Real-time Cell Analysis of Human Cancer Cell Lines after Chemotherapy with Functionalized Magnetic Nanoparticles

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Abstract. *Background: Magnetic drug targeting is a new and innovative approach in cancer treatment. In order to avoid the adverse effects of chemotherapy, the therapeutic agent is linked to superparamagnetic nanoparticles which are injected into a tumour-supporting artery and is focused by an external magnetic field to the tumour region in order to provoke maximum local impact. Analysis of nanoparticles and chemotherapeutic substances in human cancer cell culture is necessary to provide respective information for in vivo applications. Materials and Methods: The effect of pure mitoxantrone and mitoxantrone bound to nanoparticles was tested on human cancer cell lines using real-time cell analysis (RTCA) and lactate dehydrogenase (LDH) assays. RTCA was performed by impedance measuring. The impedance is expressed as the cell index (CI), which is a parameter of cell viability. Results: RTCA showed that mitoxantrone when bound to nanoparticles was more toxic than the drug alone. The CI clearly decreased faster after adding the chemotherapeutic bound to nanoparticles than when adding the pure drug alone. However, in the first experiments, the particles themselves showed no toxicity at therapeutically relevant concentrations. These results were confirmed by LDH assays. Conclusion: The toxic effects of chemotherapeutic agents (e.g. mitoxantrone) on human cancer cell lines (e.g. MCF-7) can be enhanced if these drugs are bound to magnetic nanoparticles. These preliminary data show a dependency on the different application modes of RTCA. The results presented here are a first step for a better understanding of the effectiveness of magnetic drug targeting as a new and innovative cancer treatment.*

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Drug delivery by nanoparticles is the most promising approach in nanomedicine (1). Especially in cancer therapy, this will be of great importance. Cancer is one of the scourges of humanity. Nearly 7.6 million people died from cancer in 2008 in the world and by 2030, this number will have increased to over 11 million (2). Cancer treatment, basically, is a threefold therapy: surgery, radiation and systemic chemotherapy. In recent years, monoclonal antibodies have been developed and a clear advancement in some cancer entities was obtained, but the crucial breakthrough has not yet been achieved (3). Surgery often requires long-standing and complex procedures. Sometimes a difficult reconstruction *e.g.* with free flaps has to be performed. But even this high-tech approach often cannot prevent the patient from serious functional limitations. Moreover, surgery often causes long-term hospitalization. Finally, a substantial proportion of tumours are inoperable because of their dimensions or localization. Radiation has also clearly improved over the past years. For example, intensity modulated radiation therapy (IMRT) tries to spare the tissues surrounding the tumour from damage. However, after radiation, patients suffer from distinct side effects, *e.g.* difficulty in swallowing can be a problem after the radiation of a head and neck squamous cell carcinoma (4). Systemic chemotherapy is often only poorly tolerated. In particular, overwhelming nausea, which is still not sufficiently controllable, is a great problem. For these reasons, creating new therapy modalities is a worthwhile aim. One of these new and innovative approaches is magnetic drug targeting (MDT). Superparamagnetic iron oxide nanoparticles (SPION) are coated and linked to a therapeutic agent. The suspension is then injected into a tumour-supporting artery and is focused by an external magnetic field to the tumour region. By this method, it is possible to reduce the overall

dose and to avoid the adverse effects of chemotherapy, but still obtain a maximum local impact on the tumour (5-10). A pivotal factor of MDT is the particular chemotherapeutic agent bound to the particles. To gain pre-therapeutic information concerning the impact of each component and the respective compound, *in vitro* investigations are required. This is also very important in order to reduce the necessary animal experiments and to provide a basis for the implementation of MDT to the clinic.

Materials and Methods

Cell lines. MCF-7, a breast cancer cell line, and PC-3, a prostate cancer cell line (both from ATCC/LGC Standards GmbH, Wesel, Germany) were examined.

Nanoparticles. The nanoparticles are an in-house production by the Section for Experimental Oncology and Nanomedicine itself. They are SPIONs with a hydrodynamic diameter from 15 to 100 nm. The SPIONs were synthesized according to a protocol by Hodenius (11) and are coated with a biocompatible layer (*e.g.* fatty acids).

Chemotherapeutic agent. As chemotherapeutic agent, the anthracenedione mitoxantrone (MTO) (Mitoxantrone NC 2 mg/ml; NeoCorp AG, Weilheim, Germany) was used. MTO is a cytotoxic drug, acting by intercalation (hydrogen bonds) into DNA, which causes crosslinks and strand breaks (12), and additionally inhibits the enzyme topoisomerase II (13). The agent was bound to the nanoparticles in an aqueous solution (ferrofluid).

Real-time cell analysis (RTCA). To analyze the cells continuously over time, the xCELLigence-system™ (Roche Diagnostics GmbH, Mannheim, Germany) was used. This system affords continuous monitoring of adherent cell growth directly from the point in time of the experiment commencement. Thereby, no intervention that can perturb the cells (*e.g.* addition of reagents) is needed. The xCELLigence-System™ works by measuring the electrical impedance in each well. This was enabled by covering the bottom of the well with gold electrodes. In this way, modifications of cell adhesion, cell spreading and cell proliferation can be detected. The shift of the electrical impedance is expressed as the cell index (CI), which is therefore a parameter of cell viability. The CI is a non-dimensional factor, reflecting the number of cells and their adhesive behaviour. Of course, a pre-condition is the use of adherent growing cells (14). The most important advantage of RTCA is the opportunity of continuous documentation of the growth behaviour of a cell population for several days. The outcome of the experiments is not dependent up-on the point in time that the assay is carried out. In contrast, the point in time of making the final examination is and is selected according to the development of the cells. In this way, the influence of, for example, chemical substances, gene products or siRNA, can be monitored with greater precision. MTO, pure as well as bound to SPION, was analyzed. Additionally, the nanoparticles were also tested without MTO to examine their effect on the cells. Cells were seeded in three 16-well plates and their growth was recorded by the xCELLigence-System™. After 24 hours, the respective test substance was added, at which usually a marginal shift of the CI can be observed. Modifications of volume and temperature slightly influence the electrical impedance and therefore the CI.

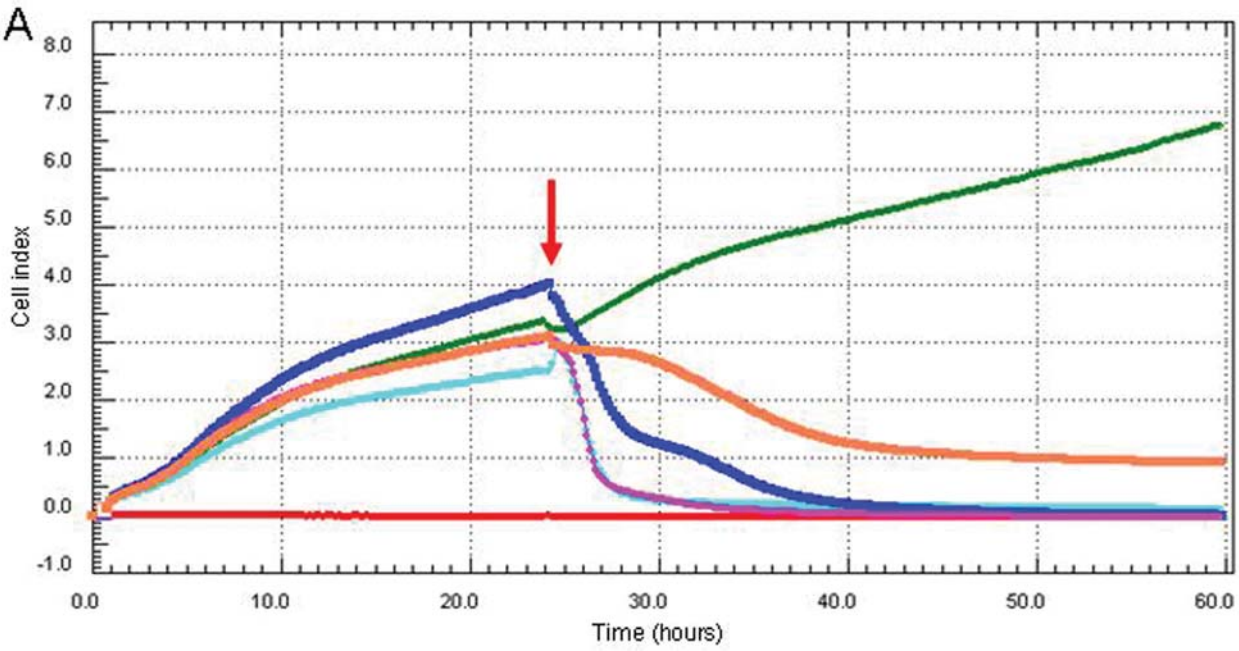
Table I. *Concentrations of mitoxantrone (MTO) and superparamagnetic iron oxide nanoparticles (SPION) used in this study.*

Concentration (μg/ml)			
MTO		SPION	
MTO	SPION+MTO	SPION+MTO	SPION
0.02	0.02	0.7	0.7
0.2	0.2	7	7
2	2	70	70
20	20	700	700
40	40	1400	1400

Cytotoxicity assay. After ending the experiment in the xCELLigence-System™, a lactate dehydrogenase (LDH) assay using the Cytotoxicity Detection KitPLUS (LDH) (Roche Diagnostics GmbH, Mannheim, Germany) was performed in order to confirm the results.

Results

RTCA. The effect of pure MTO as well as of the agent bound to SPIONs, and SPIONs without MTO, on two human cancer cell lines (MCF-7, PC-3) was examined. As there was no considerable difference in the results atributed to the cell lines, only the experiments on the MCF-7 cells are shown. As expected, the analysis of the pure MTO led to a concentration-related behaviour of the cells. Judging from the clinical setting, where MTO is used in a solution of 2 mg/ml, we examined corresponding dilutions. Cells exhibited clear reactions to concentrations of more than 2 μg/ml (1:1000). No considerable effects were detected using a concentration of 0.2 μg/ml (1:10000). High concentrations of 1000 μg/ml (1:2) and 200 μg/ml (1:10) resulted in nearly complete reduction of the CI within the first six hours. At the concentration of 20 μg/ml (1:100), the CI suddenly dropped also within the first six hours but reached zero after 40 hours. After adding MTO at a concentration of 2 μg/ml (1:1000), the CI remained stable for six hours and then began to decrease more slowly than at the higher analyzed concentrations. After a further 12 hours, it stabilized at a value 30% at which point in time the drug was added (Figure 1A). SPIONs without the linked chemotherapeutic agent were also tested to assess their impact on the cells. To allow for a better comparability, the concentration of the unlinked particles used was related to the concentration of MTO in the corresponding MTO-linked ferrofluid (Table I). For example, a concentration of 2 μg/ml (1:1000) MTO correlated approximately to a concentration of 70 μg/ml of SPIONs. The MTO content of the SPION stock solution was measured by HPLC (Waters Alliance model; Waters,



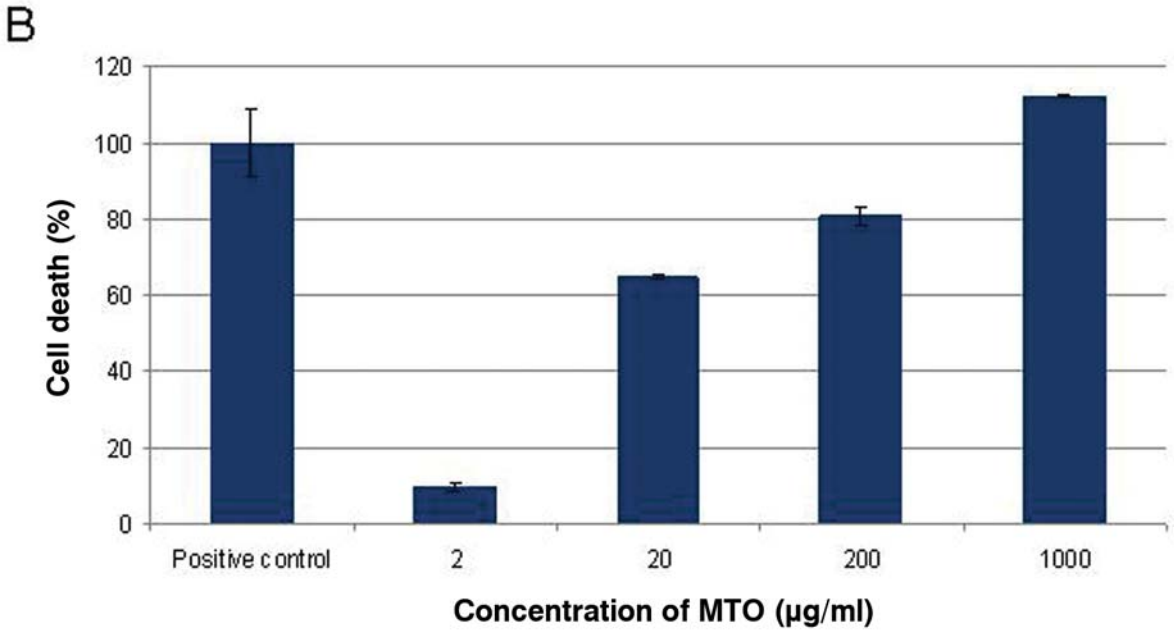


Figure 1. *A: Effect of pure mitoxantrone (MTO) on MCF-7 cells (red curve: pure cell medium as baseline; green curve: cells without test substance; orange curve: 2 µg/ml MTO; dark blue curve: 20 µg/ml MTO; pink curve: 200 µg/ml MTO; light blue curve: 1000 µg/ml MTO; red arrow: substance addition). B: Lactate dehydrogenase (LDH) assay carried out after the ending of the real-time cell analysis (RTCA) experiment.*

Eschborn, Germany). Only the high concentrations of 700 µg/ml and 1400 µg/ml of pure SPION (without MTO) led to a nearly complete decrease of the CI. The dilution of 700 µg/ml SPIONs showed an interesting curve progression. The CI rose sharply to a maximum, 10 hours after adding the pure particles, which was followed by a rapid decrease during the subsequent 12 hours. The highest concentration of pure SPIONs tested in this experiment (1400 µg/ml) led to an immediate fall of the CI within 24 hours after their addition to the cell culture medium. No essential impact was

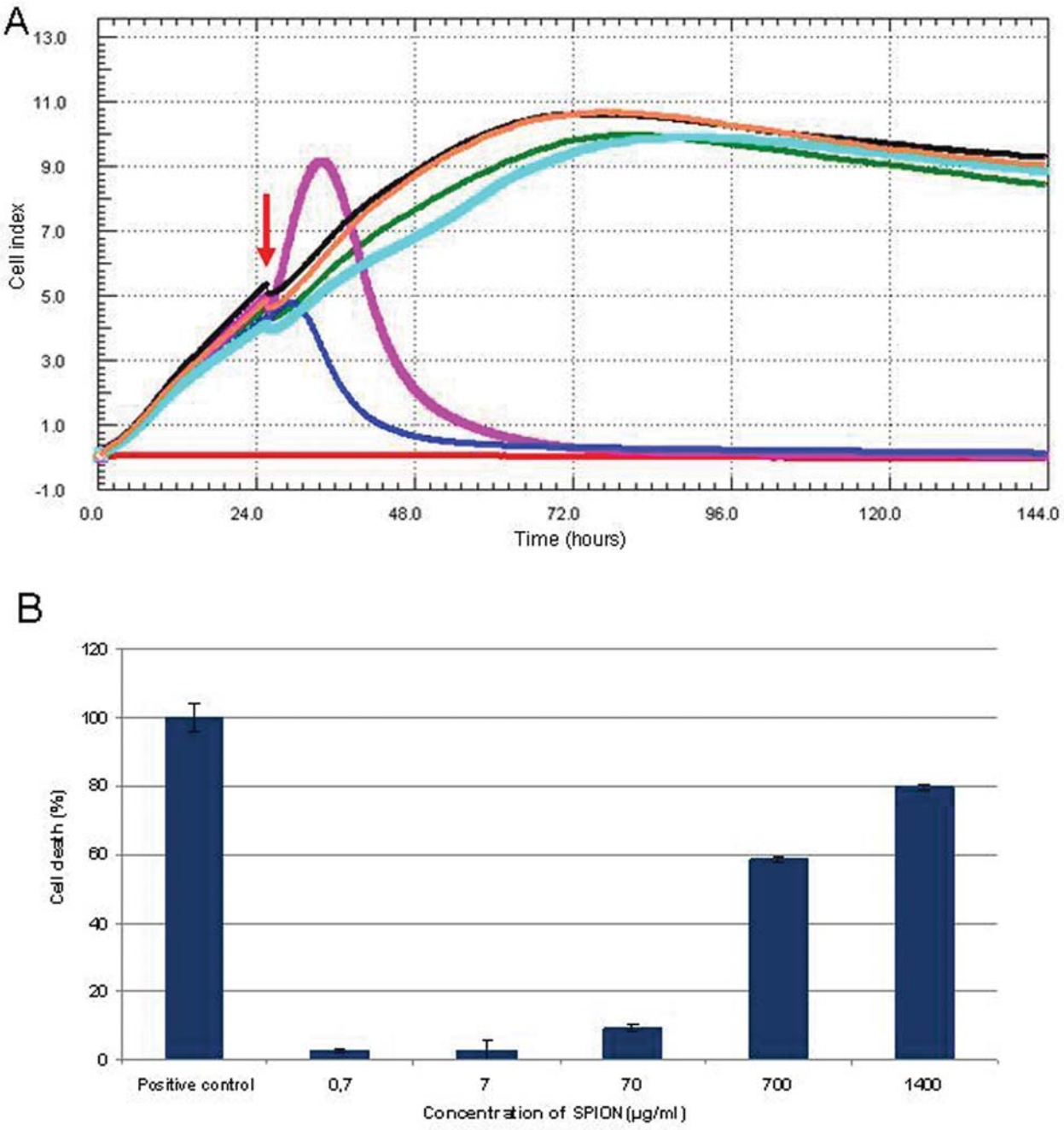


Figure 2. *A: Effect of pure superparamagnetic iron oxide nanoparticles (SPION) on MCF-7 cells (red curve: pure cell medium as baseline; green curve: cells without test substance; orange curve: 0.7 µg/ml SPIONs; black curve: 7 µg/ml SPIONs; light blue curve: 70 µg/ml SPIONs; pink curve: 700 µg/ml SPIONs; dark blue curve: 1400 µg/ml SPIONs; red arrow: substance addition). B: LDH assay carried out after the ending of the RTCA experiment.*

detected by RTCA using the lower SPION concentrations of 70 µg/ml, 7 µg/ml and 0.7 µg/ml (Figure 2A). Taken together, SPIONs that were not linked to MTO had toxic effects only at very high concentrations. In MDT the SPIONs are linked to a drug such as MTO and are applied into a tumour supporting artery. Therefore, SPIONs linked to MTO, as used in MDT, were examined on the tumour cell lines and the measured effects were compared to those of pure chemotherapeutic agent and pure SPIONs. At a concentration of 4 µg/ml pure MTO, the CI decreased to a value of 30% during the first 24 hours after adding the drug and was nearly completely reduced after 48 hours. Compared to this, the

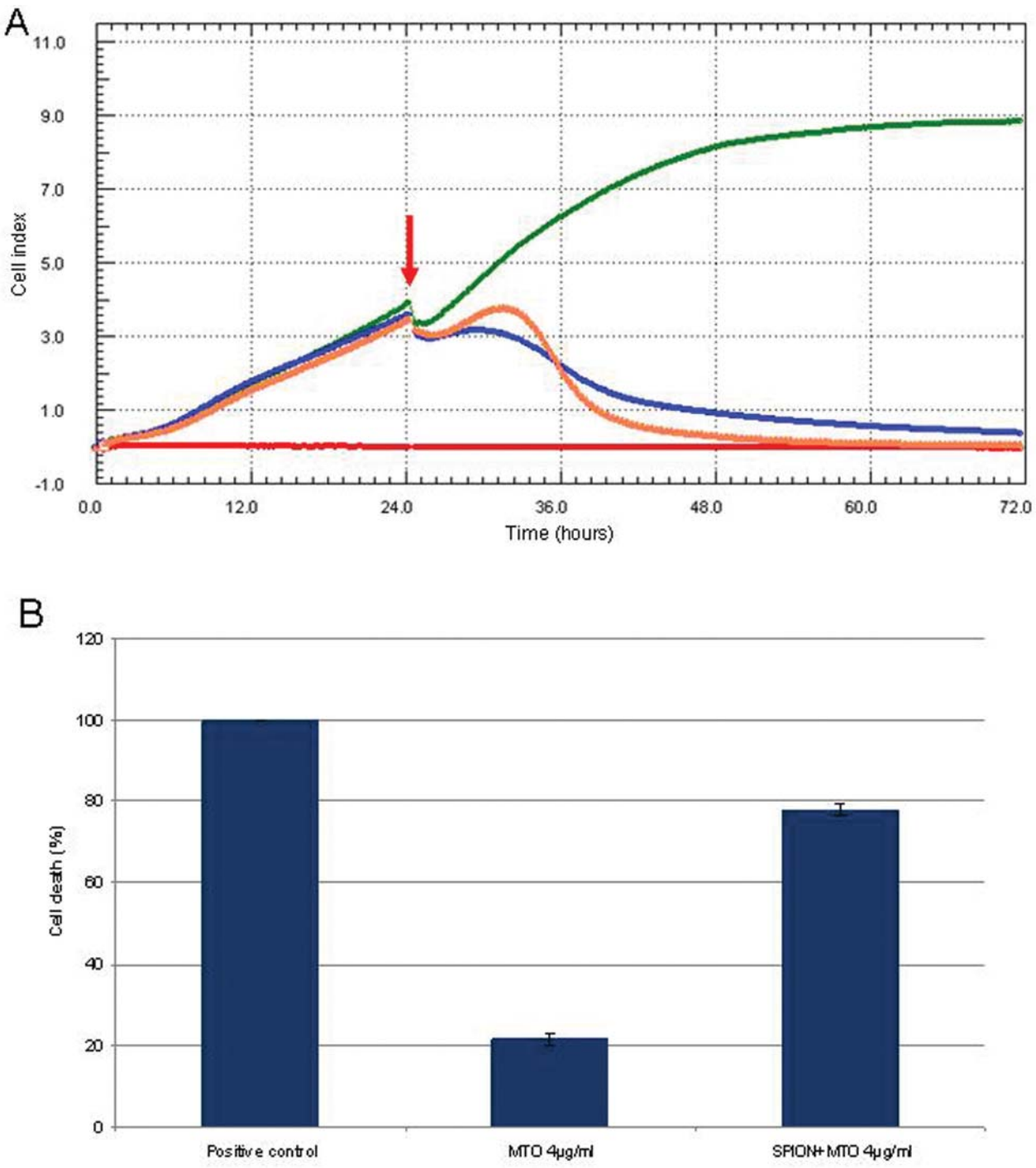


Figure 3. *A: Effect of pure MTO and SPIONs+MTO, as used in magnetic drug targeting, on MCF-7 cells (red curve: pure cell medium as baseline; green curve: cells without test substance; dark blue curve: 4 µg/ml MTO, orange curve: SPIONs+MTO 4 µg/ml; red arrow: substance addition). B: LDH assay carried out after the ending of the RTCA experiment.*

corresponding amount of nanoparticles conjugated with MTO caused an even faster decline of the CI, reaching a value of 30% after 16 hours and a complete reduction 36 hours after the addition of the compound (Figure 3A). Interestingly, its curve also showed a slight rising of the CI during the first hours after adding the nanoparticle MTO compound. This phenomenon was not as pronounced as that described for the addition of 700 µg/ml pure nanoparticles

to the culture medium. Taken together, the RTCA experiments reveal that all tested substances exhibited a rising toxicity with the rise of concentration. The pure nanoparticles did not lead to considerable toxicity at MDT-relevant concentrations. Nanoparticles linked to MTO had an even higher toxicity at a concentration of 4 μg/ml MTO than did MTO alone.

Cytotoxicity assay. After ending the RTCA experiments in the xCELLigence-System™ , an LDH assay was performed on the corresponding plates. The LDH assay confirmed the cell behaviour according to the concentration of the pure MTO that was used. However, it became apparent that a total decrease of the CI did not mean complete cell death, but was due partly to cell death and partly to a poorer adhesion of the cells to the plate. This discrepancy between the results of the RTCA and the cytotoxicity assay was documented by cell microscopy. This phenomenon was also apparent when testing the pure chemotherapeutic agent as well as the pure SPIONs and the pure chemotherapeutic agent *vs.* SPIONs linked to MTO (Figure 1B, 2B, 3B). The increase of the cytotoxic effect of MTO when linked to SPIONs became even more evident in the LDH assay. Adding pure MTO to a dilution of 4 μg/ml and MTO linked to SPIONs at the same concentration, the LDH assay revealed 22% and 78% dead cells respectively after 72 hours, whereas in the RTCA, the curves did not differ as much (Figure 3B).

Discussion

In this work, we investigated the toxicity of MTO, SPIONs and of MTO-loaded SPIONs on the human cancer cell lines MCF-7 and PC-3 (data not shown). This analysis is a question of particular scientific and practical importance, as such nanoparticles linked to a chemotherapeutic agent are already used for regional cancer treatment (15). MTO is an anthracenedione and is structurally related to the group of the anthracyclines including doxorubicin, daunorubicin, idarubicin and epirubicin. Its first clinical application was reported in 1980 (16). Like the latter drugs, it is most commonly used as a chemotherapeutic agent in cancer treatment, *e.g.* in breast (17) and prostate cancer (18). Its application can be systemic, but has also been described for locoregional use (19). SPIONs have already found their way into medicine. They are used for interventious different medical, *e.g.* as colloidal solutions (ferrofluid) for contrast media in magnetic resonance imaging (20, 21). Commonly, SPIONs are well tolerated (22). An essential aspect for their *in vivo* application is the size of the particles. If they are bigger than 100 nm, most of them are removed by phagocytes, usually in the liver and spleen, especially after intravenous application (23). The particles used for this study had a hydrodynamic diameter of 10 to 150 nm with a maximum of 50 nm as detected by dynamic light scattering. The toxicity of SPIONs has been repeatedly examined and discussed in the literature in the last few years. Generally, the toxicity of SPION depends on the concentration used; thus the basic principle "*Dosis sola facit venenum*.", dating from Paracelsus in the 16th century, is still in force. Another crucial aspect is to differentiate between coated and uncoated SPIONs. Examining bare SPIONs, Hussain *et al.* found no effect on cell culture up to a concentration of 100 μg/ml, and at 250 μg/ml they detected a loss of viability of only 30%. Hence, the authors assessed these results as "less or no toxicity at the doses tested" (24). Gupta and Gupta compared SPIONs coated with the polysaccharide pullulan and bare SPIONs in six concentrations from 50 up to 2000 μg/ml. Regarding the uncoated SPIONs, a loss of 20% and of 60% viability was noted at the lowest and the highest concentrations, respectively. No substantial toxicity was found regarding the pullulan coated SPIONs, meaning a loss of viability less than 10% (25).

In our experiments, a toxicity of SPIONs was only apparent at very high concentrations (700 μg/ml and 1400 μg/ml). Yet an enhancement of the toxic effect of the chemotherapeutic agent through linking to SPIONs was seen at much lower concentrations (7 μg/ml and 70 μg/ml). The results of RTCA and of the cytotoxicity assay did not differ in quality and differed only by a little in quantity. However, it should be taken into account, that these two methods determine different parameters. The LDH assay detects the number of dead cells and the RTCA detects a combination of cell number and adhesion of the cells to the bottom of the culture plates. Indeed, it should be noted that adhesion has a crucial impact on important cell characteristics, such as growth, migration, differentiation and survival (26). An interpretation of the course of a CI curve needs to bring under consideration several aspects, and depends on the compound, the duration of exposure, and the mechanism of action. A possibility to explain an uncommon course is to compare it with a curve of a substance whose mechanism is known, because there are specific kinetic profiles. The curve of SPIONs at a concentration of 700 μg/ml with its sharp rise and rapid decrease (Figure 2) is similar to the curve of 5-fluoruracil, which causes apoptosis (27). Therefore, it could be concluded that SPIONs also cause apoptosis at high concentrations. The ascent of the electrical impedance can be explained by a clinging of the cells to the plate. Nevertheless, further investigation of this point is necessary. The essential advantage of applying a method affording a continuous monitoring over the whole experiment compared to one only affording measurements at a specific point of time is obvious. In our opinion, a combination of both methods leads to the best result. For locoregional chemotherapy with MDT, the results of our experiments are very informative. In contrast to conventional therapies, the major advantage of MDT is the enhancement of the local concentration of a drug using magnetic force. In this context,

it is reasonable to have high toxicity at a high concentration to destroy the tumour, but nearly no or low toxicity at low concentrations. Using nanotechnology, and nanoparticular drug delivery in particular, scientists hope to combat the increasing problem of cancer. The presented results encourage us to continue and contribute in bringing a new and innovative therapeutic approach to the clinic.

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