ANTICANCER RESEARCH *25*: 1023-1028 (2005)

Frequency and Irradiation Time-dependant Antiproliferative Effect of Low-power Millimeter Waves on RPMI 7932 Human Melanoma Cell Line

AMERIGO BENEDUCI[1], GIUSEPPE CHIDICHIMO[1], RENATA DE ROSE[1], LUIGI FILIPPELLI[1], SERAFINA V. STRAFACE[2] and S. VENUTA[2]

[1]*Department of Chemistry, University of Calabria, 87036 Arcavacata di Rende (CS);*
[2]*Department of Experimental and Clinical Medicine, University of Magna Grecia, Catanzaro, Italy*

Abstract. *The biological effects produced by low power millimeter waves (MMW) were studied on the RPMI 7932 human melanoma cell line. Three different frequency-type irradiation modes were used: the 53.57-78.33 GHz wide-band frequency range, the 51.05 GHz and the 65.00 GHz monochromatic frequencies. In all three irradiation conditions, the radiation energy was low enough not to increase the temperature of the cellular samples. Three hours of radiation treatment, applied every day to the melanoma cell samples, were performed at each frequency exposure condition. The wide-band irradiation treatment effectively inhibited cell growth, while both the monochromatic irradiation treatments did not affect the growth trend of RPMI 7932 cells. A light microscopy analysis revealed that the low-intensity wide-band millimeter radiation induced significant morphological alterations on these cells. Furthermore, a histochemical study revealed the low proliferative state of the irradiated cells. This work provides further evidence of the antiproliferative effects on tumor cells induced by low power MMW in the 50-80 GHz frequency range of the electromagnetic spectrum.*

Previous studies from our laboratory showed that low-power wide-band frequency range millimeter irradiation could be used as a means to cause selective inhibition of tumor cell growth in culture (1, 2). We showed that such a frequency band inhibits the MCF-7 and K-562 tumor cell growth and weakly stimulates that of human healthy lymphomonocytes and primary mammary epithelial cells. We also showed that millimeter waves (MMW) irradiation has a smoothing effect on the membranes of MCF-7 and K-562 cells, as revealed in a scanning electron microscopy study (2). The nature of the interaction between MMW and biological systems has not yet been understood. In this context, our MMW spectroscopy observations, in the 50-80 GHz frequency range, showed that the absorption phenomena observed in cellular systems are entirely due to water molecules (2, 3). Two relatively large absorption bands, whose maximum intensities are centered on the frequencies 65 GHz and 51 GHz, characterize the absorption curves (2).

Furthermore, our previous ^{2}H-NMR study (3) and recent unpublished data show that, when a model membrane is subjected to MMW irradiation, the absorption mechanism of the radiation affects the chemical equilibrium at the water/membrane polar interface.

In this work, we studied the response of the RPMI 7932 human melanoma cell line to the millimeter radiation. Other than the large band mode (52-78 GHz), two monochromatic frequency modes were used. In this case, the frequencies of the two absorption maxima previously mentioned were chosen to irradiate the cells.

Materials and Methods

Wide-band irradiation mode. The wide-band irradiation experiments were carried out using instruments produced by the UWOM Company of Nihznj Novgorod (Russia). The MMW radiation apparatus AMFIT 32 comprises a microwave noise generator diode and an opportune waveguide connected to a conical antenna needed to irradiate in the free space of the frequency band 53.57-78.33 GHz. The mean radiating power at the output of the generator is less than 1 μW.

Monochromatic irradiation mode. The device used to irradiate the cell samples at fixed frequencies comprises a generating head connected, through an opportune waveguide, to a conical antenna that propagates the radiation into the free space. Such devices generate monochromatic millimeter radiation with a narrow frequency band (±0.05 GHz) centered at the working frequency

Correspondence to: A. Beneduci, Department of Chemistry, University of Calabria, 87036 Arcavacata di Rende (CS), Italy. Tel.: +39 0984492117, Fax: +39 0984492041, e-mail: beneduci@unical.it

Key Words: Millimeter waves, wide-band irradiation mode, monochromatic irradiation mode, RPMI 7932, cell morphology.

51.05 GHz and 65.00 GHz. They are named IMG-51 and IMG-65, respectively, and are produced by the MicroMedTech Company of Nihznj Novgorod (Russia). The radiating powers at the output of the generators are respectively 44 mW and 46 mW. In the treatment conditions used, the cellular samples were directly irradiated on the open surface of the cell culture dishes, at a distance of 18 cm from the antenna of the instruments.

Cell culture. The RPMI 7932 cell line was obtained from the Bank of Biological Material (ICLC) CBA-Genova, Italy, and cultured in RPMI 1640 medium (Roswell Park Memorial Institute; Sigma), supplemented with 10% fetal calf serum (FCS), L-glutamine (5000 U/ml), streptomycin and penicillin (5 mg/ml), at 37˚C in a humified atmosphere with 5% CO_2 and 95% air. Cells from the stock flask were suspended after treatment with trypsin, buffered with phosphate buffer saline and counted using a hemocytometer. Cell suspensions were seeded into 35-mm plates, at a density of 0.5 x 10^5 cells/ml in 2.8 ml of medium (layer thickness ~ 2.9 mm), for the one-hour irradiation treatment and into 100-mm plates, at a density of 1.5 x 10^5 cells/ml in 10 ml of medium (layer thickness ~ 1.3 mm), for the three-hour irradiation treatment. In all the experiments, MMW irradiation treatment started at the fourth day from seeding, when the RPMI 7932 cells were in the active growth phase. Cell growth data were analysed by the Student's *t*-test assuming that there might be variations in the two groups (control and irradiated). Differences between group means were considered significant at $p<0.05$.

Optical microscopy. A Televal 3 Zeiss inverted optical microscope was used for the cell growth experiments and a Laborlux 12 Pol Leitz microscope equipped with a 50/0.85 EF objective and a 10X/18 mm periplan ocular was used to analyze the stained cell samples. A Pentax $P30_N$ with a Kodak Ultra 400 was used to photograph the stained samples.

Histochemical study. RPMI 7932 cells were seeded onto micro slides, cultured and irradiated under the same conditions as used in the growth experiments. Cells were then fixed and stained after three and eight irradiation treatments. The cells were fixed using ethyl alcohol 95% (v/v).

The staining procedure. The most widely used dye combination of hematoxylin for nuclear stain and eosin for cytoplasm stain was used (H. & E.).

Xylol was used for the clarification of the stained samples that were then embedded in Canadian Balsam.

Results

Effects on the cell proliferation

Wide-band frequency exposure (Figure 1a, b): Figure 1a shows the growth trend of the one-hour wide-band-irradiated cells compared to the non-irradiated control. As shown, the control cells exhibited a logarithmic growth and reached confluency after 11 days of incubation (cells seeded into 35-mm plates). The cell count revealed that the number of cells in the two samples was identical in the first 7 days of incubation, though on the 8th day (after three treatments) the cell number in the treated sample was 7% less than in the control ($p<0.32$). The cell growth inhibitory effect of the MMW irradiation was more obvious (though relatively weak) on day 10 of culture (after four treatments), when the cell number in the irradiated sample was 16% less than the control.

Though a weak cell antiproliferative effect was observed on irradiating the cells for one hour every other day, we chose to increase either the number of irradiation treatments (every day) or the irradiation time/treatment (three hours). In Figure 1b, the effect of the three-hour wide-band irradiation treatment on the proliferation of the RPMI 7932 cells is shown. After three treatments, a slight inhibitory effect could be observed. This effect became important after seven irradiation treatments (by day 11 of culture) when the proliferation of the irradiated RPMI 7932 cells was inhibited by about 29% compared to the control cells that indeed reached confluency (cells seeded into 100-mm plates).

Monochromatic frequency exposure (Figure 2a, b): Neither one hour (data not shown) nor three hours of monochromatic irradiation/day given to the cell samples at 51.05 GHz (Figure 2a) and 65.00 GHz (Figure 2b) produced any effect on the RPMI 7932 cell proliferation. In fact, the growth behavior of the irradiated RPMI cells was identical to the non-irradiated ones within the chosen statistical confidence interval ($p<0.05$).

Effects on the cell morphology

Light microscopy results: The H. & E. staining assay was performed only on cell samples treated with the wide-band low intensity MMW in the three-hour radiation experiment. These samples were irradiated under similar conditions to those used in the growth experiments and the cells were fixed and stained after three and seven treatments. Furthermore, identical cell samples were sham exposed every day for three hours (control samples), fixed and stained after three and seven sham exposures, too. Figure 3a and b depict the situation of the control samples (non-irradiated) after three and seven sham exposure treatments, respectively. In both Figures, all the morphological characteristics of the RPMI 7932 cells can be observed. We generally observed two categories of cell shape for this cell type: a) a spindle morphology in which an elongated cell body and two long, thick protrusions on the opposite sites could be noted; b) a triangular morphology in which the cell body is elongated in one direction (leading lamella) and two retracting tails are found opposite the leading lamella.

In any case, at least one long (two or more nuclear diameters) and thin cell protrusion could generally be observed in the non-irradiated control cells. By the staining procedure, a relatively dispersed chromatin and prominent nucleoli were evidenced within the nuclei of the control

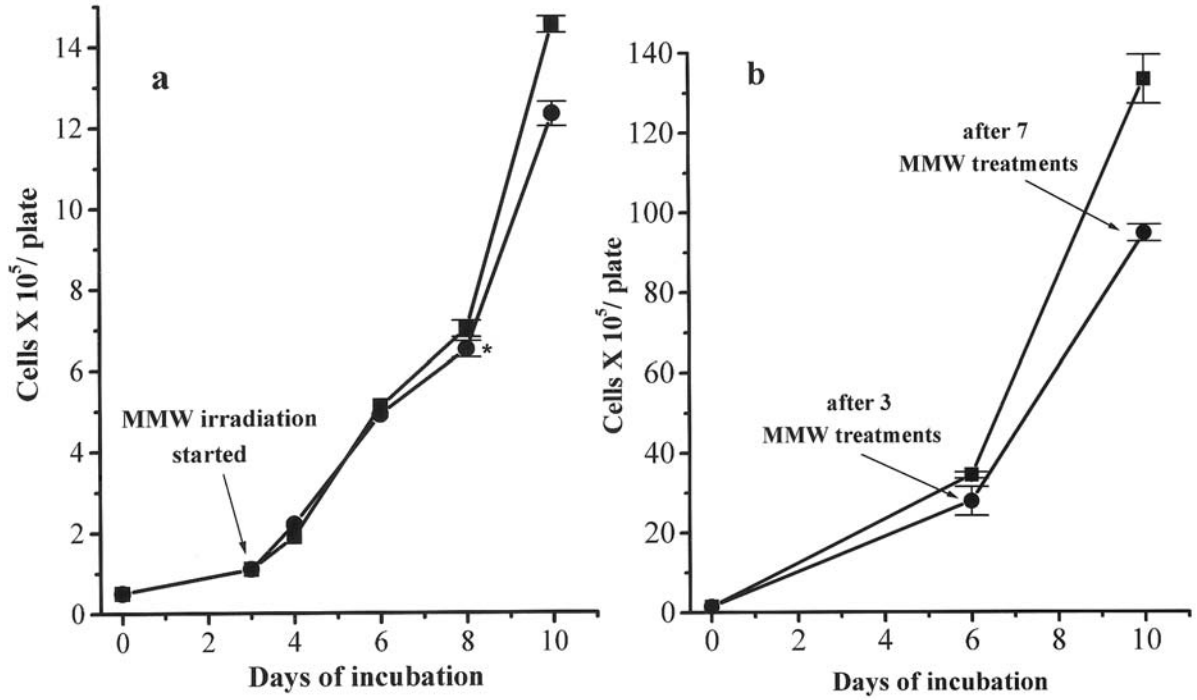

Figure 1a, b. *Wide-band frequency irradiation effects on the proliferation of the RPMI 7932 human melanoma cells: a) 1 h of radiation treatment every other day. Cells seeded in 35-mm plates and supplemented with 2.8 ml of culture medium; b) 3 h of radiation treatment every day. Cells seeded in 100-mm plates and supplemented with 10 ml of culture medium. Points, mean cell count from four plates for experiment (a) and three plates for experiment (b) of non-irradiated control cells (■) and irradiated cells (●). Determination of the total cell count and viable cell number were made by the use of a hemocytometer and trypan blue stain; bars, SEM. *, p<0.32 versus non-irradiated controls.*

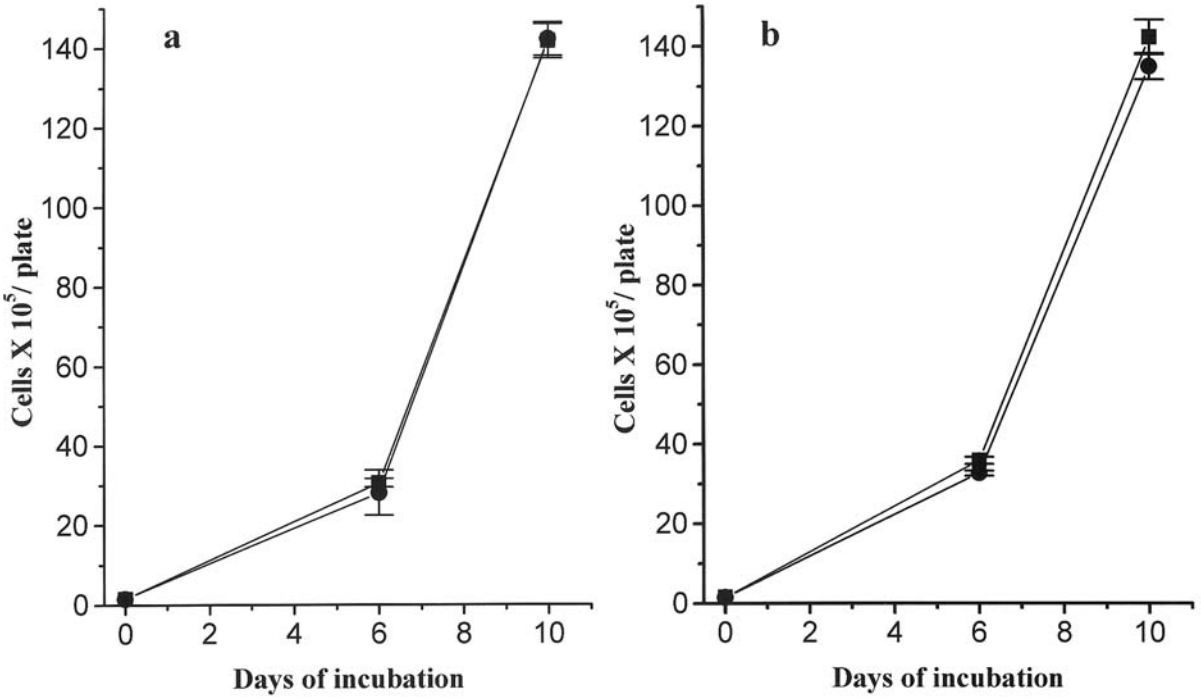

Figure 2 a, b. *RPMI 7932 melanoma cell growth under the monochromatic frequency irradiation mode: a) at 51.05 GHz and b) at 65.00 GHz. In both experiments, 3 h of radiation treatment was given every day. Cells seeded in 100-mm plates and supplemented with 10 ml of culture medium. Points, mean cell count from three plates of non-irradiated control cells (■) and irradiated cells (●). Determination of the total cell count and viable cell number were made by the use of a hemocytometer and trypan blue stain; bars, SEM.*

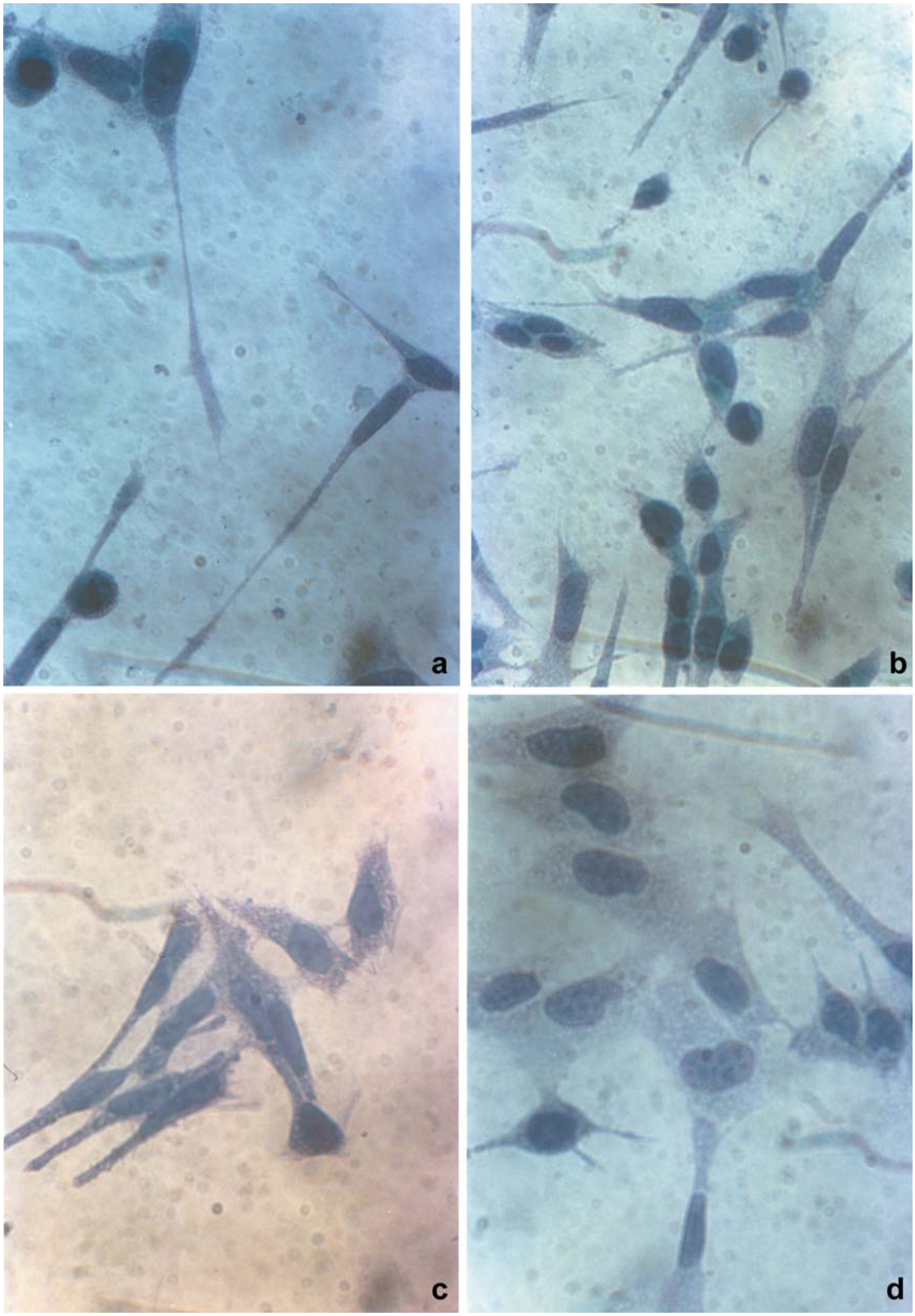

Figure 3. *Light microscopy of the RPMI 7932 cells: non-irradiated control cells after three (a) and seven (b) sham exposures; wide-band MMW-irradiated samples (3 h/treatment) after three (c) and seven (d) treatments. The cells were stained with hematoxylin and eosin. Bar, 22 μm.*

cells. Furthermore, a markedly blue-stained cytoplasm (basophilic cytoplasm rich in ribonucleoprotein) was found in most of the non-irradiated cells (Figure 3a, b). Both these cytological features are associated with an active synthetic state of the control cells.

The photograph in Figure 3c is representative of the state of the tumoral system subjected to three MMW irradiation treatments. At this stage of the irradiation treatment, the RPMI 7932 cells began to change their dimensions, especially with a reduction in the length of their cell protrusions. A slight variation in the nucleus/cytoplasm ratio was also noted. The group of cells in the photograph in Figure 3d show the representative morphological state of the irradiated cells after seven irradiation treatments. In this case, a global increase of the irradiated cells dimensions was observed compared to the non-irradiated control cells; in particular a cytoplasm/nuclear ratio increment could be estimated. Most of the cytoplasm of the MMW-irradiated cells tended to be lavender in color under light microscopy. These effects could be due to the swelling of the cells and to a low active synthetic state that agrees with the low proliferative efficiency. Furthermore, most of the irradiated cells definitely lost their spindle or triangular shape after seven irradiation treatments and assumed a quite polygonal morphology, characterized by very short protrusions.

Discussion

The effects of three different millimeter radiation exposure conditions on the RPMI 7932 human melanoma cell line were studied. The wide-band frequency irradiation mode markedly inhibited the human melanoma cells growth. This inhibitory effect was correlated to the number of irradiation treatments and to the irradiation time of a single treatment. It markedly increased when the cells were subjected to seven irradiation treatments of three hours each. Furthermore, the wide-band low-power irradiation mode affected the melanoma cell morphology, as revealed by light microscopy. A "swelling" of the cell nuclei, associated with an overall increase of the cell dimensions, was observed. Particularly, a net increase of the cytoplasm volume and a net decrease of the length of the cell protrusions were observed.

The morphology alterations appeared to be correlated to the inhibitory effect (Figure 1b) of the wide-band millimeter waves on the cells growth, since both the effects were enhanced with the number of irradiation treatments. The low proliferative state of the wide-band-irradiated melanoma cells was also evidenced by the histochemical analysis.

Based on the millimeter absorption spectra cited in the introduction, we irradiated the cells in the single frequency mode choosing the frequencies at maximum intensity of the absorption bands (51 GHz and 65 GHz). We would have expected, therefore, to find some relevant effects in correspondence to these absorption maxima. By contrast, the monochromatic irradiation modes did not produce detectable biological effects on the RPMI 7932 cells. Active millimeter monochromatic frequencies, able to induce specific biological effects, have not yet been identified.

This work provides further evidence that the low-power wide-band millimeter waves, in the 50-80 GHz frequency range, act as inhibitor of tumor cell growth *in vitro*, in non-thermal conditions. The inhibitory effect was shown to depend on the irradiation time per treatment and on the number of treatments. Furthermore, this study shows that the antiproliferative effect is frequency-dependent in the specified electromagnetic range. This conclusion agrees with the hypothesis of Prof. Devyatkov (5) and other researchers (6-11), who showed that low-power MMW could exert profound effects on biological systems, depending on the frequency and power of the radiation, as well as on the irradiation time.

A probable interaction mechanism, coherent with other findings concerning the effects produced by MMW irradiation on model membranes (3), is proposed. Water molecules, which are either bound to some supramolecular structures or freely diffusing inside the system, may absorb the millimeter radiation. Due to the different physical states in which the water molecules can be found in a biological system (free and bound water), frequency-specific absorption phenomena could arise (2, 4-10). Our results show that one of the most probable targets of the radiation is the delicate chemical equilibrium involved at the polar interface between the water molecules and the polar head group of the membrane molecules (3). The alteration of such an equilibrium could be the primary cause of the swelling and smoothing effects observed on the irradiated cells, because it could influence the osmotic transmembrane equilibrium.

Finally, it should be noted that there are no practical limitations for applying the wide-band irradiation methodology directly on the melanoma *in vivo*.

Acknowledgements

This work was supported by Lega Italiano per la Lotta Contro i Tumori Sezione Cosenza.

References

1 Chidichimo G, Beneduci A *et al*: A novel and selective physical anticancer agent. Anticancer Res *21*: 1531, 2001.

2 Chidichimo G *et al*: Selective inhibition of tumoral cells growth by low power millimeter waves. Anticancer Res *22*: 1681-1688, 2002.

3 Beneduci A, Chidichimo G and Filippelli L: Water as "primary antenna" in the interaction between millimeter waves and biological systems. "XIV Conference-Workshop: Horizons in Hydrogen Bond Research, Torino, Italy, September 2001.

4 Devyatkov ND, Sevastyanova LA, Vilenskaya RL, Smolyanskaya AZ, Kondrateva YF, Chistyakova EN, Shmakova IF, Ivanova NB, Treskunov, A A, Manoilov SE, Zalyubovskaya VA, Koselev RJ, Gaiduk VI, Khurgin YI and Kudryashova VA: Scientific session of the division of general physics and astronomy, USSR Academy of Sciences. Sov Phis-Usp *16*: 568-579, 1974.

5 Sevastyanova LA and Vilenskaya RL: A study of the effect of millimeter-band microwaves on the bone marrow of mice. Sov Phis-Usp *16*: 570, 1974.

6 Sevastyanova LA: Specific influence of millimeter waves on biological objects. *In:* Nonthermal Effects of Millimeter Wave Irradiation (Devyatkov ND, ed.), USSR: Acad Sci USSR, pp. 86-113, 1981.

7 Smolyanskaya AZ: Influence of electromagnetic waves on microorganism. *In:* Nonthermal Effects of Millimeter Wave Irradiation (Devyatkov ND, ed.), USSR: Inst Radiotech Electrotech Moscow, Acad Sci USSR, 1981.

8 a) Grundler W and Keilmann F: Sharp resonances in yeast growth prove non-thermal sensitivity to microwaves. Phys Rev Lett *51*: 1214-1216, 1983.
b) Grundler W, Jeutzsch U, Keilmann F and Putterlik V: Resonance cellular effects of low intensity microwaves. *In:* Fröhlich H (ed.), Biological Coherence and Response to External Stimuli, pp. 65-85. Berlin Heidelberg: Springer Verlag, 1988.

9 Kaiser F: Theory of resonant effects of RF and MW energy. *In:* Biological Effects and Dosimetry of Nonionizing Radiation (Radiofrequency and microwave energies) (Grandolfo M, Michaelson S M and Rindi A eds.), New York and London, Plenum Press: NATO ASI Series, pp. 251-281, 1983.

10 a) Fröhlich H: Long range coherence and energy storage in biological system. Int J Quantum Chemi, *2*: 641-649, 1968.
b) Fröhlich H: Long range coherence and the action of enzymes. Nature *228*: 1093, 1970.
c) Fröhlich H: The biological effects of microwaves and related questions. Adv Electronic Electron Phys *53*: 85-152, 1980.
d) Fröhlich H: Evidence for coherent excitation in biological system. Int J Quantum Chem *23*: 1589-1595, 1983.

11 Pakhomov G *et al*: Current state and implication of research on biological effects of millimeter waves: a review of the literature. Bioelectromagnetics *19*: 393-413, 1998.

Received November 3, 2004
Accepted February 25, 2005