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# NMR exposure sensitizes tumor cells to apoptosis

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**NMR technology has dramatically contributed to the revolution of image diagnostic. NMR apparatuses use combinations of microwaves over a homogeneous strong (1 Tesla) static magnetic field. We had previously shown that low intensity (0.3–66 mT) static magnetic fields deeply affect apoptosis in a $Ca^{2+}$ dependent fashion (Fanelli *et al.*, 1999 FASEBJ., 13;95–102). The rationale of the present study is to examine whether exposure to the static magnetic fields of NMR can affect apoptosis induced on reporter tumor cells of haematopoietic origin. The impressive result was the strong increase (1.8–2.5 fold) of damage-induced apoptosis by NMR. This potentiation is due to cytosolic $Ca^{2+}$ overload consequent to NMR-promoted $Ca^{2+}$ influx, since it is prevented by intracellular (BAPTA-AM) and extracellular (EGTA) $Ca^{2+}$ chelation or by inhibition of plasma membrane L-type $Ca^{2+}$ channels. Three-days follow up of treated cultures shows that NMR decrease long term cell survival, thus increasing the efficiency of cytocidal treatments. Importantly, mononuclear white blood cells are not sensitised to apoptosis by NMR, showing that NMR may increase the differential cytotoxicity of antitumor drugs on tumor vs normal cells. This strong, differential potentiating effect of NMR on tumor cell apoptosis may have important implications, being in fact a possible adjuvant for antitumor therapies.**

*Keywords:* apoptosis; blood cells; $Ca^{2+}$ influx ; etoposide.; NMR; static magnetic field; tumor cells .

## Introduction

The putative lack of hazard of NMR technology has contributed to its dramatic diagnostic impact: indeed, the static and microwaves magnetic fields used for NMR imaging do not possess the dangerous cytocidal and mutagenic properties of ionising radiations. As expected, after many years of monitoring, no harmful effects was reported in frequent NMR users, patients or operators. The impact of magnetic fields on living matter is becoming an issue of extreme interest, due to the inevitable exposure of practically everyone to modern technology.[1,2] The initial skepticism on the actual effects of magnetic fields on living matter is now replaced by more critical studies with mechanistic approaches that, though excluding direct cytocidal or mutagenic effects,[3,4] do indicate reproducible alterations at the cellular level.[5–7] These studies are not so easy to interpret as a whole, since they hugely differ from many crucial points of view, *i.e.*, they span from bacteria to humans, from observation (epidemiologic) to experimental conditions, from static to oscillating fields (with special attention to extremely low frequencies, ELF), to microwaves, and within the same type of field from very low to very high intensities (spanning about nine orders of magnitude). However, perhaps unexpectedly, many converging effects of different types of fields are being reported. Much effort is now being expended on the comprehension of the mechanisms involved in such alterations, one consensus concerning the behaviour of $Ca^{2+}$ ion, one of the major intracellular messengers, and more precisely $Ca^{2+}$ influx from the extracellular environment through plasma membrane.[8–10] Interestingly, $Ca^{2+}$ influx is increased by all types of magnetic fields analysed so far (*i.e.*, static[11] or oscillating fields,[9,10]) thus excluding the hypothesis that the cyclotronic resonance effect,[12] null for static fields, may be the mechanism through which magnetic fields increase $Ca^{2+}$ entry.

We have deeply analysed such effects, showing that magnet-produced static magnetic fields in the range of 0.3–66 mT affect apoptosis induced by cellular damaging agents such as chemotherapics, peroxides or high temperature, on a set of "sensitive" tumor cells, whereas another set of "insensitive" cells are not affected.[13,14] These effects are completely dependent on $Ca^{2+}$ influx,[13,14] and are not merely a delay of apoptosis, but a real rescue.[13]

This prompted us to analyse the possible effects of the much stronger static magnetic fields of NMR apparatuses ($\geq$1 T) on apoptosis. The rationale of the present study is to examine whether NMR can alter apoptosis induced by chemotherapic agents on reporter tumor cell lines. The impressive result we obtained was the strong increase

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(about 2.5 fold) of chemotherapic-induced apoptosis by NMR. This is the first evidence to our knowledge that NMR exposure may increase the efficiency of cytocidal treatments, implying a possible therapeutic use of NMR.

## Material and methods

### Cell culture

U937 (human tumour monocytes) and Jurkat (human tumour T lymphocytes) cells were cultured in standard conditions (RPMI 1640 plus 10% heat inactivated fetal calf serum, hi-FCS) as described.[13,15] All experiments were performed in the logarithmic phase of growth, in condition of excellent viability (>98% propidium iodide excluding cells).

Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood samples of healthy individuals by collection with Ficoll-Hystopaque (Sigma-Aldrich) density gradient centrifugation. Red cells were removed by hypotonic lysis. PBML were then plated in RPMI 1640 plus 10% human AB serum in culture flasks (pretreated with the same human AB serum) to promote monocytes adhesion. After 2 h, floating cells (lymphocytes) were washed out, spinned down and separately plated in RPMI 1640 plus 10% hi-FCS. Adherent monocytes were washed 3 times with RPMI to remove residual lymphocytes, detached by cell scraping, and resuspended in RPMI 1640 plus 10% hi-FCS. Treatments (see below) were performed at 20–24 h post-separation. Cell viability in both fractions was >98% as assessed by trypan blue exclusion test. Purity of the enriched fractions was controlled by labelling (20 min at room temperature) with cell-type specific antibodies: FITC-conjugated-anti-CD45 (from Bekton-Dikinson) for lymphocytes; and PE-conjugated-anti-CD14 (from Bekton-Dikinson) for monocytes. Cells were then washed with PBS and labelling evaluated. Both monocyte and lymphocyte fractions were >95% pure.

### Induction and detection of apoptosis

Apoptosis was induced with the protein synthesis inhibitor puromycin (PMC, 10 $\mu$g/ml); or the topoisomerase II inhibitor etoposide (VP16, 100 $\mu$g/ml), as described,[13–18] or 1 mM H2O2.[19–23] The three agents were kept throughout the experiments. Apoptosis was measured on U937 at 3.5 h for PMC and VP16 and at 5 h for H2O2; on freshly isolated monocytes and lymphocytes, at 5 h of treatment.

Apoptosis was evaluated by double labelling (same samples) of: a) apoptotic nuclear morphology (vesiculation (see[15]) or shrinkage, see below) detectable by fluorescence microscopy on cells stained with the cell permeant DNA-specific dye Hoechst 33342; and b) in situ DNA digestion detected by the TUNEL assay. TUNEL assays (from Roche, FITC-labelled dUTP) were performed following the manufacturer's protocol and analyzed under fluorescence microscopy. 5 ul samples of the labelled cells of the different treatments were placed on a microscopic slide, and examined at a fluorescence microscope. The fraction of cells with apoptotic nuclei (detected by Hoechst or TUNEL) among the total cell population, was calculated by counting three hundred cells per sample in at least 10 randomly selected microscopic fields[13,16–23]; the results are expressed as percentage of apoptotic cells among the total cells counted. All TUNEL assay positive cells (showing FITC fluorescence) showed apoptotic nuclear morphology, and *viceversa* (total overlap; Figure 1). Apoptotic nuclear morphology of U937 was previously deeply analysed by ultrastructural and dynamic studies,[15,16] showing nuclear vesiculation by at least two different pathways;[15] similar structures are present in apoptotic circulating monocytes. Jurkat cells in apoptosis shrink their nuclei; apoptotic circulating lymphocytes show a moderately shrunken nucleus with a characteristic "bite".

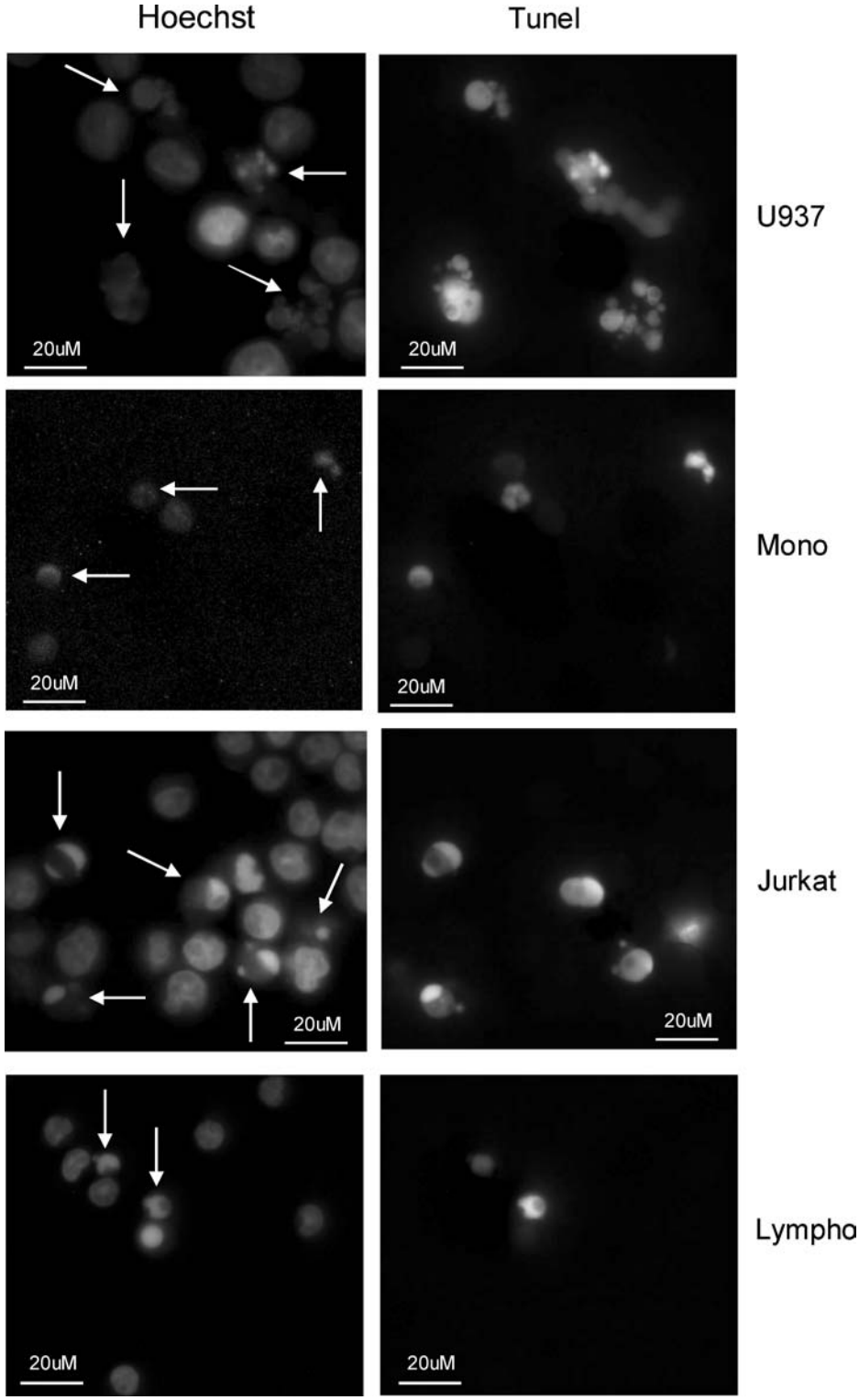
### Other treatments

The extracellular $Ca^{2+}$ chelator EGTA (650 $\mu$M, equimolar to the $Ca^{2+}$ concentration in the standard RPMI 1640 cell culture medium) was added 15 min before the apoptogenic treatments. Nifedipine (10 $\mu$M) was added 15 min prior to treatments. 10 $\mu$gM BAPTA-AM were added for 20 min at 37°C; then cells were washed, resuspended in fresh medium, and apoptosis was induced.

### Magnetic field application

a. NMR: Cells were exposed to the static component of magnetic field (1 T) generated by the diagnostic NMR apparatus. The standard NMR apparatus, with a working temperature of 18°C was made suitable for cell culture experiments, that strictly require 37°C. Since no metal objects such as thermostatic equipment may be placed in the NMR area, a system of circulating warm water was deviced, to guarantee that the waterbath with the cell flasks placed in the NMR area was stably maintained at 37±0.1°C (continually monitored by an alcohol thermometer). Cells were placed in the position where a homogenous nominal 1 T magnetic field was guaranteed by the manufacturer instructions. A second set of cells was placed in another waterbath at 37°C outside the NMR area, in a nominally magnetic field-null area (as stated by the manufacturer instructions and actually measured, not shown), and considered as the sham-exposed. Cells were placed in the waterbathes immediately after the addition of the apoptogenic treatments.
b. Low intensity static magnetic fields (6 mT) were produced by metal magnetic disks placed under the cell flasks concomitantly with the apoptogenic treatments,

**Figure 1.** Detection of apoptosis. Apoptosis was detected by nuclear Hoechst staining (left column) and TUNEL assay (right column). The same microscopic field was doubly labelled as described in materials and methods. All TUNEL positive cells display a typical apoptotic nuclear pattern (see arrows in the left column).

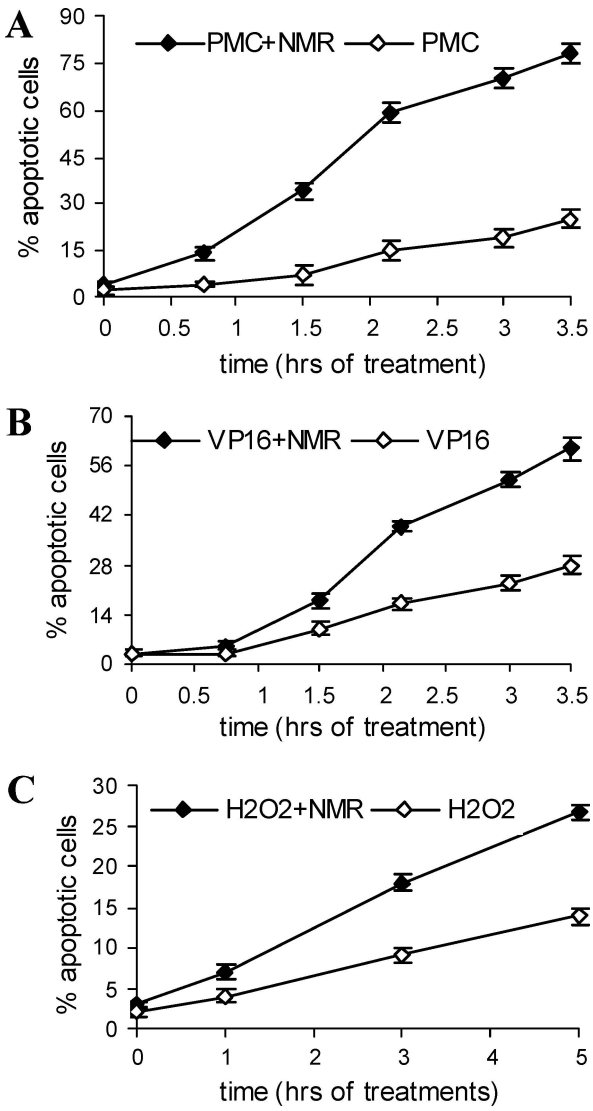


as described.[13,14] Cells were placed in the waterbath outside the NMR area.

### Analysis of cell proliferation rate

Cells were induced to apoptosis by puromycin as described, either within or outside NMR. After 3.5 h of PMC, cells were washed and plated for recovery at the concentration of 100,000/ml in fresh medium (outside NMR in regular culture conditions); the concentration of viable cells was estimated at the indicated time points by cell count at the haemocytometer by the trypan blue exclusion test, each value being the average of three independent measures.

**Figure 2.** NMR potentiates apoptosis. Time course of apoptosis induced on U937 by puromycin (**A**; PMC) or etoposide (**B**; VP16) or hydrogen peroxide (**C**; H2O2) in the presence of the static component of the NMR apparatus (NMR), or sham exposed. The increase in apoptosis due to NMR is highly significant (T-student test: $p<<0.05$). The values are the average of three experiments±SD.




### Statistical analysis

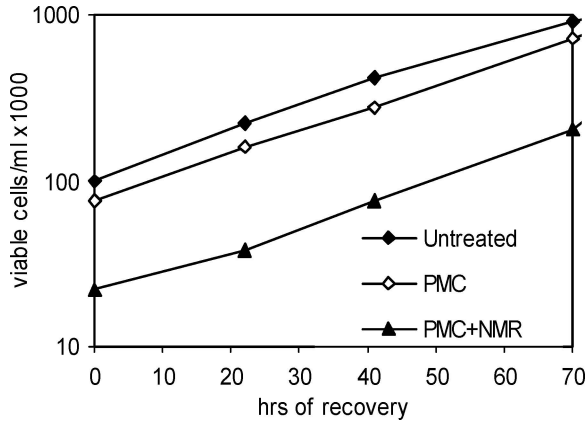
Statistical analysis were performed using Student's *t*-test for unpaired data and $P$ values $<0.05$ wereconsidered significant. Data are presented as mean±SD.

## Results

NMR strongly sensitises U937 human monocytic tumor cells to apoptosis induced by three different cell damaging agents acting with unrelated mechanisms (Figure 2). The difference between exposed and sham is already detectable as early as at 1 h of treatment and is highly significant ($p\ll0.05$) for puromycin starting from 1.5 h; for etoposide starting from 2.25 h; for H2O2 starting from 3 h (Figure 2A–C). NMR was ineffective in triggering apoptosis on its own (*i.e.*, without apoptotic stimulus, see belolw), indicating that NMR-dependent increase of damage-induced apoptosis is a potentiation of an already ongoing apoptotic signaling.

We then analysed whether the difference in viability between cells treated with puromycin inside or outside NMR was maintained in the ensuing hours, by evaluating the number of viable cells at different times of recovery from puromycin (see[13,16]). Figure 3 shows that the proliferation rate of cells recovering from puromycin is parallel to control cells, indicating that cells resume a regular growth rate immediately after puromycin removal. The curve related to cells recovering from puromycin performed within

**Figure 3.** NMR reduces survival of damaged cells. U937 cells were induced to apoptosis by PMC in the presence or absence of NMR; PMC was then washed out and cells were reseeded for recovery at the concentration of 100,000/ml; concentration of viable cells was evaluated at the indicated time points. Untreated cells were used as a control for growth rate. Since only viable cells were considered, the different concentration between the different treatments at $t = 0$ is due to the presence of apoptotic cells in the treated samples (PMC within NMR > PMC outside NMR, see also Figure 2) at that point. Essentially no apoptosis was found at later time points in any sample (degradation of existing apoptotic cells occurs within few hours in U937 cells,[18] indicating that apoptosis stopped taking place after drug removal. One of two independent experiments (each in triplicate) with similar results is shown.



NMR never reaches the one related to cells recovering from puromycin performed outside NMR, showing that NMR produces a real decrease in the number of cells surviving the apoptogenic treatment.

Figure 4 compares the different effects exerted by low intensity (6 mT) magnetic fields vs. NMR on apoptosis on U937 cells, showing that they act in the opposite way of reducing vs potentiating apoptosis, respectively.

We had described cells "sensitive" vs "insensitive" to the modulation of apoptosis by low intensity magnetic fields.[13] This difference is maintained also for the high magnetic fields of NMR. Jurkat T lymphocytic tumor cells, insensitive to magnet-induced 6 mT magnetic fields, are also insensitive to the NMR-produced magnetic fields (Figure 4). We repeated the same type of analysis on the non tumor-equivalent of U937 and Jurkat cells, *i.e.*, freshly isolated peripheral blood monocytes and lymphocytes, respectively. Both types of normal blood cells turned out insensitive to both NMR and low intensity magnetic fields, which did not alter the rate of apoptosis induced by puromycin (Figure 5).

Next, we analysed the mechanisms by which NMR alters apoptosis on sensitive cells. It is known that magnetic fields favour $Ca^{2+}$ influx; if strong, this may lead to dangerously high cytosolic $Ca^{2+}$ levels. Thus, we explored whether the pro-apoptotic effect may be due to an influx of $Ca^{2+}$ from the extracellular environment. To this purpose, we probed if NMR is still able to exert its effect when $Ca^{2+}$ influx is impeded. This can be obtained by specifically chelating extracellular $Ca^{2+}$ ions with EGTA, or by specifically inhibiting L-type plasma membrane $Ca^{2+}$ channels with nifedipine, which was shown to inhibit capacitative $Ca^{2+}$ entry in U937 cells[13] and to revert the apoptosis-modulating effect of low intensity magnetic fields.[13] EGTA or nifedipine by themselves do not induce apoptosis, nor they affect apoptosis induced by etoposide or puromycin outside NMR. However, in the presence of EGTA or nifedipine, NMR is no longer able to exert any potentiation of apoptosis either with PMC (Figure 6A) or VP16 (Figure 6B), showing that the pro-apoptotic effect of NMR depends on $Ca^{2+}$ influx. In order to analyse whether the apoptosis potentiating effect of NMR was due to cytosolic $Ca^{2+}$ overload following $Ca^{2+}$ influx, we chelated cytosolic $Ca^{2+}$ level with BAPTA-AM, which is internalised within cells due to the AM moiety, which is then removed by intracellular esterases thus

**Figure 4.** NMR vs low intensity magnetic fields on apoptosis: sensitive and insensitive cells. The extent of apoptosis at 3.5 h of PMC was evaluated in the presence/absence of NMR or low intensity magnetic fields (6 mT; MF) in U937 (**A**) and Jurkat cells (**B**).The values are the average of three experiments±SD.

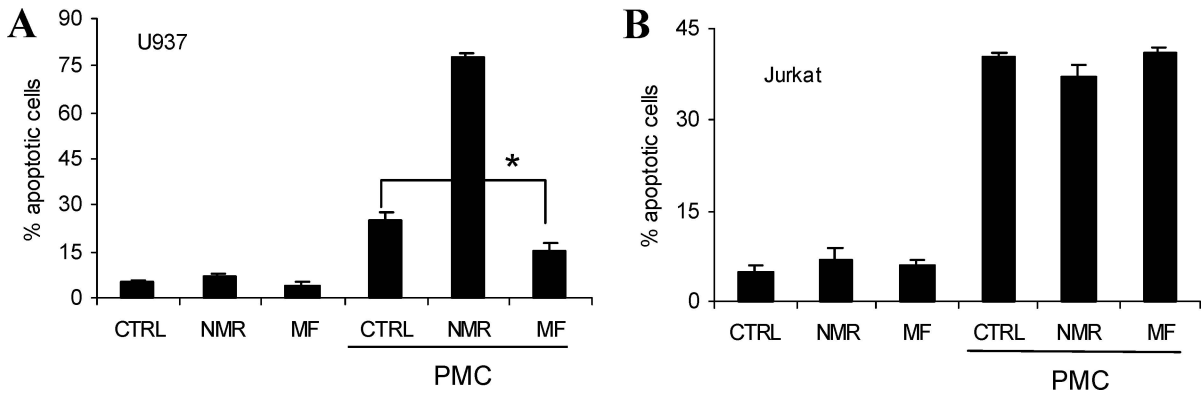



**Figure 5.** Peripheral blood mononuclear cells are not sensitised to apoptosis by NMR. The extent of apoptosis at 5 h of PMC was evaluated in the presence/absence of NMR or low intensity magnetic fields (MF) in monocytes (**A**) and lymphocytes (**B**). Similar results were observed for VP16-induced apoptosis (data not shown). The values are the average of three experiments±SD.

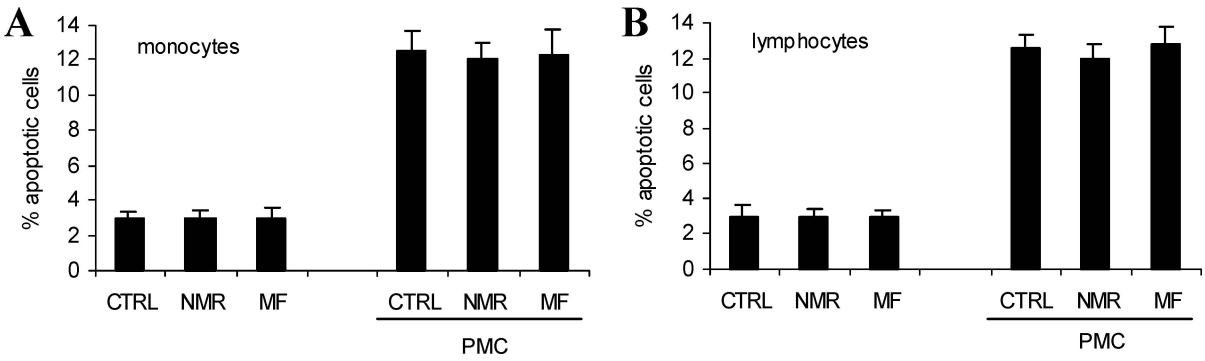
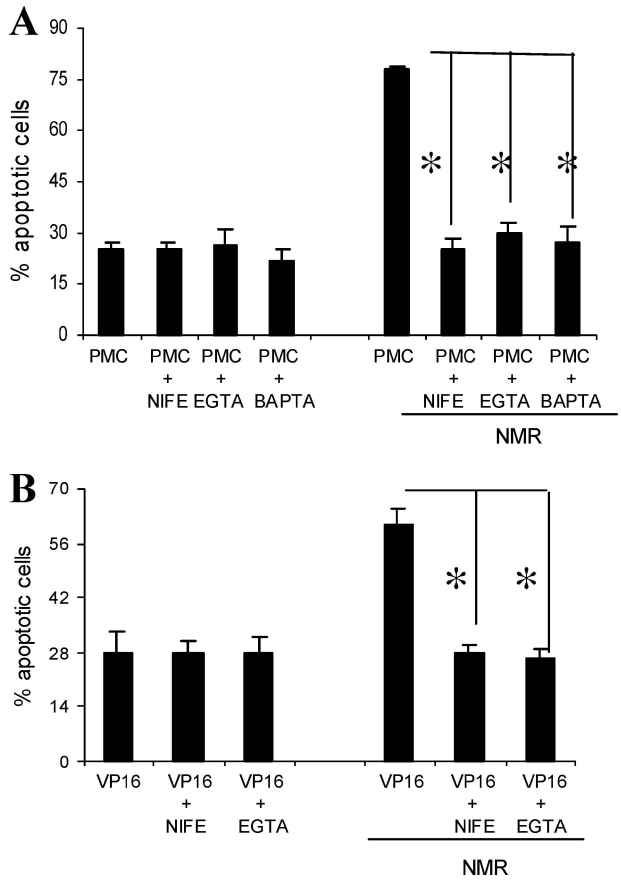


**Figure 6.** The potentiation of apoptosis by NMR depends on $Ca^{2+}$ influx. Apoptosis was induced by PMC (**A**) or VP16 (**B**) outside (left) or inside (right) NMR in U937 cells, in the presence/absence of the $Ca^{2+}$ chelating agent EGTA or the $Ca^{2+}$ L-type channels blocker nifedipine (NIFE), or the intracellular $Ca^{2+}$ chelating agent BAPTA-AM. The reversion of pro-apoptotic effect of NMR observed with EGTA, nifedipine and BAPTA is highly significant (T-student test: $p \ll 0.05$). Apoptosis was measured after 3.5 h of treatment. The values are the average of three experiments±SD.




allowing cytosolic $Ca^{2+}$ chelation. As shown in Figure 6A, NMR does not increase damage-induced apoptosis on BAPTA-loaded cells, showing that the potentiation effect of NMR depends on cytosolic $Ca^{2+}$ overload.

## Discussion

The striking ability of magnetic fields of high intensity (NMR) of potentiating apoptosis induced by cell damaging agents is opposite to the anti-apoptotic effect we[13,14] and others[24,25] described for low intensity magnetic fields. In spite of this, cell response to the high or low intensities shares many characteristics. Indeed, cells that are insensitive to the anti-apoptotic effect of low intensity magnetic fields are also insensitive to the potentiation effect of NMR. Intriguingly, both the anti-apoptotic and the potentiating effect are mediated by $Ca^{2+}$ influx. Preliminary studies concerning the mechanisms involved in the modulation of apoptosis by magnetic field, indicate that both the NMR-induced potentiation, and the 6 mT decrease of apoptosis, are similarly mediated by signal transduction events, and by redox modulating enzymes (in preparation). Thus, two supposedly similar signal transductions end up with two opposite cell responses. We wish to comment this paradoxical result. The opposite effects on apoptosis may be due to the overlapping of two opposite, $Ca^{2+}$ mediated effects, beginning at a lower or higher threshold, and triggering a pro-survival or a pro-apoptotic pathway, respectively. This is conceivable, and reports are accumulating on such dualistic effect of $Ca^{2+}$ on cell survival.[26] To this purpose, a parallel study we are performing on alternate extremely low frequency (50 Hz) magnetic fields gives the same result, showing an intensity-response curve where lower intensities exert a strong antiapoptotic effect, that declines to finally revert to a potentiating effect with increasing intensities (Albertini *et al.* in preparation). The lack of a direct intensity-response curve may explain why a) there are so many contradictory reports in the literature (*i.e.*, pro vs anti-apoptotic effects of magnetic fields) and b) the epidemiological analysis of the effects of magnetic fields on public health is so difficult and controversial.

The existance of sensitive and insensitive cells indicates a possible way where to look in order to identify the primary target(s) of magnetic fields action on cell metabolism. We reported[13] that static magnetic fields of low intensity (6 mT) stimulates a nifedipine-sensitive $Ca^{2+}$ influx in U937 sensitive cells. We have also preliminary evidences that i) signal transduction inhibitors inhibit MF-induced $Ca^{2+}$ influx; ii), that the 6 mT magnetic field does not stimulate $Ca^{2+}$ influx on insensitive Jurkat cells; and that iii) Jurkat may be rendered sensitive by proper treatments (in preparation). Thus, we may speculate that some cells have an active signal transduction step that "senses" magnetic fields and allows MFs-mediated promotion of $Ca^{2+}$ influx, whereas other cells do not. The finding that normal circulating monocytes and lymphocytes proved insensitive to the apoptosis-potentiating effect of NMR means that these quiescent, inactive cells may not have the primary target of the pro-apoptotic effect of NMR expressed. PBML are among the most important, unwanted casualties of systemic antitumor therapies, and chemotherapy-dependent transient immunodepression is one of the main concern of oncologists. Thus, a differential potentiation of the effectivness of chemotherapy against the target tumor cells might allow to limit the doses of the drugs, thereby limiting PBML killing.

The strong potentiating effect of NMR on induced apoptosis may thus have very important implications. A supposedly inoffensive exposure such as that to the static magnetic fields of NMR, might increase the efficiency of therapeutic treatments aimed at cell killing by apoptosis; to this purpose, it will be important to establish the feature(s) cells have to possess in order to be sensitive to the pro-apoptotic effect, focusing on possible histotypic differences. This study

indicates a possible development for the use of NMR apparatuses, with essentially no modifications with respect to the standard ones, as adjuvant for increasing the efficiency of antitumor therapies.

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