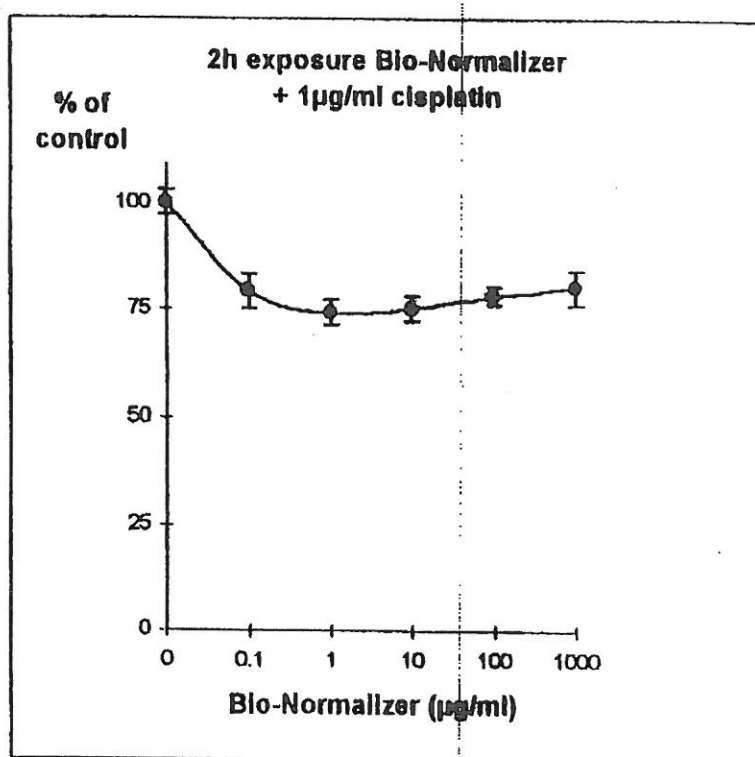


Figure 7. Effects of different concentrations of Bio-Normalizer on IGROV1 cell growth (expressed as % of control), after 2h with simultaneous exposure to 1 $\mu\text{g/ml}$ cisplatin.

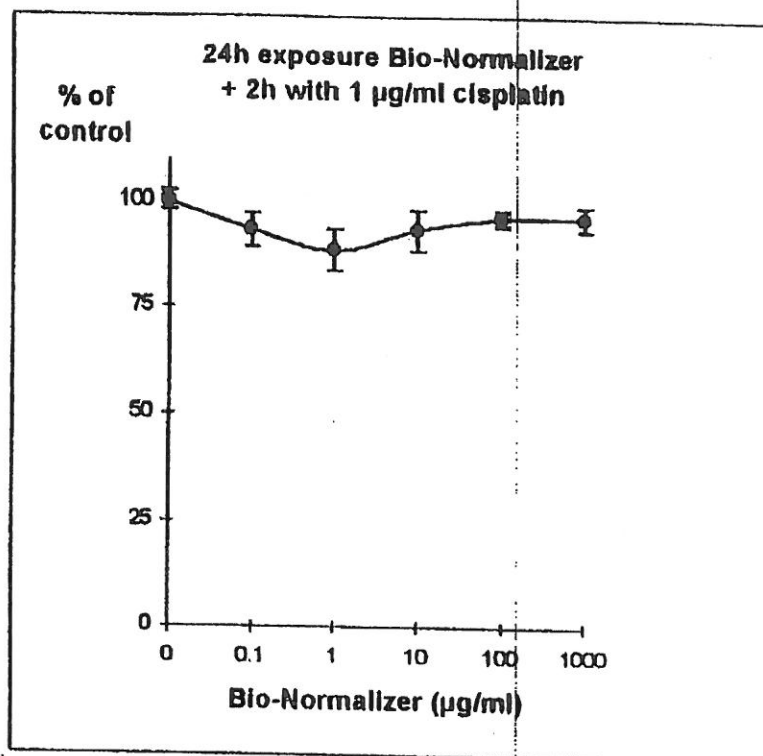


Figure 8. Effects of different concentrations of Bio-Normalizer on IGROV1 cell growth (expressed as % of control), after 24h pre-treatment and 2h exposure to 1 µg/ml cisplatin.

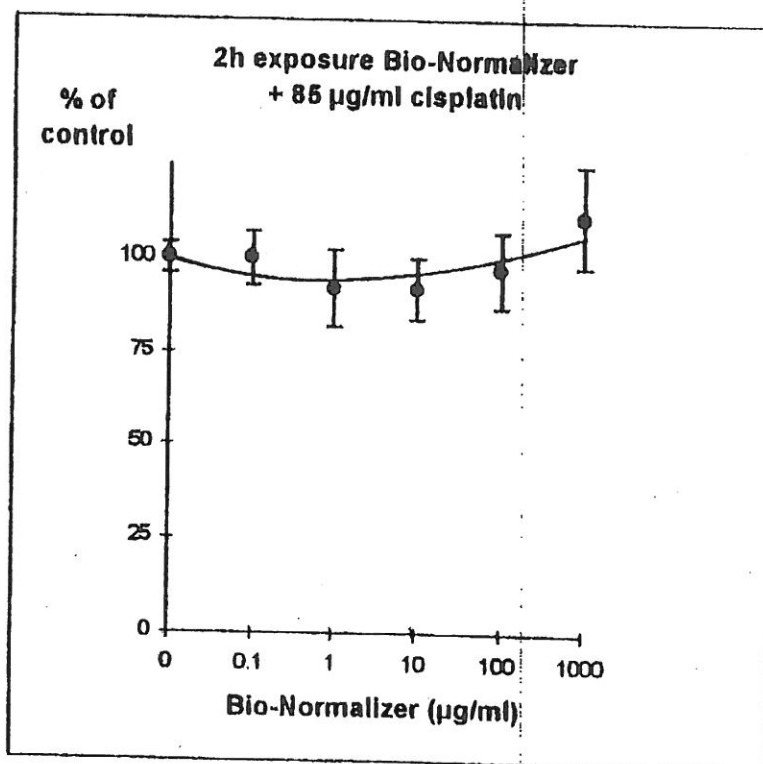


Figure 9. Effects of different concentrations of Bio-Normalizer on IGROV1-DDP cell growth (expressed as % of control), after 2h with simultaneous exposure to 85 µg/ml cisplatin.

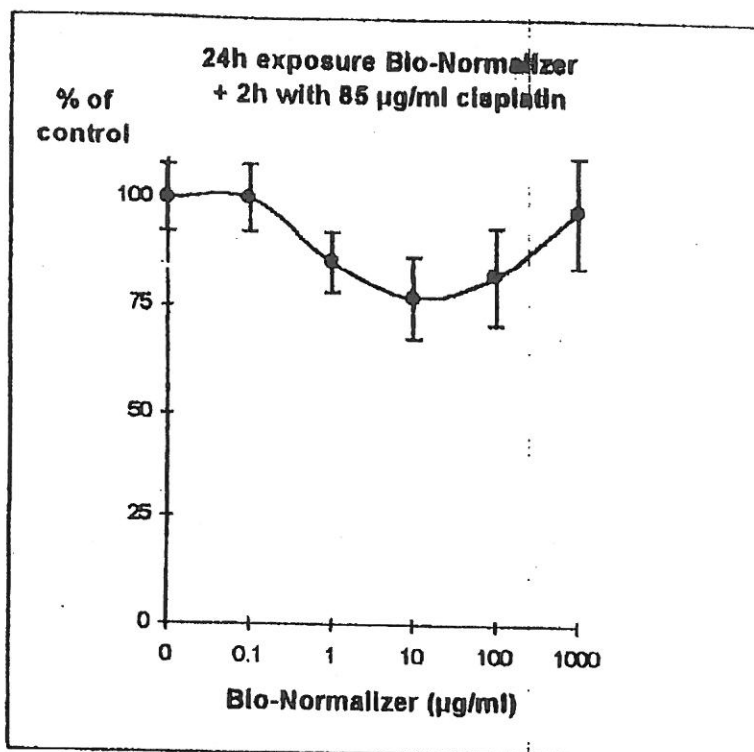


Figure 10. Effects of different concentrations of Bio-Normalizer on IGROV1-DDP cell growth (expressed as % of control), after 24h pre-treatment and 2h exposure to 85 µg/ml cisplatin.

II.1.d. Determination of melphalan IC₅₀. Melphalan concentration inhibiting 50% of cell proliferation (IC₅₀) was determined on IGROV1 cells, after 2h drug exposure (Fig. 11). For IGROV1, IC₅₀ = 95 µg/ml melphalan.

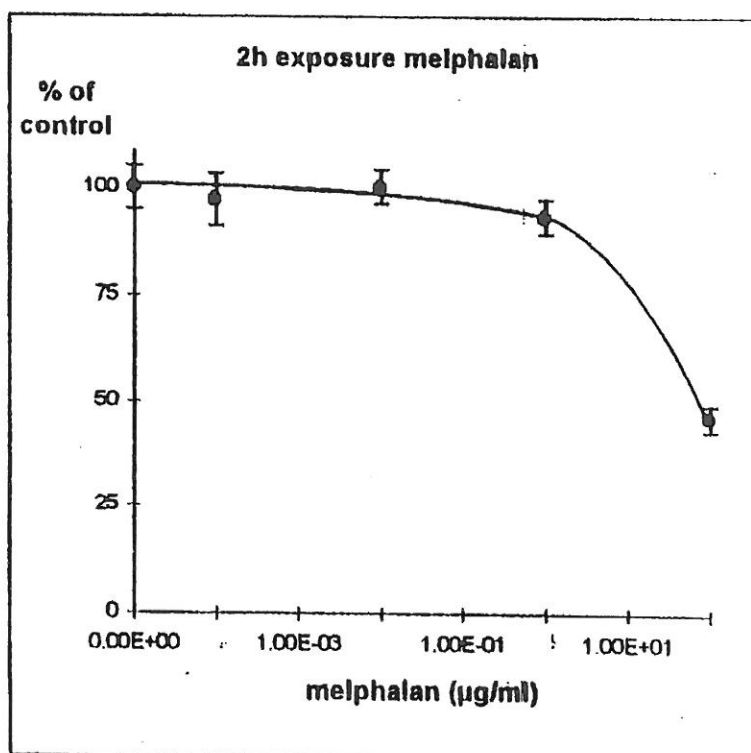


Figure 11. Effects of different concentrations of melphalan on IGROV1 cell growth (expressed as % of control), after 2h exposure.

II.1.e. Bio-Normalizer and melphalan. IGROV1 cells were incubated during 2h with 100 $\mu\text{g/ml}$ melphalan, and simultaneously with various concentrations of Bio-Normalizer (0, 0.1, 1.0, 10, 100, 1000 $\mu\text{g/ml}$), or pre-incubated with these concentrations of Bio-Normalizer during 24h. During simultaneous exposure with Bio-Normalizer and melphalan, the resultant cytostatic activity of melphalan was significantly modified (Student's t-test $P < 0.05$) (Fig. 12). Indeed, a moderate enhancement of cytotoxicity was noticed with a cell proliferation rate ranging from $33 \pm 7\%$ to $40 \pm 5\%$, when cells were simultaneously exposed to 100 $\mu\text{g/ml}$ melphalan and various concentrations of Bio-Normalizer. In comparison, the corresponding value was $46 \pm 3\%$ when cells were exposed only to 100 $\mu\text{g/ml}$ melphalan. However, melphalan cytotoxicity was not modified after 24h pre-incubation with various concentrations of Bio-Normalizer. In this case, cell proliferation varied between 44 ± 3 and $54 \pm 3\%$ for melphalan and Bio-Normalizer combinations ($46 \pm 3\%$ for melphalan alone) (Fig. 13). This observation was confirmed by long treatment with high doses of Bio-Normalizer (Fig. 14). Effectively, A 24h pre-incubation of IGROV1 cells with 4000 $\mu\text{g/ml}$ of Bio-Normalizer in the culture medium did not change cell proliferation rates after 2 h exposure to 100 $\mu\text{g/ml}$ melphalan.

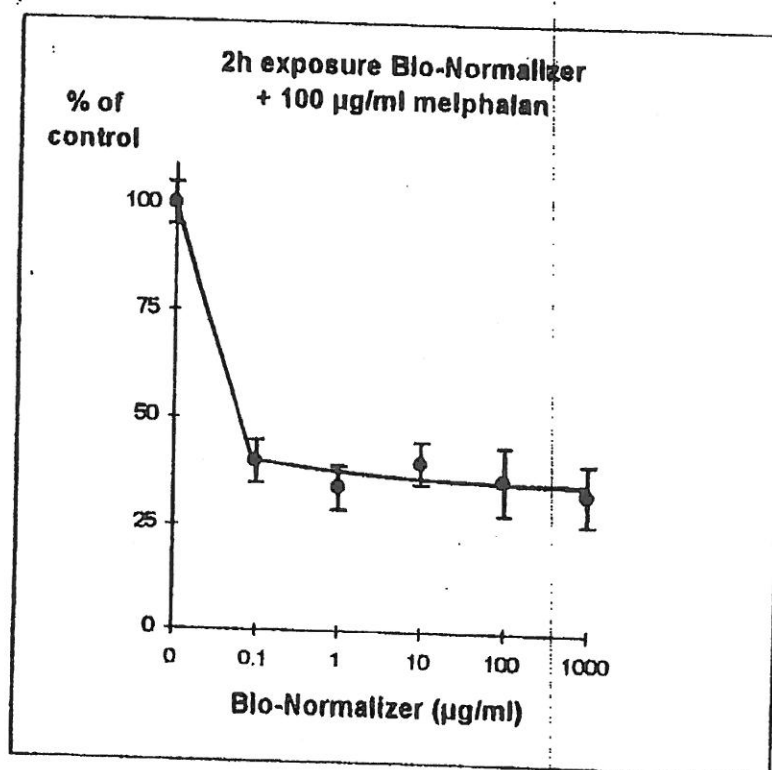


Figure 12. Effects of different concentrations of Bio-Normalizer on IGROV1 cell growth (expressed as % of control), after 2h with simultaneous exposure to 100 $\mu\text{g/ml}$ melphalan.

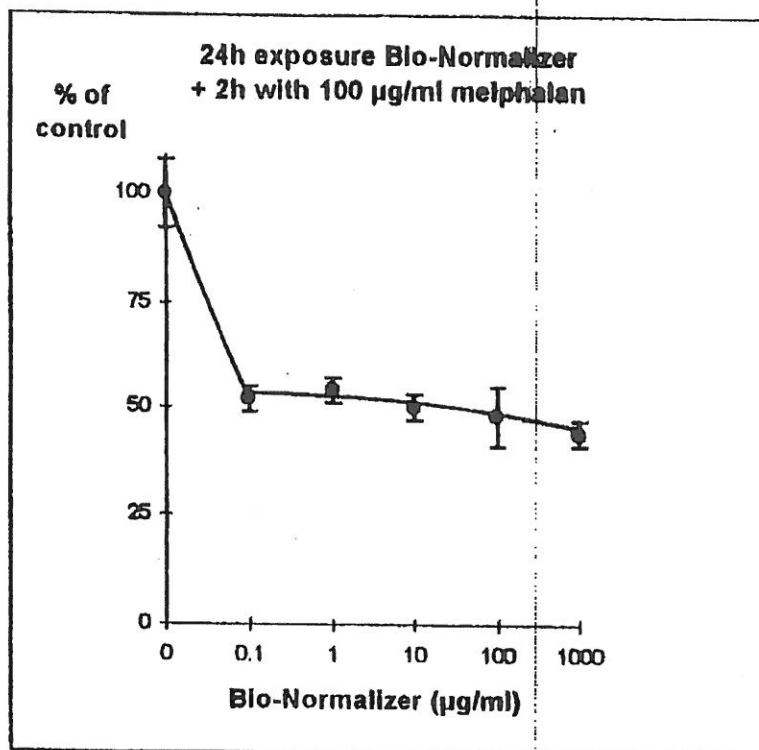


Figure 13. Effects of different concentrations of Bio-Normalizer on IGROV1 cell growth (expressed as % of control), after 24h pre-treatment and 2h exposure to 100 µg/ml melphalan..

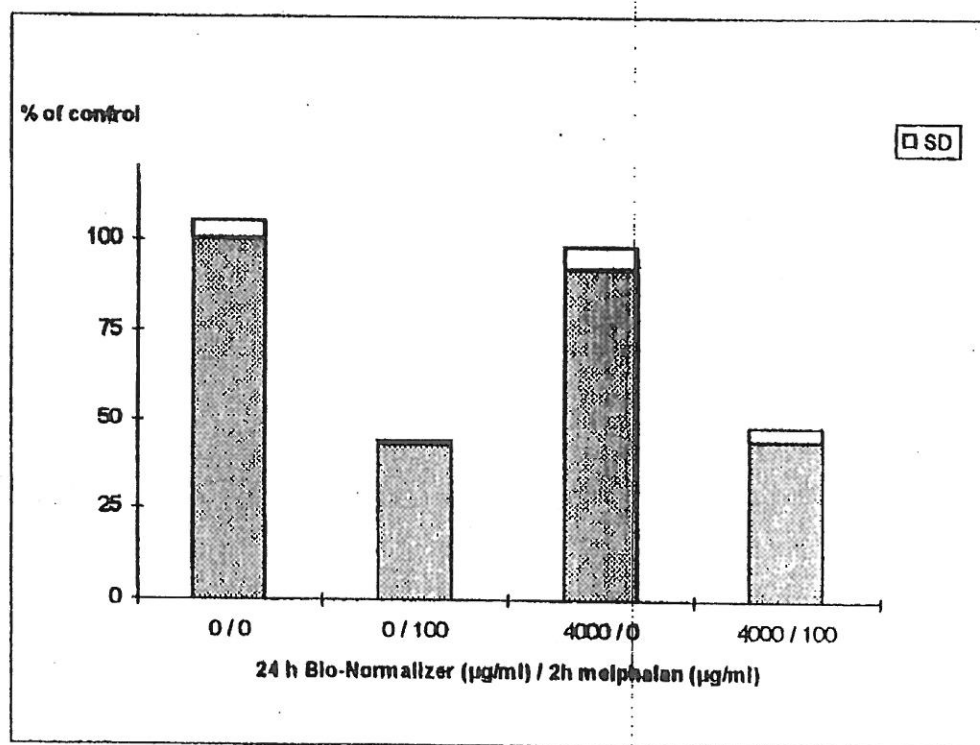


Figure 14. Effects of Bio-Normalizer (4000 µg/ml) on IGROV1 cell growth (expressed as % of control), after 24h pre-treatment with/without 2h exposure to 100 µg/ml melphalan.

II.2. Chemical analysis

II.2.a. Retinoids, β -carotene and α -tocopherol. The quantities of retinol, β -carotene and α -tocopherol extracted from solutions of 10 mg/l Bio-Normalizer in 5 mM K_2HPO_4 were under the limit of detection. Respective limits of detection for retinol, β -carotene and α -tocopherol were approximately 500, 100 and 3600 $\mu\text{g/g}$ of Bio-Normalizer. Retinoic acid has been detected within lipid extracts at $752 \pm 13 \mu\text{g/g}$ of Bio-Normalizer. Characteristic chromatograms corresponding to HPLC analysis of Bio-Normalizer extracts from 10 mg/ml solutions in K_2HPO_4 , and to control solutions with standards of retinoic acid and retinyl acetate are presented in Fig. 15 & 16.

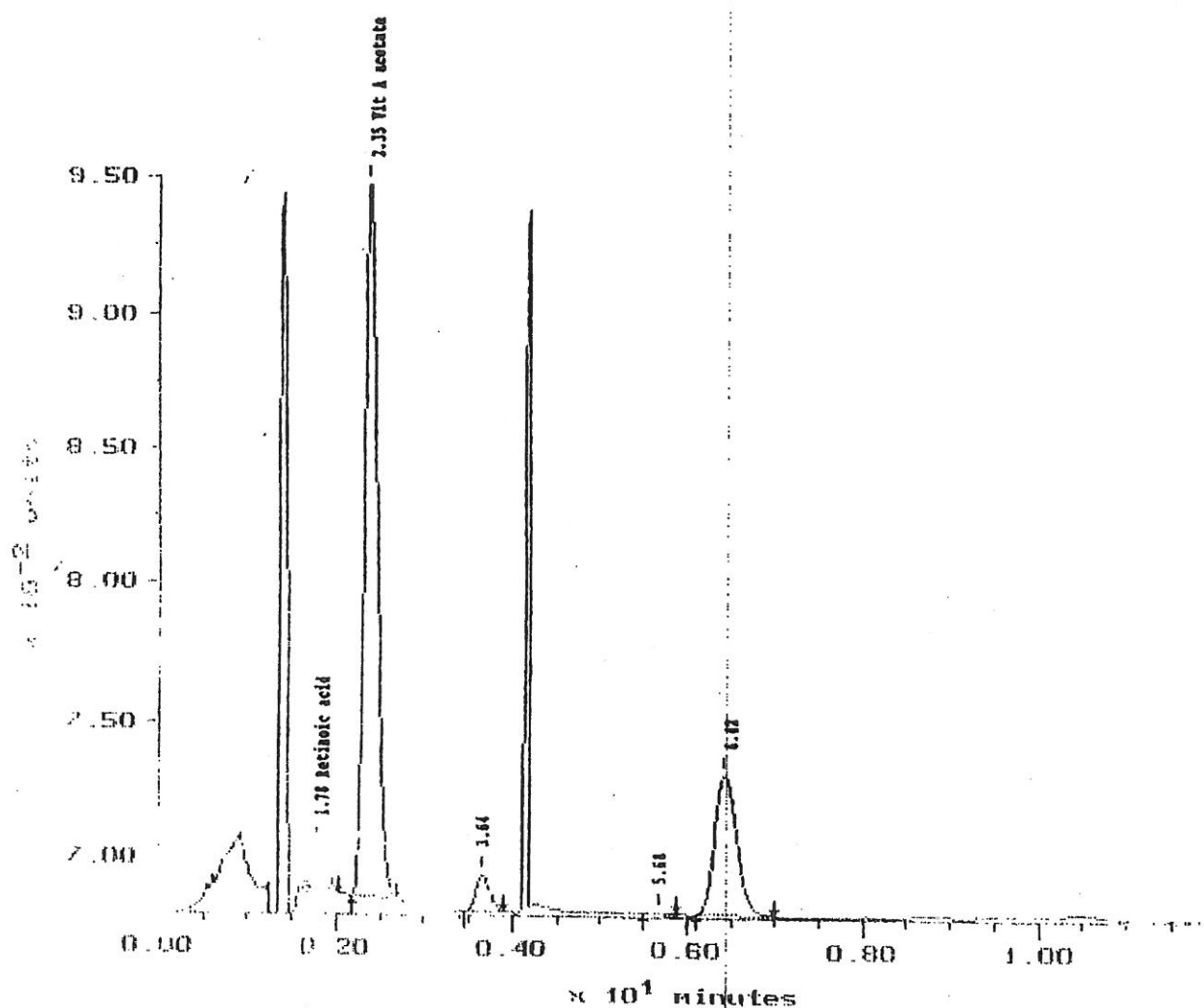


Figure 15. Chromatogram of Bio-Normalizer solution lipid extracts with retinyl acetate acetate and tocopherol acetate internal standards. Peak at 1.78 min = retinoic acid, 2.35 min = retinyl acetate, 6.42 min = tocopherol acetate.

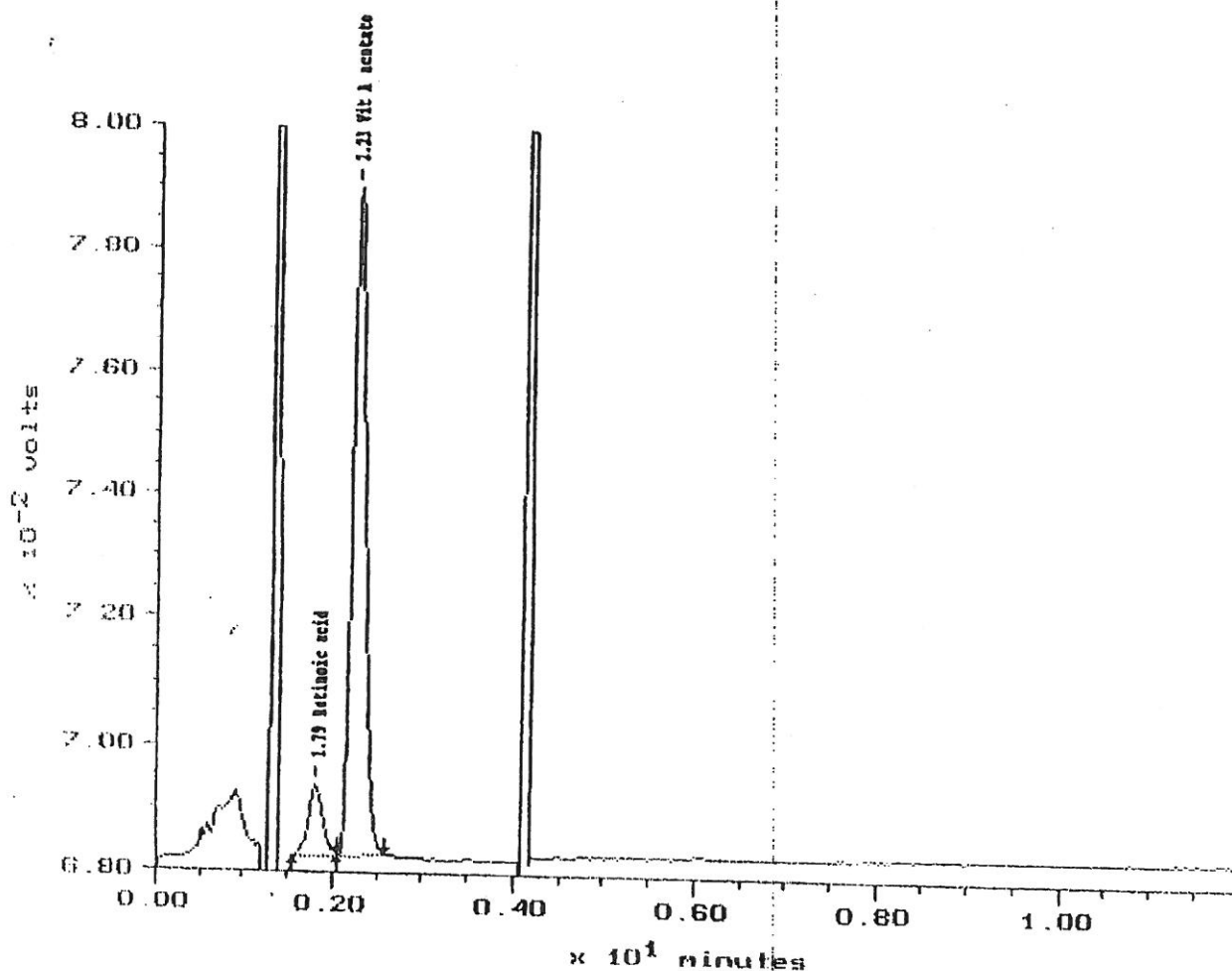


Figure 16. Control chromatogram with retinoic acid and retinyl acetate internal standards.
Peak at 1.79 min = retinoic acid, 2.23 min = retinyl acetate.

II.2.b. Selenium. Selenium content of Bio-Normalizer was inferior to $1 \mu\text{g/g}$, limit of detection obtained after pre-concentration and PIXE analysis.

II.2.c. Fatty acids. The total mass of fatty acids in Bio-Normalizer is relatively low (inferior to 0.1%). The main forms encountered in Bio-Normalizer lipid extracts were C13:0, C14:1, C16:0, C17:0, C18:0, C18:1, C18:2 and C18:3 α (Fig. 17). Their quantity within Bio-Normalizer ranged from approximately $10 \mu\text{g/g}$ (dry mass), for C18:0 and C18:3 α , to $150 \mu\text{g/g}$ for C14:1 and C18:2. This composition is classical of plant extracts.

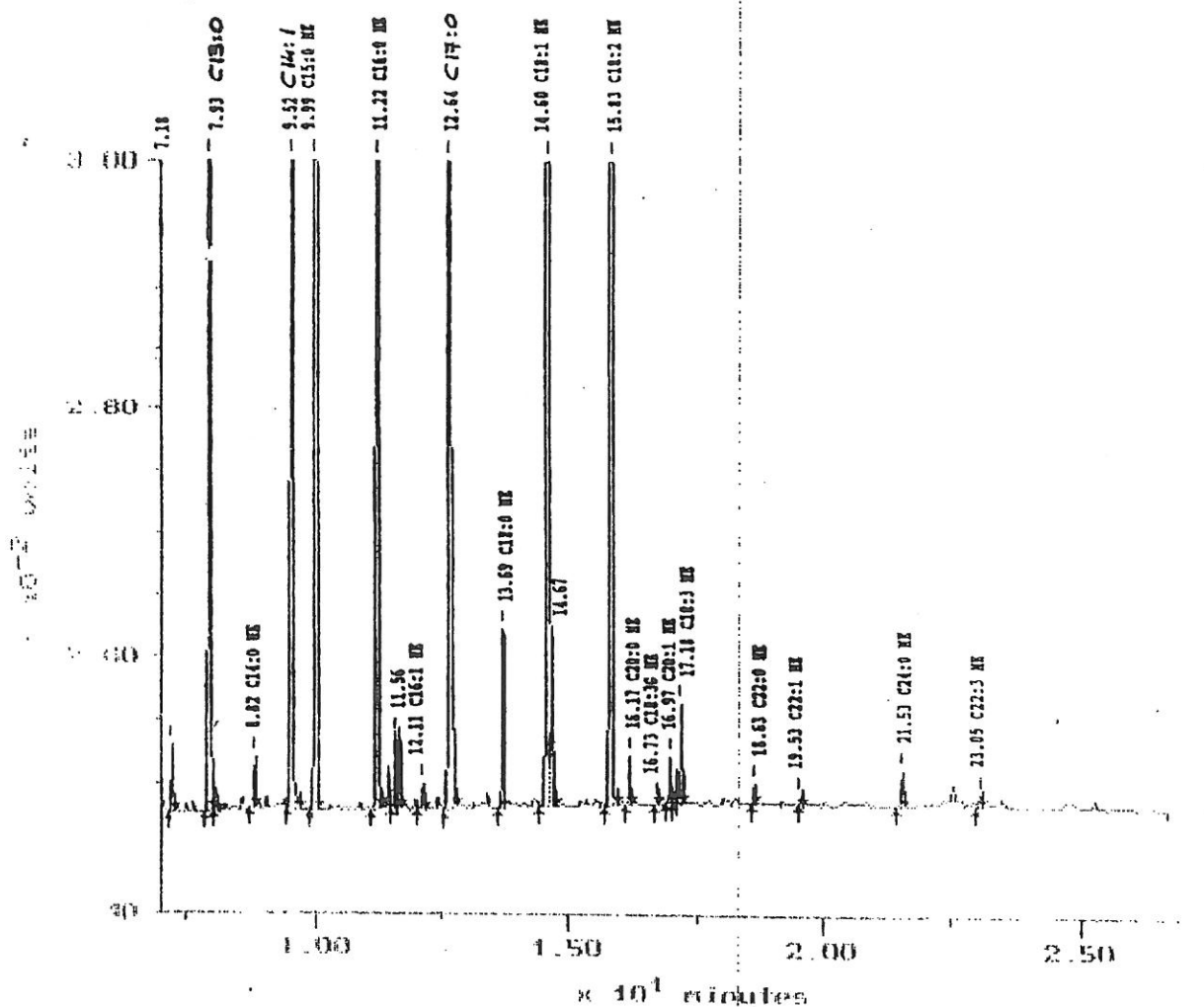
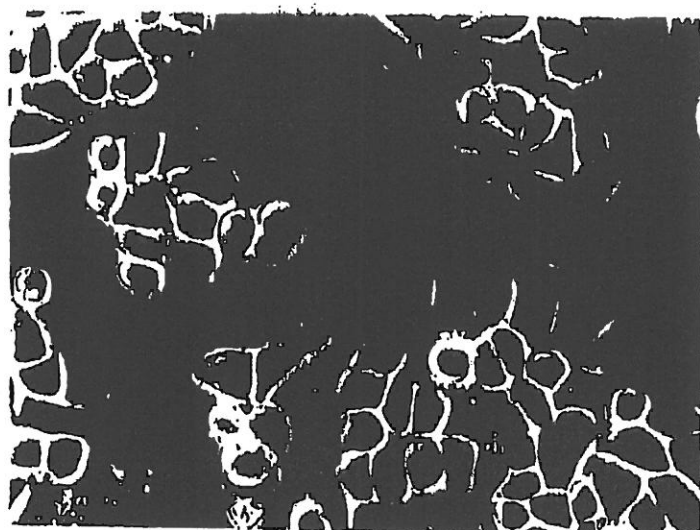
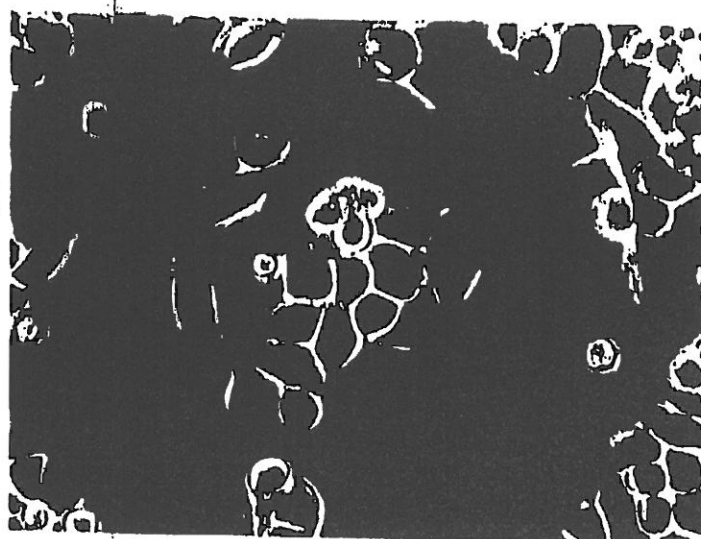


Figure 17. A typical chromatogram of fatty acids distribution within Bio-Normalizer lipid extracts. The main components detected are C13:0, C14:1, C16:0, C17:0, C18:0, C18:1, C18:2 and C18:3 α .

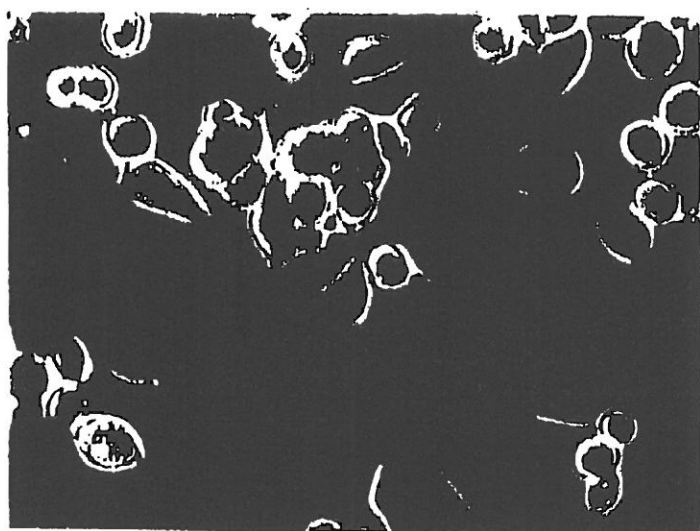
II.3. Cytological observations. After two weeks incubation with 4 mg/ml Bio-Normalizer, no modification of cellular morphology could be noticed on IGROV1, or on IGROV1-DDP cells (Fig. 18). However, a slight increase in the growth rate of cisplatin-resistant cells was remarked, whereas IGROV1 growth remained unchanged. Effectively, when IGROV1-DDP cells were exposed continuously to 1 μ g/ml cisplatin and 4 mg/ml Bio-Normalizer, passages occurred after 7 days instead of 9 days. A decreased efficacy in cell separation with trypsin/EDTA solutions was also noticed during continuous incubation with Bio-Normalizer.



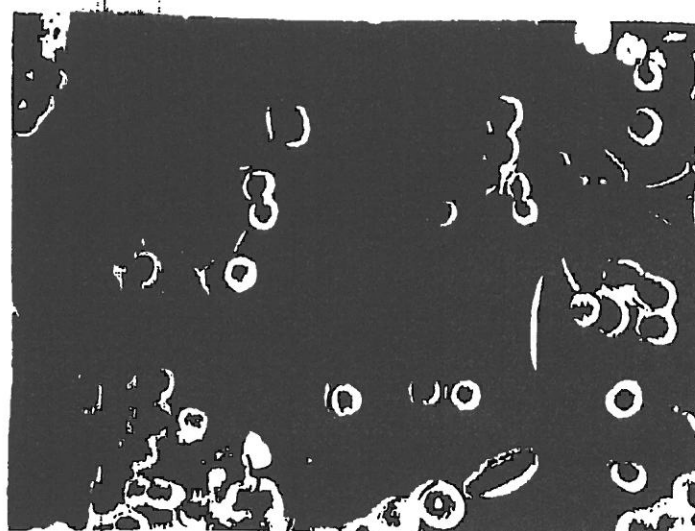
(a)



(b)



(c)



(d)

Figure 18. Phase contrast light microscopy video images of IGROV1 cells (a, b), and IGROV1-DDP cells (c, d), cultured with Bio-Normalizer free medium (a, c), or with 4 mg/ml Bio-Normalizer in complete medium (b, d); bar, 50 μ m.

II.4. Nuclear Micro-Probe Analysis. Essential trace metal (Mn, Fe, Cu, Zn) and platinum were quantified in IGROV1 cells after 5h incubation with 10 µg/ml cisplatin with, or without, 1000 µg/ml Bio-Normalizer (Table 1). Only one analysis could be carried out on cells exposed to Bio-Normalizer, therefore these results must be considered as preliminary and confirmed by further analysis. According to these first experiment, platinum uptake in IGROV1 cells appeared to be reduced by the action of Bio-Normalizer with an approximate decreased of 75 % of incorporation.

Table 1. Platinum and trace metal concentrations (µg/g dry mass) in IGROV1 cells with/without 5 h exposure to 10 µg/ml cisplatin, and to 10 µg/ml cisplatin + 1 mg/ml Bio-Normalizer.

Cellular Content (µg/g dry mass)	IGROV1 ^a	IGROV1 ^b 10 µg/ml cisplatin	IGROV1 ^c 10 µg/ml cisplatin 1 mg/ml Bio-Normalizer
Mn	9.5 ± 1.6	12.7 ± 7.0	11.2 ± 1.0
Fe	143 ± 19	167 ± 11	126 ± 2
Cu	11.7 ± 3.8	10.5 ± 1.3	7.8 ± 0.8
Zn	212 ± 23	252 ± 53	187 ± 3
Pt	-	283 ± 8	66.6 ± 7.0

a, b; n = 5, mean ± SD.

c : n = 1, concentration ± error

Scanning dimensions : 500x500 µm² (corresponding to nearly 500 IGROV1 cells).

DISCUSSION

Bio-Normalizer at concentrations ranging from 0.1 to 1000 µg/ml have demonstrated no inhibition of cell proliferation on IGROV1 or IGROV1-DDP cells, after a short or a long treatment (2 or 24 hours exposure). This observations suggest that Bio-Normalizer alone does not present any toxicity at pharmacological or supra-pharmacological concentrations on IGROV1 or IGROV1-DDP cells. This lack of toxicity was confirmed by long term incubations (2 weeks), of IGROV1 cells with high doses of Bio-Normalizer (4 mg/ml). In these conditions no changes were apparent on IGROV1 cell morphology and growth. These results corroborate the fact that Bio-Normalizer is a natural non-toxic nutritional health food (Santiago *et al.*, 1992b).

However, several other studies have demonstrated that some natural antioxidants can exhibit antiproliferative activity (Elegbede *et al.*, 1986; Shklar *et al.*, 1987; Schwartz & Shklar, 1988; Schwartz *et al.*, 1989; Shklar *et al.*, 1989; Schwartz & Shklar, 1992). It was hypothesized that antioxidants inhibit tumor cell growth through a process that reduces free radical production. Therefore *in vitro* inhibition of growth is a function of the oxygen state of the tumor cell target. At normal oxygen pressure (like in these experiments 5% CO₂, 95% air), inhibition of tumor cell survival would required very high levels of antioxidants (Schwartz *et al.*, 1993). Thus, it would be particularly important to determine Bio-Normalizer antiproliferative activity on cancer cells in an oxygen-restricted environment. As a matter of

fact, the level of reactive molecules should then be controlled by Bio-Normalizer free radical scavenger components, resulting in a decreased expression of transcription factors and membrane kinase activity, and ultimately a decreased cell proliferation. This outcome would represent a fundamental breakthrough as regions of low oxygen (hypoxia) are very common features of solid tumors (Vaupel & Hockel, 1995).

Cisplatin-resistant cells, IGROV1-DDP, exhibited an increased growth rate when incubated with Bio-Normalizer. This phenomenon appeared in short treatment assays at high doses (1000 $\mu\text{g/ml}$), and was more clearly noticed after long treatments, even at low doses of Bio-Normalizer (0.1 $\mu\text{g/ml}$). These observations may explain the increased growth rate of IGROV1-DDP cells noticed during continuous exposure with 4 mg/ml Bio-Normalizer. As IGROV1-DDP cells are cultivated in presence of 1 $\mu\text{g/ml}$ cisplatin, in order to maintain the cisplatin-resistant phenotype, it can be hypothesized that Bio-Normalizer interacts with cisplatin, and decreased its cytostatic effect. Cell proliferation assays with combinations of cisplatin and Bio-Normalizer supported this hypothesis. Simultaneous exposure of IGROV1 cells to 1 $\mu\text{g/ml}$ cisplatin during 2h (IC50), and 0.1 to 1000 $\mu\text{g/ml}$ Bio-Normalizer resulted only in 25% inhibition of cell proliferation. During long treatments, this effect was enhanced and the maximum inhibition was only 12%, with 10 $\mu\text{g/ml}$ cisplatin and 10 $\mu\text{g/ml}$ Bio-Normalizer, instead of 50% expected when cisplatin was administered alone. The same was true for the cisplatin-resistant cell line IGROV1-DDP. For this cell line, IC50 corresponded to 2h exposure with 85 $\mu\text{g/ml}$ cisplatin. Simultaneous exposure of IGROV1-DDP cells with 85 $\mu\text{g/ml}$ cisplatin and Bio-Normalizer (0.1 to 1000 $\mu\text{g/ml}$) during 2 hours resulted in a nearly total inhibition of cisplatin antiproliferative effects. Similarly, during long treatments, the inhibitory effect of Bio-Normalizer on cisplatin cytotoxicity was also remarked. Therefore, it can be concluded that Bio-Normalizer interferes *in vitro* with cisplatin pharmacology, even at low doses, and prevents its cytotoxic effects.

Additionally, preliminary nuclear probe microanalysis results suggested that Bio-Normalizer interacts with cisplatin in the extracellular environment. Effectively, reduced intracellular platinum concentrations were measured in cultured cells after simultaneous exposure with cisplatin and Bio-Normalizer. Protection against organoplatinum toxicity, and more generally against heavy metals toxicity, could represent an unexpected application of Bio-Normalizer. It would result of particular interest to continue these analysis in order to confirm the first observation. Effectively, this property would support the efficacy of Bio-Normalizer as a natural health food, enlarging its domain of action to the protection against heavy metals. Moreover, in the case of a strong interaction between cisplatin and Bio-Normalizer involving stable metal-binding, platinum would represent an exogenous tracer that would facilitate the identification of Bio-Normalizer active components.

Chemical analysis of Bio-Normalizer revealed that among the natural antioxidants analyzed such as retinoids, β -carotene, α -tocopherol, or selenoproteins, only retinoic acid was detected. The concentration measured (750 $\mu\text{g/g}$) is theoretically able to induce retinoic acid-associated effects such as activation of cell differentiation. However, IGROV1 cell proliferation is not affected by retinoic acid, even at high concentration (Caliaro *et al.*, 1994). Therefore an other cellular model should be used to test pro-differentiative effects of Bio-Normalizer.

After 2h of simultaneous treatment with Bio-Normalizer and melphalan, a slight, but significant, enhancement in melphalan cytostatic effect was noticed. This phenomenon was remarked even at low concentrations of Bio-Normalizer (0.1 $\mu\text{g/ml}$). The maximum effect was measured at 1000 $\mu\text{g/ml}$ Bio-Normalizer and corresponded to 28% enhancement of growth inhibition. However, IGROV1 cell proliferation, after melphalan exposure, was unchanged when pre-incubation with various concentrations of Bio-Normalizer were performed. Therefore, further studies seem necessary to confirm the potentiation of melphalan antiproliferative activity by Bio-Normalizer.

CONCLUSION - PERSPECTIVES

In this study we have investigated whether the natural free radical scavenger, Bio-Normalizer, could inhibit *in vitro* human ovarian carcinoma cells proliferation, and modulate cisplatin or melphalan anticancer activity.

We have demonstrated that Bio-Normalizer exhibits no cytotoxicity on human ovarian adenocarcinoma cells, even at supra-pharmacological concentrations. This lack of toxicity may represent a very positive characteristic if the hypothesis of anticancer activity through reduction of free radical production is confirmed in hypoxic conditions. It is now important to complete the investigation of Bio-Normalizer anticancer capabilities by cell proliferation assays in oxygen restricted conditions. These additional experiments would permit to conclude definitely on the potential role of Bio-Normalizer for inhibition of tumor cell growth.

Bio-Normalizer contains retinoic acid, and maybe other unidentified differentiative compounds, in pharmacological quantity. Differentiation studies should be carried on with adequate protocols and cellular models.

Unexpected interaction of Bio-Normalizer with cisplatin pharmacology results in the inhibition of its cytotoxic action. Therefore, Bio-Normalizer is not able to reverse cisplatin cellular resistance but demonstrates heavy metal protective capabilities. It would result very interesting to identify the mechanism involved in these interference. Preliminary experiments, by nuclear probe microanalysis, on cells incubated with Bio-Normalizer and cisplatin seemed to reveal a reduced uptake of platinum. If this result is confirmed, it will mean that the interaction occurs in the extracellular medium, thus providing evidence for metal chelation properties of Bio-Normalizer. Moreover, the fact that this interaction involves platinum would allow an easier identification of the active component(s) responsible for metal binding, and maybe for the free radical protection, if these two phenomena were linked.

Finally, Bio-Normalizer do not reduce the anticancer activity of alkylating agents such as melphalan. In contrast, a moderate enhancement of melphalan growth inhibition has been observed following simultaneous incubation with Bio-Normalizer. This result could be of major interest in order to modulate alkylating agents cytotoxicity, and reverse cellular resistance. Further studies should be performed on alkylating agents sensitive and resistant cell lines in order to determine the conditions for an optimal combination.

REFERENCES

- Allen R.G. Oxygen-reactive species and antioxidant responses during development: the metabolic paradox of cellular differentiation. *Proc Soc Exp Biol Med*, 1991, 196: 117-129
- Allen R.G., Venkatraj V.S. Oxidants and antioxidants in development and differentiation. *J Nutr*, 1992, 122(suppl 3): 631-635
- Ames B.N., Swirsky Gold L., Willet W.C. The causes and prevention of cancer. *Proc Natl Acad Sci USA*, 1995, 92: 5258-5265
- Amstad P., Crawford D., Muehlethaler D., Zbinden I., Larsson R., Cerutti P. Oxidants stress induces proto-oncogenes c-fos and c-myc in mouse epidermal cells. *Bull Cancer*, 1990, 77: 501-502
- Bénard J., Da Silva J., De Blois M.C., Boyer P., Duvillard P., Chiric E., Riou G. Characterization of a human ovarian adenocarcinoma line, IGROVI, in tissue culture and in nude mice. *Cancer Res*, 1985, 45: 4970-4979

- Bertram J.S., Bortkiewicz H. Dietary carotenoids inhibit neoplastic transformation and modulate gene expression in mouse and human cells. *Am J Clin Nutr*, 1995, 62(suppl): 1327-1336
- Bienvenu P., Caron L., Gasparutto D., Kergonou J.-F. Assessing and counteracting the prooxidant effects of anticancer drugs. In: *Free Radicals and Aging*, ed. I. Emerit & B. Chance, Birkhauser Verlag, Basel, 1992, pp. 257-265
- Caliaro M.J., Marmouget C., Guichard S., Mazars Ph., Valette A., Moisand A., Bugat R., Jozan S. Response of four human ovarian carcinoma cell lines to all-trans retinoic acid: relationship with induction of differentiation and retinoic acid receptor expression. *Int J Cancer*, 1994, 56: 743-748.
- Cornic M., Agadir A., Degos L., Chomienne C. Retinoids and differentiation treatment: a strategy for treatment in cancer. *Anticancer Res*, 1994, 14: 2339-2346
- Elegbede J.A., Elson C.E., Tanner M.A., Qureshi A., Gould M.N. Regression of rat primary mammary tumors following dietary d-limonene. *J Natl Cancer Inst*, 1986, 76: 323-325
- German J.B. Micronutrients and cancer: vitamins, antioxidants and nutrients in cancer prevention. In: *Nutrition and Cancer*, ed CERIN, Paris, 1995, pp. 167-193
- Lepage G., Roy C.C. Direct trans-esterification of all classes of lipids in one-step reaction. *J Lipid Res*, 1986, 27: 114-120
- Llabador Y., Bertauld D., Gouillaud J.C., Moretto Ph. Advantages of high speed scanning for microprobe analysis of biological samples. *Nucl Inst Meth*, 1990, B49: 435-440
- Maxwell J.A., Campbell J.L., Teesdale W.J. The Guelph PIXE software package. *Nucl. Inst Meth*, 1989, B43: 218-230
- Moretto Ph., Razafindrabe L. Simulation of RBS spectra for quantitative mapping of inhomogeneous biological tissue. *Nucl. Inst Meth*, 1995, B104: 171-175
- Moretto, Ph., Llabador, Y., Ortega, R., Simonoff, M., Razafindrabe, L. PIXE microanalysis in human cells: physiology and pharmacology. *Nucl. Inst. and Meth.*, 1993, B75: 511-517
- Nagy K., Pasti G., Bene L., Nagy I. Induction of granulocytic maturation in HL-60 human leukemia cells by free radicals: a hypothesis of cell differentiation involving hydroxyl radicals. *Free Radical Res Commun*, 1993, 19: 1-15
- Nagy K., Pasti G., Bene L., Nagy I. Involvement of Fenton reaction products in differentiation induction of K562 human leukemia cells. *Leuk Res*, 1995, 19: 203-212
- Ortega R., Moretto Ph., Llabador Y., Simonoff M., Bénard J. Intracellular platinum and trace elements in cisplatin-resistant human ovarian adenocarcinoma cells: a PIXE microanalysis study. In: *Metal Ions in Biology and Medicine*, eds Ph. Collery, L.A. Poirier, N.A. Littlefield, J.C. Etienne, John Libbey Eurotext, Paris, 1994, vol. 3, pp. 359-364
- Pfahl M., Apfel R., Bendik I., Fanjul A., Graupner G., Lee M.O. La Vista N., Lu X.-P., Piedrafita J., Ortiz M.A., Salbert G., Zhang X.-K. Nuclear retinoid receptors and their mechanism of action. In: *Vitamins and Hormones*, ed. G. Litwack., Acad. Press, San Diego, 1994, vol. 49, pp. 327-381
- Santiago L.A., Osato J.A., Hiramatsu M., Edamatsu R., Mori A. Free radical scavenging action of Bio-Catalyzer $\alpha.p$ No. 11 (Bio-Normalizer) and its by-product. *Free Radical Bio Med*, 1991, 11: 379-383
- Santiago L.A., Osato J.A., Hiramatsu M., Mori A. Bio-Catalyzer $\alpha.p$ No. 11, Bio-Catalyzer 2B, BIO No. 66 quenched free radicals and inhibited lipid peroxidation in iron-induced epileptic focus in rats. In: *Oxygen Radicals*, eds. K. Yagi, M. Kondo, E. Niki, T. Yoshikawa, Elsevier, 1992, pp. 405-408

- Santiago L.A., Osato J.A., Mori A. Stability of the hydroxyl radical scavenging components of the health food "Bio-Normalizer". *Med Sci Res*, 1992, 20: 27-28
- Schwartz J.L., Shklar G. Regression of experimental oral carcinomas by local injection of β -carotene and canthaxanthin. *Nutr Cancer*, 1988, 11: 35-40
- Schwartz J.L., Sloane D., Shklar G. Prevention and inhibition of oral cancer in the hamster pouch model associated with carotenoid immune enhancement. *Tumor Biol*, 1989, 10: 297-309
- Schwartz J.L., Shklar G. The selective cytotoxic effect of carotenoids and α -tocopherol on human cancer cells in vitro. *J Oral Maxillfac Surg*, 1992, 50: 367
- Schwartz J.L., Tanaka J., Khandekar V., Herman T.S., Teicher B.A. β -carotene and/or vitamin E as modulators of alkylating agent in SCC-25 human squamous carcinoma cells. *Cancer Chemother Pharmacol*, 1992, 29: 207-213
- Schwartz J.L., Antoniadou D.Z., Zhao S. Molecular and biochemical reprogramming of oncogenesis through the activity of prooxidants and antioxidants. In: *Ann NY Acad Sci*, 1993, pp. 262-279
- Shklar G., Schwartz J.L., Trickler D.P., Nukian K. Regression by vitamin E of experimental oral cancer. *J Natl Cancer Inst*, 1987, 78: 987-992
- Shklar G., Schwartz J.L., Trickler D.P., Reis S. Regression of experimental cancer by oral administration of combined α -tocopherol and β -carotene. *Nutr Cancer*, 1989, 12: 321-325
- Simonoff M., Hamon C., Moretto Ph., Llabador Y., Simonoff G. High sensitivity PIXE determination of selenium in food and biological samples using a pre-concentration technique. *Nucl Inst Meth*, 1988, B31: 442-448
- Simonoff M., Sergeant C., Garnier N., Moretto Ph., Llabador Y., Simonoff G., Conri C. Antioxidant status (selenium, vitamins A and E) and aging. In: *Free Radicals and Aging*, ed. I. Emerit & B. Chance, Birkhauser Verlag, Basel, 1992, pp. 368-397
- Stahelin H.B. Critical reappraisal of vitamins and trace minerals in nutritional support of cancer patients. *Support Care Cancer*, 1993, 1: 295-297
- Stoler A.B. Genes and Cancer. *Br Med Bull*, 1991, 47: 64-75
- Sundstrom H., Korpela H., Sajanti E., Kauppila A. Supplementation with selenium, vitamin E and their combination in gynaecological cancer during cytotoxic chemotherapy. *Carcinogenesis*, 1989, 10: 273-278
- Takenaga K., Honma Y., Hozumi M. Inhibition of differentiation of mouse myeloid leukemia cells by phenolic antioxidants and α -tocopherol. *Cann*, 1981, 72: 104-112
- Teicher B.A., Schwartz J.L., Holden S.A., Ara G., Northey D. In vivo modulation of several anticancer agents by β -carotene. *Cancer Chemother Pharmacol*, 1994, 34: 235-241
- Vasavi H., Thangaraju M., Sachdanandam P. Effect of α -tocopherol on lipid peroxidation and antioxidant system in fibrosarcoma bearing rats. *Mol Cel Biochem*, 1994, 131: 125-129
- Vaupel P.W., Hockel M. In: *Tumor Oxygenation*, eds. P.W. Vaupel, D.K. Kelleher, Gunderoth M., Gustav Fisher, Stuttgart, 1995, pp. 219-232.
- Warell R.P., Frankel S.R., Miller W.H., Scheinberg D.A., Itri L.M., Hittelman W.N., Vyas R., Andreff A., Tadufi A., Jakubowski A., Gabrilove J., Gordon M., Dmitrovsky E. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N Engl J Med*, 1991, 323: 795-801