Invest New Drugs (2013) 31:333–344
DOI 10.1007/s10637-012-9870-2

PRECLINICAL STUDIES

Enhanced killing of cervical cancer cells by combinations of methyl jasmonate with cisplatin, X or alpha radiation

Elad Milrot · Anna Jackman · Eliezer Flescher · Pinhas Gonen · Itzhak Kelson · Yona Keisari · Levana Sherman

Received: 3 June 2012 / Accepted: 16 August 2012 / Published online: 6 September 2012

Summary Current therapies for treatment of advanced cervical cancer involve the use of cisplatin, often in combination with radiotherapy. These treatments do not lead to a high survival rate and furthermore, serious side effects are dose-limiting factors. Methyl jasmonate (MJ) was recently identified as potent and selective cytotoxic agent towards cervical cancer cells. In the present study we evaluated the effectiveness of combined treatments of MJ with cisplatin or X-irradiation on a variety of cervical cancer cells including SiHa, CaSki, HeLa and C33A. Cytotoxicity of alpha particles, emitted from ^{224}Ra atoms, was also evaluated as a single agent and in combination with MJ. Cooperation between MJ and cisplatin in reducing cell viability (XTT assays) and survival (clonogenicity assays) was exhibited towards several cancer cell lines at a range of combination doses. MJ effectively cooperated also with X-ray irradiation, significantly lowering the radiation doses required to inhibit cell survival (ID50) of all tested cells lines. We show for the first time, that alpha irradiation selectively reduced cell viability and survival of cervical cancer cells. Lower doses of α irradiation were required as compared to X-irradiation to inhibit cell survival. Cooperation with MJ was demonstrated in part of the cancer cell lines. In conclusion, our studies point to α irradiation and MJ, novel anticancer agents, as potent candidates for treatment of cervical cancer, in single agent regiments and in combination. MJ can be added also to conventional X-ray and cisplatin therapies to increase their cytotoxic effect while lowering the effective dose.

Keywords Cervical cancer · Therapies for cervical cancer · Methyl jasmonate · Cisplatin · X-ray radiation · Alpha radiation · Combination treatments

Dr Eliezer Flescher passed away on 16 May 2011 when this study was in full progress.

E. Milrot · A. Jackman · E. Flescher · P. Gonen · Y. Keisari · L. Sherman (✉)
Department of Clinical Microbiology and Immunology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel
e-mail: lsherman@post.tau.ac.il

I. Kelson
School of Physics and Astronomy, Raymond and Beverly Sackler School of Exact Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

Introduction

Cervical cancer is the third most common cause of female cancer death worldwide and the second common cancer in women in developing countries [1, 2]. It is well established that persistent infections with oncogenic (or "high risk") types of human papillomavirus (HPV) contribute to the development of cervical cancer. The most common oncogenic types are HPV16 and HPV18 which are responsible for 70 % of cases of cervical cancer. HPV DNA is found in more than 90 % of cervical cancers [3, 4]. Recently, two prophylactic vaccines against HPV16 and HPV18 were registered, Gardasil by Merck and Co. and Cervarix by GlaxoSmithKline [5]. However, preventive vaccines are likely to impact on HPV prevalence and cancer rates many years from now and women already infected with the virus or women with cervical cancer will not benefit from these vaccines [5, 6]. Thus, new strategies to treat cervical cancer are still needed.

Current treatments for cervical cancer are platinum based chemotherapy (cisplatin) and radiotherapy [7–9]. Although cisplatin is the drug of choice for treatment of a wide variety of tumors, serious side effects are dose limiting factors [9] and accumulating resistance to cisplatin cytotoxicity need also to be considered [10]. Radiotherapy or surgery, are

considered for women with small localized tumors in the cervix [7]. For bulky or locally advanced cervical cancer, the primary treatment consists of concurrent chemoradiation with platinum-based chemotherapy as radiation alone fails to control the disease [7]. Chemoradiotherapy based treatments improve the survival of patients, but this treatment is limited by toxicity and side effects in the long term [8, 9]. A series of phase II studies has been performed in locally advanced or recurrent/metastatic cervical cancers in order to evaluate the effectiveness of a number of compounds of recent development as single and combination agents [11]. So far however, cisplatin was more effective as a single agent or in combination with other compounds. Novel chemotherapy approaches using inhibitors of survival pathways and activators of apoptotic pathways are currently being investigated as potential options for the treatment of cervical cancer [7, 12].

Studies in recent years discovered that plant stress hormones called jasmonates, which regulate cell death in stressed plants, possess anticancer activities in vitro and in vivo (Reviewed in [13, 14]). Jasmonates induced suppression of cell proliferation and death in a variety of cancer cell lines as well as in leukemia cells from patients with chronic lymphocytic leukemia [15]. Three potential mechanisms of action have been proposed to explain the anticancer activity of jasmonates; the bioenergetics mechanism that involves severe depletion of ATP via mitochondrial perturbation, the induction of re-differentiation via mitogen-activated protein kinase (MAPK) activity and the induction of apoptosis via the generation of reactive oxygen species (ROS) and elevation of the proapoptotic Bcl-2 proteins (Reviewed in [13, 14]). Our recent studies with cervical cancer cell lines identified MJ as a potent cytotoxic agent acting on a range of cancer cells irrespective of the presence of HPV DNA [16, 17]. Cytotoxicity of MJ was selective to the cancer cells, primary human keratinocytes (PHKs) were almost resistant to the agent. The effect of MJ was dose and time dependent, and associated mainly with the induction of cell death and to a lesser extent with inhibition of cell growth. Cell death induced in cervical cancer cells displayed features characteristic of both apoptosis and necrosis [16]. MJ acts via different and common pathways to induce cell death in cervical cancer cells. In some cell lines, MJ caused elevation of the mitochondrial superoxide anion. Different changes in the expression levels of apoptosis control proteins p53, bcl-2 and bax were induced by MJ in the various cervical cancer cell lines, however, reduced expression of the apoptosis inhibitor, survivin, was observed in all tested cell lines [16, 17].

In the present study we asked whether MJ could potentiate the effectiveness of currently used treatments for cervical cancer namely, cisplatin and X-irradiation. Curative therapy regiments for cancer employ multi agent treatments. Combining different anticancer agents may overcome cancer cells resistance to a specific treatment, slow or prevent the development of newly resistant tumor cells and maximize cancer cell cytotoxicity while minimizing host cytotoxicity [18]. Several studies performed recently indicated the ability of MJ to cooperate with chemotherapeutic drugs or anticancer plant products. Cooperative cytotoxicity was demonstrated in several types of cancer cell lines including pancreases, breast, prostate and lung [19–21], as well as cancer cells derived from chronic lymphocytic leukemia [19].

Recently, a novel method which introduces alpha particles into the tumor, named diffusing alpha emitters radiation therapy (DaRT) was described [22]. The method consists of interstitial radioactive wires loaded with radium- 224 (^{224}Ra). The decay of ^{224}Ra releases by recoil its daughter atoms into the tumor, which disperse in the tumor and spray the tumor cells with lethal alpha particles. In vivo treatment of tumor bearing mice with DaRT achieved high levels of local tumor control and prolongation of survival [22–25]. In vitro studies with α particles showed anti proliferative effects and apoptosis in the murine SQ2 squamous cell carcinoma cell line [24], and inhibition of proliferation and reduction of cell survival in various human carcinoma cell lines (26).

As an initial step to evaluate the effectiveness of α irradiation against cervical cancer we investigated the cytotoxic effects of α irradiation toward various cervical cancer cell lines. Cytotoxicity of α irradiation alone and in combination with MJ was investigated.

Materials and methods

Reagents

Methyl jasmonate (MJ) was from Sigma Chemicals (Sigma-aldrich, St Louis, MO). The solution (95 %) was diluted in ethanol absolute to give a stock solution of 500 mM. Further dilutions were performed in culture medium. The final concentration of ethanol in cultures did not exceed 0.6 % [19]. Cis-Diamminedichloroplatinum (II) (cisplatin): ABIPLATIN® (active ingredient cisplatin 1 mg/ml) was obtained from ABIC Biological Laboratories TEVA Ltd, Israel.

Cells

CaSki, SiHa, HeLa, C33A cervical carcinoma cell lines, were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Foreskin primary human keratinocytes (PHKs) were prepared freshly as described previously [27]. PHKs were maintained in keratinocyte serum free medium (KSFM) supplemented with epidermal growth factor (EGF) (5 ngml-1) and bovine pituitary extract (BPE) (50 μgml-1) (all from Invitrogen, Paisely, Scotland). All cervical cancer cell

lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat inactivated foetal bovine serum and penicillin/Streptomycin.

Viability assays for cells treated with cisplatin and MJ

Cervical cancer cells, SiHa, CaSki, HeLa and C33A were seeded into 96-plate dishes at 1×10^4/well. After 24 h, cells were treated with cisplatin (3–10 μM) or/and MJ (1–3 mM) for 24 h. Cell viability was determined with the XTT reagent kit (Biological Industries, Israel, Beit Haemek, Ltd) [16]. This assay allows the development of a reaction in which dehydrogenases reduce the XTT in metabolically active cells. Absorbance of the soluble XTT product was measured at 490 nm. Absorbance is directly proportional to the number of the live cells in culture. The relative cell viability in percentage was calculated as absorbance of drug-treated cells/absorbance of control cells x100 and given as mean of the quadruplicate samples (<10 % standard deviation).

Survival assays for cells treated with cisplatin, X-irradiation and MJ

Clonogenicity assays were carried out as described (17). For X-irradiation, cervical cancer cells were seeded sparsely into 6 cm dishes at concentrations ranging from 500–2,000 cells per well, dependent on the cervical cancer cell line (HeLa, 2,000 cells, CaSki, 1,000 cells, SiHa, 1,000 cells, C33A, 500 cells) and allowed to adhere overnight. Cells were irradiated with X- irradiation (160Kv, MG165, Philips industrial X- ray, Hamburg, Germany) at doses of 0.25-3 Gy. For combination treatment with MJ, after irradiation, medium containing 0.1-1 mMMJ was added for 24 h. MJ was then removed and cells were allowed to grow in medium without MJ for 2 weeks to allow the formation of colonies. Colonies were stained using 1 % methylene blue. Colonies of at least 50 cells were scored and the survival fraction (%) was calculated as mean number of colonies of treated cells/mean number of colonies of control cells X100. For experiments evaluating cisplatin, 24 well plates were used for plating 300–600 cells per well. Cisplatin (0.1–0.5 μM) and MJ (0.1–1 mM) were added at the same time for 24 h.

Viability assays for cells irradiated with α particles in vitro

An in vitro irradiation setup in microplates was used as described before [24]. Cervical cancer cells were seeded at 10^4 cells/well in a 96-well plate implanted with escalating ^{224}Ra activities ranging from 0.063 to 2 Bq/mm^2. Cells were allowed to grow for 48–72 h under continuous irradiation by α particles emitted along the decay chain of ^{224}Ra atoms implanted on the well bottoms. ^{224}Ra implantation took place before cells were seeded and was carried out inside a vacuum chamber, using a column of eight unsealed surface sources of ^{228}Th, fitting a single column of the plate. The ^{224}Ra activity densities on the well bottoms were set by the duration of exposure to the ^{228}Th sources [24]. For combination treatment with MJ, medium was removed after 48 h and replaced with medium containing 1–2 mMMJ. Cell viability was measured after 24 h with the XTT reagent kit (described above in Viability assays for cells treated with cisplatin and MJ).

Survival assays for cells treated with α irradiation, the Kapton wells irradiation setup

Cervical carcinoma cells seeded on a thin (7.5 μm) (polyimide) foil were irradiated by alpha particles traversing the foil from below in a device described before [23, 24, 26]. Before seeding, the Kapton wells were sterilized with UV light for at least 1 h. Cells were seeded on the foil at a density of 3×10^4 cells per well and allowed to adhere overnight. Cells were exposed to the α particle flux by positioning the cells seeded on the foil 10 mm above a ^{228}Th source in air. The dose of irradiation (Gy) was determined according to the exposure time to the alpha particles flux [23, 26]. Immediately after being irradiated in the Kapton wells, the cells were harvested by trypsinization and plated sparsely into 6 cm culture dishes. Cells were allowed to grow and proliferate for 10–14 days with medium replaced twice a week. For combination treatment with MJ, irradiated cells were seeded into 6 well plates in medium containing 0.1 mMMJ. The drug was removed 24 h later and cells were allowed to grow for 2 weeks without the drug until colonies were formed.

Statistical analysis

Differences between treated cells compared to control cells or between the combined treated cells compared to each of the treatments alone were subjected to two-tailed Student's *t*-test. Significance was accepted at $P<0.05$. One-way ANOVA was used when differences in the response to MJ among groups were analyzed. Significance was accepted at $P<0.05$. The combination index (CI) was calculated using the equation described before $CI=\frac{C_{A,x}}{IC_{x,A}}+\frac{C_{B,x}}{IC_{x,B}}$ [28] where CA,x and CB,x are the doses of agent A and agent B used in combination to achieve x % agent effect. ICx,A and ICx,B are the concentrations for single agents to achieve the same effect. A CI of less than, equal to, or more than 1 indicate synergy, additivity, and antagonism, respectively. The CI values of doses indicating synergy or additive effect were presented in the Results. The CI was not presented in case where the CI was more than 1 or could not be calculated because the individual drug effect at the maximal dose used did not reach the combinatorial effect of the drugs.

Results

Long and short term effects of cisplatin in combination with MJ

Cisplatin is currently used for the treatment of advanced cervical cancer, often in combination with radiotherapy. Cisplatin is believed to act via the formation of inter and intra strand cross-links in DNA, culminating in the initiation of cell death via caspases [10]. Recent data have shown that cisplatin may have direct interaction with mitochondria and mitochondrial DNA which can induce mitochondrial damage that may account for a significant portion of the clinical activity associated with this drug [29]. MJ was shown to cooperate with cisplatin in the induction of cytotoxicity in different cancer cell lines including murine prostrate adenocarcinoma, human pancreas carcinoma, and breast cancer cell lines [19, 20]. The combined effect of MJ with cisplatin was not tested before on cervical cancer cells. Both, short and long term cytotoxicity of the agents was evaluated (XTT and clonogenicity assays, respectively). The cytotoxic effect was tested on a variety of cervical cancer cell lines including HPV positive, CaSki, HeLa, and SiHa, and HPV negative, C33A cells. For short term cytotoxicity assays, cells were treated for 24 h with a range of drug doses previously shown to be effective in these cells in single drug regiments, 1–3 mMMJ and 3–10 μM cisplatin [16, 17]. The agents were applied alone and in combination. Representative results from three cell lines are shown in Fig. 1a. The cytotoxic effects of both drugs were dose dependent. SiHa and CaSki (not shown) were less sensitive to the drugs as compared to C33A and HeLa. 10 μM cisplatin (the maximal dose) caused only 18 % reduction in cell viability in SiHa cells and 28 % reduction in CaSki cells. MJ alone caused 10–20 % reduction in cell viability at concentration of 1 and 2 mM. Higher doses of MJ, 3 mM, caused prominent reduction in cell viability. Combined treatment of SiHa and CaSki with cisplatin (1–10 μM) and MJ (1–3 mM) caused only small additional reduction beyond that of cisplatin or MJ alone. C33A and HeLa cells were more sensitive to MJ and cisplatin. MJ at 3 mM reduced cell viability significantly reaching 44 % in C33A and 31 % in HeLa whereas cisplatin alone at concentration of 10 μM reduced cell viability to 48 % and 39 % in the respective cell lines. The combined treatment caused additional decrease in cell viability over that of cisplatin or MJ alone. The effect was evident at combinations of sub-optimal doses. The calculated ID50 values of cisplatin in the short term viability assays are shown in Table 1. Consistent with the above data, reduced ID50 values for cisplatin were observed in C33A, HeLa and CaSki cells in combination treatments with certain MJ doses. The ID50 of other MJ/cisplatin dose combinations could not be determined because the individual effect of MJ or cisplatin at the concentrations used was strong, causing less than 50 % viability (ND).

The long term cytotoxic effect of cisplatin in combination with MJ was tested in clonogenicity assays. Cells were seeded at sparse, clonogenic, concentrations and treated with the drugs 24 h afterwards. Lower doses of MJ were used in these assays ranging from 0.1 to 1 mM. Similar doses were previously shown to be effective in reducing cervical cancer cell survival [17]. Cisplatin was applied at 0.1 –0.5 μM. The drugs were added for 24 h either as single treatments or in combination. Colony formation was evaluated after 2 weeks. Representative results from three cell lines are shown in Fig. 1b. Treatment with 0.1 or 0.3 μM cisplatin alone caused significant reduction in cell survival ($P<0.05$) in HeLa, C33A and SiHa (not shown) cells, with almost no effect in CaSki cells. Increasing the dose of cisplatin to 0.5 μM caused marked reduction in CaSki cells survival (data not shown). Even though cisplatin at 0.1 or 0.3 μM had little effect on CaSki cells, its activity was significantly enhanced upon combination with 0.25 mMMJ, reducing cell survival from 83 % and 94 % to 33 % and 30 %, respectively ($P<0.05$). Significant combinatorial effects ($P<0.05$) were also observed in C33A and HeLa cells while in SiHa cells only a moderate combined effect was observed (not shown). The long term ID50 values for cisplatin as a single agent and in combination with MJ are shown in Table 1. The ID50 values of cisplatin decreased upon combination with MJ. Significant reduction was observed in all cervical cancer cells including CaSki that were relatively resistant to cisplatin. Determination of the combination index (CI), which provides qualitative information on the nature of drug interactions was performed using the equation described previously [28]. A CI of less than, equal to, or more than 1 indicate synergy, additivity, and antagonism, respectively. Table 2 shows CI values of cisplatin and MJ in concentrations where additive or synergistic effects were observed. As indicated, synergistic effects were observed in CaSki, HeLa and C33A cells treated with MJ doses of 0.1 or 0.25 mM combined with cisplatin dose of 0.1 μM. With other drug doses the calculated CI could not be determined because the individual drug effect at the maximal dose used did not reach the combinatorial effect of the drugs, or the calculated CI was more than 1.

The overall results of the cisplatin/MJ combination experiments indicated that MJ and cisplatin can act in a synergistic mode to reduce survival of a variety of cervical cancer cell lines, thus potentially allowing the reduction of the dose of cisplatin required to achieve the same effect. In addition, MJ could be also used to replace cisplatin in case of cisplatin resistant cervical cancers such as CaSki.

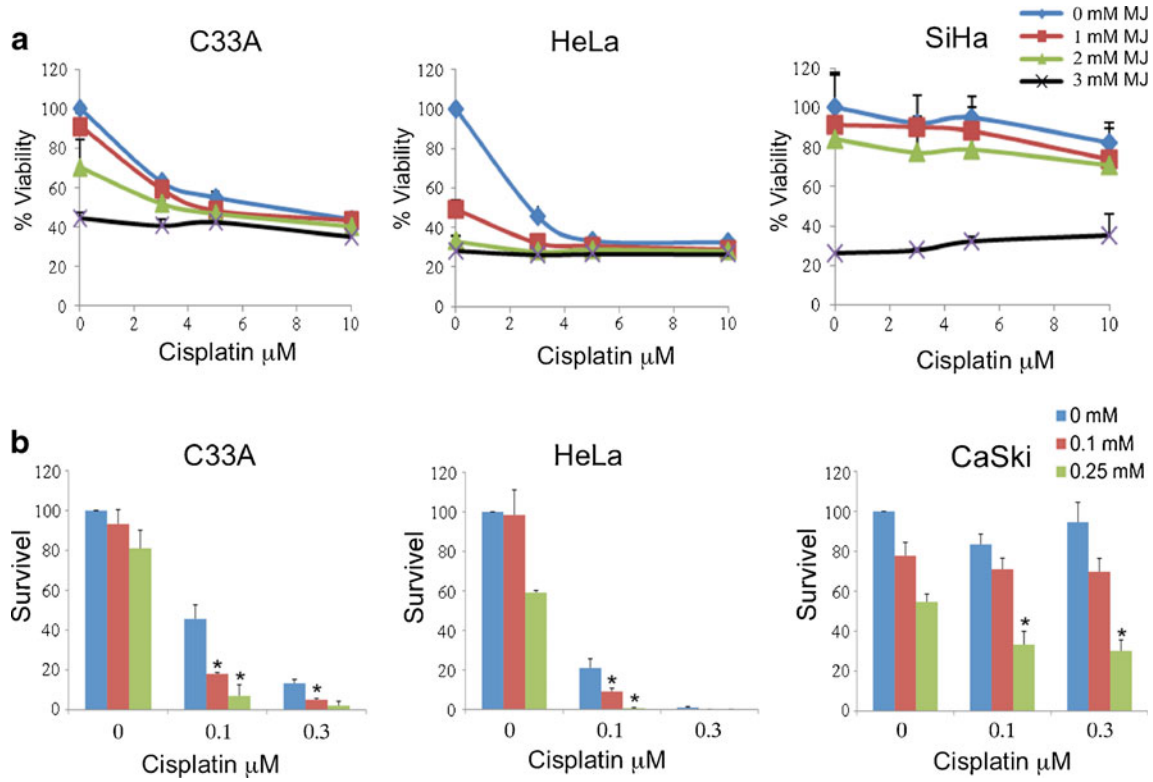

Fig. 1 Effect of combined treatment with cisplatin and MJ on cervical cancer cell viability (**a**) and survival (**b**). **a** Cervical cancer cells, *SiHa*, *CaSki*, *HeLa* and *C33A* were seeded into 96-plate dishes at 1×10^4/well. After 24 h, cells were treated with MJ and cisplatin at the indicated doses. Cell viability was determined with the XTT proliferation assay and normalized to untreated cells. Results obtained with three of the tested cell lines are shown. Results are presented as average percentage of cell viability obtained from 3 independent experiments carried out in quadruplicates. **b** Cervical cancer cells were seeded into 24 well plates at clonogenic concentrations (300–600 cells per well) and allowed to adhere overnight. Cells were treated for 24 h with 0.1–0.5 μM cisplatin and 0.1–1 mM MJ, alone and in combination. Medium was then removed and fresh medium without the drugs was added. Cells were allowed to grow and proliferate for 2 weeks until colonies were formed. Graphical presentation of the survival fraction (percent of untreated control, 100 %) of 3 of the tested cell lines from experiments using 0.1 and 0.3 μM cisplatin is shown. The data are mean ± SEM of duplicates from 2 to 3 independent experiments. Drug combinations showing significant reduction in cell survival ($P<0.05$) as compared to both individual treatments were marked with *asterisk*

Long term effect of X-irradiation in combination with MJ on cervical cancer cell

Radiotherapy plays an important role in cervical cancer therapy. Radiotherapy of cervical cancer involves exposure of the tumor to high-energy radiation, X- ray irradiation. X-ray radiotherapy is delivered by brachytherapy, localized radiotherapy, where the source of radiation is within the patient or via external beam radiation (EBRT) where radiation beam is generated from a source outside the patient [30]. Many agents have been developed to improve cytotoxicity of radiation. Chemotherapy in conjunction with radiation allows reduced radiation doses and limits normal tissue damage. Identifying biological compounds that enhance the efficiency of radiotherapy is of great interest [31]. We evaluated the effect of ionizing X- ray radiation in combination

Table 1 Short and long term ID50 of cisplatin (μM) towards different cervical cancer cells in single treatment and in combination with MJ

Cell line	Viability MJ dose (mM)				Survival MJ dose (mM)			
	0	1	2	3	0	0.1	0.25	0.5
SiHa	>10	>10	>10	ND	0.09	0.06	<0.01	<0.01
CaSki	>10	>10	>10	3.4	0.42	0.38	0.14	<0.01
HeLa	2.7	0.01	N.D	ND	0.06	0.04	<0.01	<0.01
C33A	7	4.2	3.4	ND	0.09	0.04	<0.01	<0.01

Table 2 Doses of MJ (mM) acting in cooperation with cisplatin and X-irradiation to inhibit cell survival

Cell line/doses of MJ (mM) and Cisplatin	CI Synergism/additive	Cell line/doses of MJ (mM) and X-irradiation	CI Synergism/additive
CaSki		CaSki	
0.1 mM+0.1 μM	0.99	0.1 mM+0.5 Gy	0.78
0.25 mM+0.1 μM	0.94	0.25 mM+0.5 Gy	0.81
		0.25 mM+1 Gy	0.86
HeLa		HeLa	
0.1 mM+0.1 μM	0.67	0.1 mM+1 Gy	0.82
0.25 mM+0.1 μM	0.83	0.25 mM+0.25 Gy	0.81
		0.25 mM+1 Gy	1
C33A		C33A	
0.1 mM+0.1 μM	0.59	0.25 mM+0.5 Gy	1

with MJ. For irradiation treatments, cervical cancer cells and primary human keratinocytes (PHKs) were seeded at clonogenic concentrations in 6 cm plates. The following day, the cells were irradiated and then MJ was added for 24 h. Medium was removed and replaced with fresh medium without the drug. Cells were allowed to grow for 2 weeks until colonies were formed. Figure 2 show representative results. Radiation doses of 0.25 or 0.5 Gy had small effect on cell survival, whereas higher doses of irradiation,1 and 3 Gy, significantly reduced cell survival ($P<0.05$). The various cervical cancer cell lines showed different sensitivities to X -ray irradiation as a single treatment with SiHa, CaSki and HeLa being more sensitive than C33A cells (Fig. 2). Addition of MJ significantly increased the efficacy of X-irradiation in all cervical cancer cells. Low doses of MJ (0.1–0.25 mM) were sufficient to improve the effectiveness of X-irradiation. Significant reduction (($P<0.05$) in cell survival, as compared to the single agent treatments was indicated at several dose combinations (Fig. 2, marked with asterix). The increased efficacy of X-irradiation was also reflected in the lower ID50 values of X-irradiation in the combined treatment (Table 3). Determination of the CI values of the combined treatments indicated synergistic or additive effects (Table 2). Synergistic effect of MJ and X-ray irradiation was exhibited at several doses in CaSki and HeLa cells. In C33A cells, an additive effect was observed with 0.25 mMMJ and 0.5 Gy X-irradiation. PHKs were relatively resistant to both treatments when given alone and in combination. These results suggest that combined treatment with X- ray irradiation and MJ can potentially reduce irradiation doses in treatment of cervical cancer.

Short and long term effects of alpha radiation on cervical cancer cells as single treatment and in combination with MJ

Brachytherapy for treatment of solid tumors relies on the use of photons (X- and γ rays). Recently a new experimental therapy approach using irradiation with alpha particles was described, termed DaRT [22]. It consists of interstitial radioactive wires that continually release short-lived alpha emitting atoms into the tumor [22–24]. This treatment was shown to be effective against tumors induced in nude mice with human (A427) and mouse (LL2) derived lung carcinoma cells [24]. Alpha irradiation from in vitro irradiation setups was demonstrated to be effective against various cultured squamous carcinoma cells [23, 24, 26]. The cytotoxic effect of α radiation on cervical cancer cells was evaluated by measuring short and long-term effects (cell viability and survival). Viability assays were carried out using 96-well radioactive microplates. Cells were allowed to grow for 24–72 h under continuous irradiation by alpha particles implanted in escalating doses on the bottom of the plate wells. Results of XTT cell proliferation assays are shown in Fig. 3. The cytotoxic response of the various cell lines to α irradiation was variable with CaSki cells being the most sensitive and SiHa cells being the most resistant (Fig. 3a). A decrease in cell number, enlargement of the nuclei and nuclear fragmentation was observed upon treatment with α irradiation (Fig. 3b). These dose dependent effects were also detected with C33A and HeLa (data not shown). No decrease in cell number or change in morphology of the nuclei was observed in PHKs (Fig. 3b). The combined effect of α irradiation and MJ was determined using the same in vitro irradiation setup. Cells were seeded into Ra-224 coated microplates and incubated for 48 h. Medium was then removed and fresh medium with MJ was administered for additional 24 h. Figure 3c shows the results of XTT assays. Consistent with the data described above, the cells showed variable sensitivities to α irradiation as single treatment, with CaSki being the most sensitive and SiHa being the most resistant. Even though α irradiation had small effect on SiHa cells, the irradiation markedly sensitized SiHa cells to MJ treatment. Addition of 2 mMMJ to 0.063–2 Bq/mm^2 doses of α irradiation reduced cell viability in a dose dependent manner reaching the maximal cytotoxic effect at 2 Bq/mm^2 (2 % viability). With the

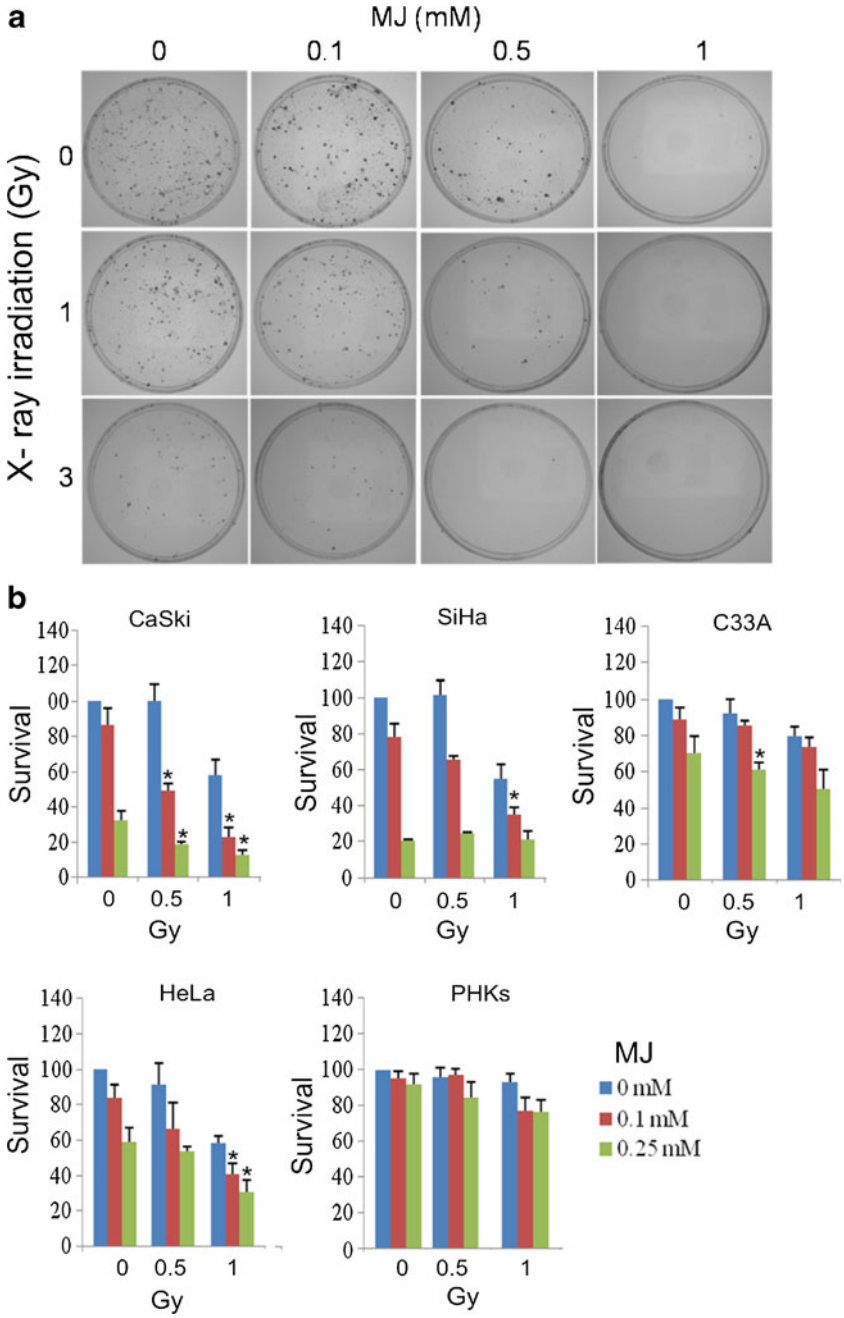

Fig. 2 Effect of combined treatment with X-irradiation and MJ on survival of cervical cancer cells and primary keratinocytes. Cervical cancer cells and PHKs were seeded at clonogenic concentration (500–2000 cells) in 6 cm plates and allowed to adhere overnight. Cells were irradiated with different X-ray doses and then medium with MJ (0.1–1 mM) was added for 24 hours. Cells were allowed to grow and proliferate for 12–14 days. Colonies of at least 50 cells were counted and the survival fraction was determined. (**a**) Representative colony assay with C33A cells treated with X-irradiation and MJ at the indicated doses. (**b**) Histograms presenting the survival fraction of different cancer cells and PHKs after treatment with combinations of MJ (0.1 and 0.25 mM) and X-irradiation (0.5 and 1 Gy). The results shown are mean ± SEM of duplicates from 2–3 independent experiments. Significant reduction in cell survival as compared to both individual treatments ($P<0.05$) was marked with asterisk

combinatorial doses used, modest cooperation was also detected in C33A and HeLa cells. The combined inhibitory effect ranged however only slightly beyond that of the individual effect of MJ or α irradiation. PHKs were resistant to both MJ and α irradiation. The ID50 values calculated for short term cytotoxicity of α irradiation alone and in combination with MJ are shown in Table 4. In SiHa and C33A cells addition of 2 mMMJ significantly reduced the ID50 of α irradiation reaching 0.05 and 0.5 Bq/mm^2, respectively. In HeLa cells, the individual effect of MJ at the doses used was strong, reducing cell viability to 35% at the low dose of 1 mM, thus the exact ID50 values of the combined treatments could not be estimated (ND). In CaSki cells, addition of MJ did not alter significantly the short term ID50 values

Table 3 Long term ID50 of X-ray irradiation (Gy) towards different cervical cancer cells in single treatment and in combination with MJ

MJ dose (mM)			
Cell line	0	0.1	0.25
SiHa	1.25	0.75	ND
Caski	1.2	0.5	ND
HeLa	1.4	0.8	0.1
C33A	1.9	1.7	1

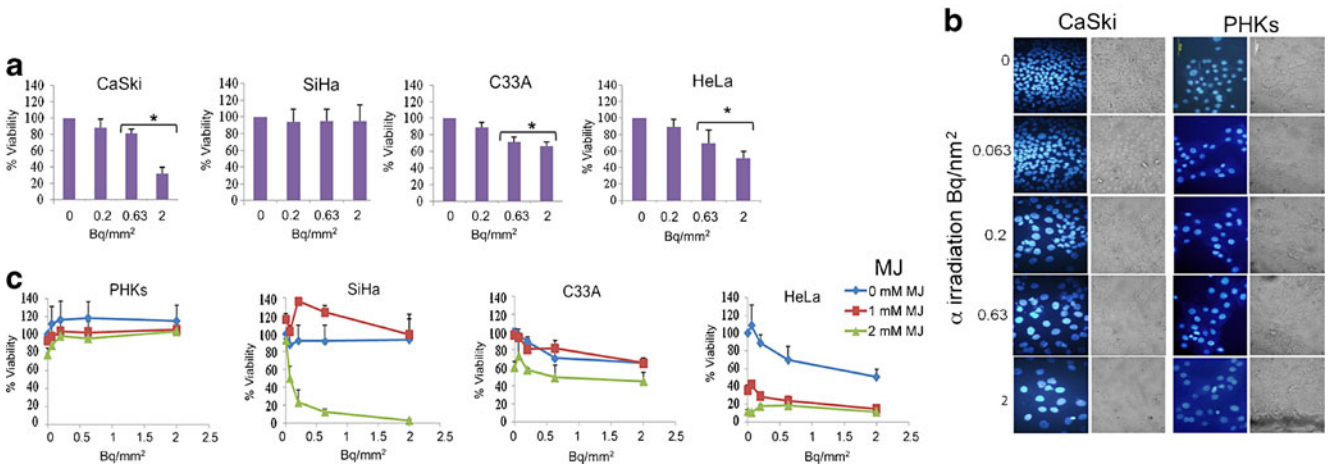

Fig. 3 Effect of α radiation as a single agent treatment and in combination with MJ on viability of cervical cancer cells and primary keratinocytes. Cells were seeded at 10^4 cells per well in 96-well plate implanted with escalating ^{224}Ra activities ranging from 0.063 to 2 Bq/mm^2 and allowed to grow for 72 h. (**a**) Cell viability was determined after 72 h by the XTT proliferation assay and normalized to untreated cells. Results are presented as average percentage of cell viability obtained from 2 to 3 independent experiments. Statistical analyses were carried out using two tailed student's *t*-test. P values less than 0.05 ($P<0.05$) were considered significant (marked with *). **b** Representative digital images of CaSki and PHKs cells treated with α irradiation and stained with DAPI. **c** 48 h after seeding into the radioactive plates MJ was added at the indicated doses and plates were incubated for additional 24 h. Dose response curves from three cancer cell lines and PHKs are shown

of α irradiation. Similarly, PHKs remained resistant to α irradiation in the combined treatment with MJ. Thus, short term cytotoxicity assays identified the selective anticancer activity of α-irradiation against most tested cervical cancer cells. Improved cytotoxic effect of α-irradiation upon combination with MJ (1 and 2 mM) was also observed in some cervical cancer cells notably, SiHa cells that were relatively resistant to α-irradiation.

The long term effect of α irradiation on cervical cancer cells was evaluated in a clonogenicity assays. A different irradiation setup was used. Cells were seeded on thin Kapton foil disks and allowed to attach overnight. Cells were irradiated with escalating doses of alpha particles in a special device (described in Materials and Methods) after which cells were trypsinized and seeded in predetermined clonogenic concentrations into 6 cm plates. Cells were monitored for the ability to form colonies within 2 weeks. The results are shown in Fig. 4a and b. The survival of all cervical cancer cells was significantly reduced at doses of 0.5–2 Gy. The long term response to α irradiation was diverse with CaSki cells being the most sensitive and SiHa cells being the least sensitive. In all cancer cells, even the minimal dose used, 0.25 Gy for HeLa, CaSki and C33A or 0.5 Gy for SiHa, caused significant decrease in cell survival ($P<0.05$). Higher doses of α irradiation completely inhibited cell survival. Table 5 shows the α-irradiation ID50 values (Gy) calculated for the various cervical cancer cells.

To evaluate if MJ cooperates with α- irradiation in reducing the survival of cervical cancer cells, their combined effect was examined in C33A, HeLa and SiHa that were less responsive to alpha irradiation. The combined treatment was tested at suboptimal doses of α irradiation (0.25 Gy) and MJ (0.1 mM). Cells were seeded in Kapton wells and irradiated with alpha particles. After irradiation the cells were harvested and plated sparsely into 6 cm dishes. The next day, cells were treated with 0.1 mMMJ for 24 h. Medium was then removed and fresh medium without the drug was added. Cells were allowed to grow for 2 weeks until colonies were formed. The results are presented in Fig. 4c. Consistent with the data presented above, the responses of the cells towards MJ and α irradiation as single treatments were variable. In C33A both MJ and α irradiation mildly decreased cell survival when given alone. However, significant decrease ($P<0.05$) was detected upon treatment with both.

Table 4 Short term ID50 of α irradiation (Bq/mm^2) towards different cervical cancer cells in single treatment and in combination with MJ

MJ dose (mM)			
Cell line	0	1	2
SiHa	>2	>2	0.05
CaSki	0.96	0.78	1.1
HeLa	2	ND	ND
C33A	>2	>2	0.5
PHKs	>2	>2	>2

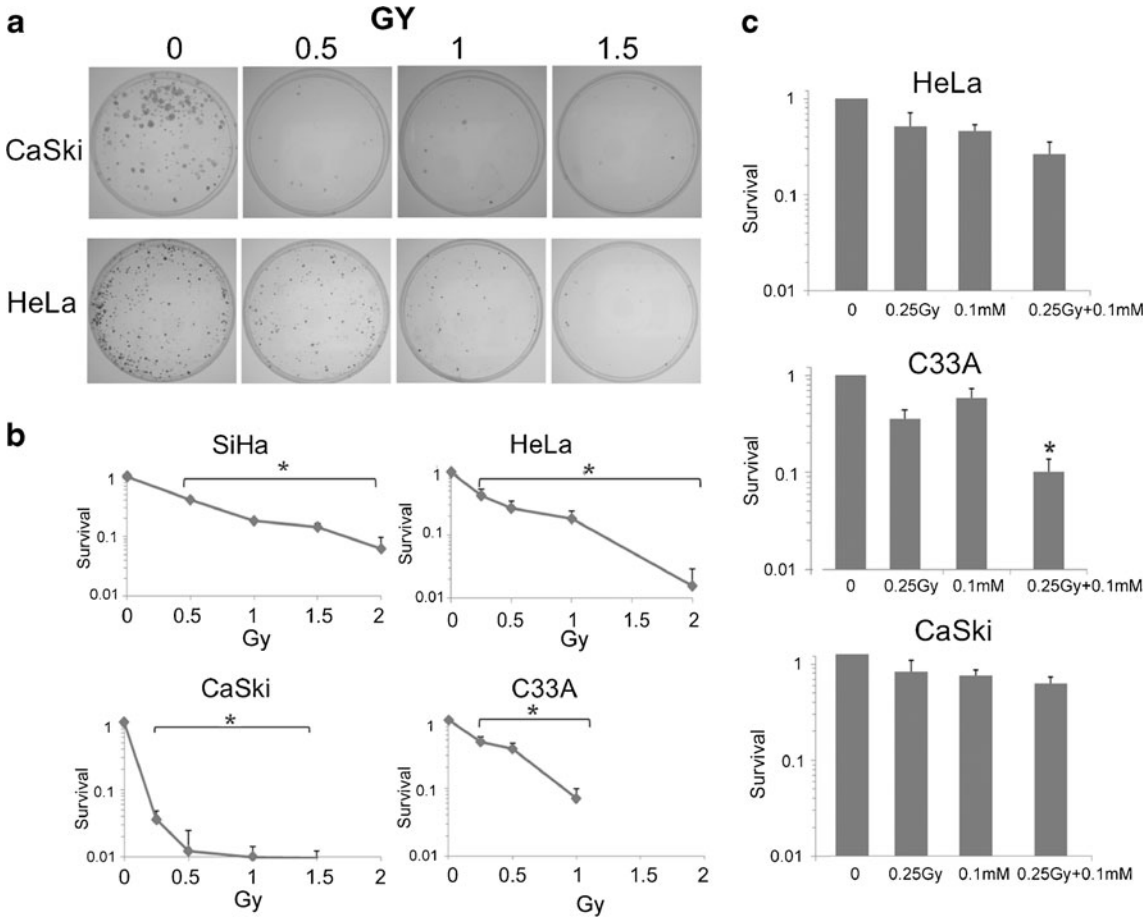

Fig. 4 Effect of α irradiation on survival of cervical cancer cells in a single agent treatment and in combination with MJ. Cervical cancer cells were seeded in Kapton wells at a density of 3×10^4 cells per well and exposed to alpha particles at the indicated doses 24 h later. After being irradiated in the Kapton wells, the cells were harvested and plated sparsely into 6 cm culture dishes. Cells were allowed to grow and proliferate for 12–14 days with medium change twice a week. **a** Representative clonogenicity assay **b** Survival curves of different cervical cancer cells after α particles irradiation with the indicated doses. The data represent the mean surviving fraction as compared to control (1)±SEM obtained from 2 to 3 independent experiments in triplicates. P values less than 0.05 ($P<0.05$) were considered significant (marked with *). **c** 24 h after irradiation and plating into 6 cm culture dishes, cells were treated with 0.1 mMMJ for 24 h and afterwards allowed to grow and proliferate until colonies were formed. The results represent the mean fraction of surviving colonies in treated cells compared to control ± SEM. Results are from 2 independent experiments in triplicates. $P<0.05$ was considered significant (marked with *)

Although α irradiation and MJ at doses of 0.25 Gy and 0.1 mM, improved cell killing in combination treatment of HeLa and CaSki, the decrease in survival was not statistically significant.

Table 5 Long term ID50 of MJ, cisplatin, X-ray and α irradiation towards different cervical cancer cells

Cell line	ID50			
	MJ (mM)	Cisplatin (μM)	X-ray (Gy)	Alpha (Gy)
SiHa	0.17	0.09	1.25	0.4
CaSki	0.27	>0.3	1.2	0.12
HeLa	0.28	0.06	1.4	0.2
C33A	0.34	0.09	1.9	0.24

In conclusion, short and long term cytotoxicity experiments identified α-irradiation as an effective and selective irradiation agent, significantly reducing cervical cancer cell viability and survival. In addition, improved killing upon combination with MJ was achieved in certain cell lines.

Discussion

In the present study we investigated the effectiveness of MJ in combination with cisplatin or X- ray irradiation on cervical cancer cells. In addition, for the first time, the effect of alpha irradiation, which is the basis for a novel treatment of solid tumors, was evaluated on cervical cancer cells as a single treatment and in combination with MJ. The effectiveness of treatments was tested in short and long-term

cytotoxicity assays namely, cell viability and survival assays. These assays evaluate different cell characteristics. The XTT assay is indicative for a short term effects of the agent on cell viability, which is the ability of a cell to preserve its physical integrity and an active respiratory metabolism, whereas the clonogenic ability of cells reflects long-term damage effects which are related to the ability to resume proliferation and growth capacity.

The aim of our studies with MJ was to determine whether MJ could be a substitute for currently used treatments in case of tumor resistance and secondly, whether MJ can cooperate with currently used and novel treatments to increase sensitivity and reduce drug or irradiation doses. Combination therapy has three important expected advantages over single agent therapy. First, it can maximize tumor cell killing while minimizing collateral damage by using agents with non-overlapping dose limiting toxicities. Second, it may increase the range of drugs against tumor cells with endogenous resistance to specific types of therapy. Finally, it may also prevent or slow the development of newly resistant tumor cells [31]. Several findings point to the advantage of MJ. Although the doses of cisplatin which induced ID50 were about 2,000 time lower than the doses of MJ (mM) (Table 5), the doses of MJ are within the in-vivo accepted pharmacological doses based on the pharmacological concentrations achieved in plasma upon administration of a well- studied, closely related plant stress hormone, salicylic acid [32]. In addition, we showed previously [16, 17] and herein, that MJ exhibits selective cytotoxicity against cervical cancer cells with almost no effect on normal human keratinocytes (Figs. 2 and 3). Most importantly, the data showed that the relative cell response to MJ among the different cervical cancer cells lines, in terms of cell survival, is different from the response hierarchy exhibited to other drug or irradiation treatments (Table 5). This suggests that MJ may replace current treatments in case of tumor resistance to commonly used therapies. CaSki cells, for example that showed marked resistance to cisplatin, exhibited significant sensitivity to MJ. Thus, in case of tumors resistant to cisplatin, MJ may be considered as an alternative.

In this study we also show the advantage of combination treatments with MJ in reducing the doses of cisplatin, X- ray irradiation and α irradiation. We addressed three different parameters in the cooperation testing assays. First, the statistical significance of the dose difference between the individual and combined treatment. Second, the ability of the combined treatment to lower the ID50 values of the individual treatment. Third, the combination index value (CI) which is a mathematical objective definition for the nature of interaction. The CI may imply on the efficiency of the combination treatments at the therapeutic level. A significant cooperation of MJ and cisplatin was observed in CaSki, C33A and HeLa cells using several concentrations (Fig. 1 and Table 1), and moderate cooperation in SiHa cells. This suggests the possibility of combination treatment with the two agents, thereby, reducing cytotoxicity of cisplatin. We found synergistic activities in certain doses that may suggest combined activity in cell killing through different molecular pathways. Previous studies also showed that MJ is capable to cooperate with cisplatin to enhance cytotoxicity in other cancer cell lines [19, 20].

We showed that high doses of X- ray irradiation (1 and 3 Gy) significantly reduced cell survival of all cervical cancer cell lines. PHKs were less affected by the irradiation treatment compared to the carcinoma cell lines, although inhibition of survival was still detected at high doses of irradiation (3 Gy, 50 % inhibition). The hierarchy of cell sensitivity to MJ and X -ray irradiation was similar (Table 5) implying common pathways of cell death. Combination of both treatments was also favorable, as indicated by the higher reduction of cell survival and reduced ID50 values of X-irradiation upon treatment in combination with MJ. Thus, MJ as adjuvant to X- ray irradiation could potentially allow lower doses of X- ray irradiation and reduced side effects.

Alpha radiation has several features which make it suitable for local treatment of tumors. Alpha particles are characterized by high energy deposition, high relative biological effect (RBE), and low track path length [33], which suggests limited irradiation of the tumor cells and no irradiation of normal cells. In addition, sensitization of cells to high linear energy transfer (LET) irradiations, such as alpha particles, causes cell death mainly by DNA double strand breaks which is independent of cell cycle progression and oxygen consumption [34]. Thus, alpha particles are potential candidate for the treatment of cervical cancer which is highly localized in its initial stage. The data described herein show, for the first time, the effectiveness of α irradiation against cervical cancer cells. When comparing α irradiation and X-ray irradiation we noted two intriguing differences. First, in general, the cervical cancer cells were much more sensitive to alpha irradiation than X -ray irradiation. At the same irradiation doses, the ID50 values for α irradiation were about 8 times lower than that of X- irradiation (Table 5). Secondly, the cervical cancer cells relative sensitivity to α and X- irradiation was different. Because of the nature of α radiation described above, and the low doses of irradiation required to cause the same effect as X- ray irradiation, α irradiation could possibly be more effective than X- ray irradiation in the treatment of cervical cancer tumor.

We observed a cooperative effect between α irradiation and MJ in some cervical cancer cell lines, indicated in the cytotoxicity (SiHa, C33A) or survival (C33A) assays results. The collaboration may be an outcome of the different main targets of each agent, mitochondria for MJ and DNA for alpha irradiation. With the suboptimal dose of MJ and α irradiation used, a significant cooperation in cell

killing was observed in C33A cells, but mild effects in SiHa and HeLa cells. Higher doses or longer treatment with MJ could possibly increase the efficacy of α irradiation in these cells. Additional studies are needed to determine the range of effective doses for α irradiation alone and in combination with MJ that induce effective killing in cervical cancer cells, as well as studies to determine the mechanisms of action of α irradiation in these cells.

In summary, our study shows that α particles, which serve as a basis for a novel irradiation modality (i.e. DaRT), and MJ, a novel anticancer agent, exhibit potent short and long term cytotoxic effects on cervical cancer cells when given alone and in combination. In addition, MJ cooperated with current therapies for cervical cancer namely, cisplatin and X -ray irradiation. This makes α irradiation and MJ promising candidates for treatment of cervical cancer. Additional studies are needed to determine the efficacy of the agents, in vivo, in xenograft tumor models of cervical cancer.

Acknowledgments This research was supported by a research grant from the Chief Scientist's Office, Ministry of Health, Israel (grant 3–3066) awarded to LS and in part by a grant from the Bernard Jacobson Fund for Cancer Research, Tel-Aviv University, awarded to LS, and a grant from the Israel Cancer Association, awarded to YK.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Parkin DM (2001) Global cancer statistics in the year 2000. Lancet Oncol 2(9):533–543
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90
3. Steben M, Duarte-Franco E (2007) Human papillomavirus infection: epidemiology and pathophysiology. Gynecol Oncol 107 (Suppl 1):S2–S5
4. DiMaio D, Liao JB (2006) Human papillomaviruses and cervical cancer. Adv Virus Res 66:125–159
5. Schiller JT, Castellsague X, Villa LL, Hildesheim A (2008) An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. Vaccine 26(Suppl 10):K53–K61
6. Lin K, Doolan K, Hung CF, Wu TC (2010) Perspectives for preventive and therapeutic HPV vaccines. J Formos Med Assoc 109(1):4–24
7. Movva S, Rodriguez L, Arias-Pulido H, Verschraegen C (2009) Novel chemotherapy approaches for cervical cancer. Cancer 115 (14):3166–3180
8. Serkies K, Jassem J (2005) Chemotherapy in the primary treatment of cervical Carcinoma. Crit Rev Oncol Hematol 54(3):197–208
9. Maduro JH, Pras E, Willemse PH, de Vries EGE (2003) Acute and long-term toxicity following radiotherapy alone or in combination with chemotherapy for locally advanced cervical cancer. Cancer Treat Rev 29(6):471–488
10. Siddik ZH (2003) Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 22(47):7265–7279
11. Savarese A, Cognetti F (2003) New Drugs in the treatment of recurrent or metastatic cervical cancer. Crit Rev Oncol Hematol 48(3):323–327
12. Hougardy BM, Maduro JH, Vander Zee AGJ, Willemse PHB, deJong S, de Vries EGE (2005) Clinical potential of inhibitors of survival pathways and activators of apoptotic pathways in treatment of cervical cancer: changing the apoptotic balance. Lancet Oncol 6(8):589–598
13. Flescher E (2007) Jasmonates in cancer therapy. Cancer Lett 245 (1–2):1–10
14. Cohen S, Flescher E (2009) Methyl jasmonate: a plant stress hormone as an anti-cancer drug. Phytochemistry 70(13–14):1600–1609
15. Rotem R, Heyfets A, Fingrut O, Blickstein D, Shaklai M, Flescher E (2005) Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. Cancer Res 65 (5):1984–1993
16. Kniazhanski T, Jackman A, Heyfets A, Gonen P, Flescher E, Sherman L (2008) Methyl jasmonate induces cell death with mixed characteristics of apoptosis and necrosis in cervical cancer cells. Cancer Lett 271(1):34–46
17. Milrot E, Jackman A, Kniazhanski T, Gonen P, Flescher E, Sherman L (2012) Methyl jasmonate reduces the survival of cervical cancer cells and downregulates HPV E6 and E7, and survivin. Cancer Lett 319(1):31–38
18. Takimoto C, Calvo E (2005) Principles of oncologic pharmacotherapy. In: Pazdur R, Coia LR, Hoskins WJ, Wagman LD (eds) Cancer management: a multidisciplinary approach. CMP Healthcare Media, Manhasset, pp 23–42
19. Heyfets A, Flescher E (2007) Cooperative cytotoxicity of methyl jasmonate with anti-cancer drugs and 2-deoxy-D-glucose. Cancer Lett 250(2):300–310
20. Yeruva L, Hall C, Abiodun J, Elegbede JA, Carper SW (2010) Perillyl alcohol and methyl jasmonate sensitize cancer cells to cisplatin. Anticancer Drugs 21(1):1–9
21. Ezekwudo D, Shashidharamurthy R, Devineni D, Bozeman E, Palaniappan R, Selvaraj P (2008) Inhibition of expression of anti-apoptotic protein Bcl-2 and induction of cell death in radioresistant human prostate adenocarcinoma cell line (PC-3) by methyl jasmonate. Cancer Lett 270(2):277–285
22. Arazi L, Cooks T, Schmidt M, Keisari Y, Kelson I (2007) Treatment of solid tumors by interstitial release of recoiling short-lived alpha emitters. Phys Med Biol 52(16):5025–5042
23. Cooks T, Arazi L, Schmidt M, MarshakG KI, Keisari Y (2008) Growth retardation and destruction of experimental squamous cell carcinoma by interstitial radioactive wires releasing diffusing alpha-emitting atoms. Int J Cancer 122(7):1657–1664
24. Cooks T, Schmidt M, Bittan H, Lazarov E, Arazi L, Kelson I, Keisari Y (2009) Local control of lung derived tumors by diffusing alpha-emitting atoms released from intratumoral wires loaded with radium-224. Int J Radiat Oncol Biol Phys 74(3):966–973
25. Horev-Drori G, Cooks T, Bittan H, Lazarov E, Scmidt M, Arazi L, Efrati M, Kelson I, Keisari Y (2012) Local control of malignant pancreatic tumors by a combined treatment with intratumoral 225Radium-loaded wires releasing alpha-emitting atoms and chemotherapy. Transl Res 159(1):32–41
26. Lazarov E, Arazi L, Efrati M, Crooks T, Scmidt M, Keisari Y, Kelson I (2012) Comparative in vitro microdosimetric study of murine-and human-derived cancer cells exposed to alpha particles. Radiat Res 177(3):280–287
27. Alfandari J, Shnitman Magal S, Jackman A, Schlegel R, Gonen P, Sherman L (1999) HPV16 E6 oncoprotein inhibits apoptosis induced during serum-calcium differentiation of foreskin human keratinocytes. Virology 257(2):383–396
28. Zhao L, Wientjes MG, Au JL (2004) Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram and combination index analyses. Clin Cancer Res 10(23):7994–8004

29. Cullen KJ, Yang Z, Schumaker L, Guo Z (2007) Mitochondria as a critical target of the chemotherapeutic agent cisplatin in head and neck cancer. J Bioenerg Biomembr 39(1):43–50
30. Ahamad A, Jhingran A (2004) New radiation techniques in gynecological cancer. Int J Gynecol Cancer 14(4):569–579
31. Mauceri HJ, Hanna NN, Beckett MA, Gorski DH, Staba MJ, Stellato KA, Bigelow K, Heimann R, Gately S, Dhanabal M, Soff GA, Sukhatme VP, Kufe DW, Weichselbaum RR (1998) Combined effects of angiostatin and ionizing radiation in antitumour therapy. Nature 394(6690):287–291
32. Katzung BG (1998) Nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, analgesics, and drugs non-opioid used in gout. In: Katzung BG (ed) Basic and clinical pharmacology. Appleton & Lange, Stamford (CT), pp 578–602
33. Pouget JP, Mather SJ (2001) General aspects of the cellular response to low- and high-LET radiation. Eur J Nucl Med 28 (4):541–561
34. Mori E, Takahashi A, Yamakawa N, Kirita T, Ohnishi T (2009) High LET heavy ion radiation induces p53-independent apoptosis. J Radiat Res 50(1):37–42