Title: Radiosensitization of head and neck cancer cell lines by a combination of poly(I:C) and cisplatin through downregulation of survivin and cIAP2

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Running title: poly(I:C) and cisplatin induce radiosenzitization

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Abstract

Purpose: Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers. Concurrent radio-chemotherapy is the standard of care for advanced tumors. However, there is still a need for more efficient treatments with less secondary effects, resulting from high doses. Therefore, we undertook to explore the therapeutic potential of ternary combinations- bringing together irradiation, cis-platinum and a TLR3 agonist, poly(I:C) with aim to reduce dosage of each treatment. This approach is based on our previous studies showing a selective cytotoxic effect of TLR3 agonists against malignant cells when it is combined with other anti-neoplastic agents.

Methods: We have explored the cell survival of the head and neck cancer cells (Detroit 562, FaDu, SQ20B and Cal27) by MTT and caspase 3/7 assay. The radiosensitization effect of poly(I:C) and cisplatin treatment was shown by western blot, cell cycle analysis, ROS determination and qPCR.

Results: We have shown here that the combination of poly(I:C) and cisplatin can downregulate cIAP2 and survivin expression, reduce cell survival, induce anti-apoptotic gene expression and apoptosis, generate ROS formation and cause G2/M phase arrest in HNSCC cell lines.

Conclusions: Our results show that poly(I:C) and cisplatin combination therapy reduces cancer cell survival and induces radiosensitivity in HNSCC cell lines thus providing a rationale for the development of a novel strategy in head and neck cancer treatment.

Keywords: poly(I:C); cisplatin; radiosensitization; head and neck cancer; therapy; TLR3

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most frequent cancer worldwide [1]. Most common etiological factors include consumption of tobacco and alcohol and infection with human papilloma virus (HPV) (for oropharyngeal cancer) [2, 3]. The therapeutic approaches vary depending on the location, stage and pathohistological properties of the disease, but commonly include surgery, radiotherapy, chemotherapy and targeted therapy with cetuximab [4]. The combination of radiotherapy and cisplatin-based chemotherapy is the standard therapeutic approach in the treatment of high-risk locally advanced HNSCC [5]. Cis-platinum brings a double benefit: it acts as a radio-sensitizer in the tumor volume while it contributes to the control of distant micro-metastases. However, concurrent chemo-radiotherapy is associated with notable toxicity, especially when used in concurrent setting and in higher doses, which is often necessary for treatment of advanced tumors. Hence the need for novel therapeutic approaches to increase the therapeutic index.

Toll-like receptors (TLRs) are transmembrane pattern-recognition receptors that are expressed on the surface of the antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, where they recognize pathogen-associated molecular patterns, or PAMPs [6, 7]. Once the ligand binds to the receptor, a signaling cascade is activated, resulting in activation of NF-κB transcription factor, as well as members of the AP-1 and IRF family, finally leading to secretion of interferons, cytokines, and chemokines [8]. TLRs were also found to be expressed on tumor tissues, including the head and neck cancer [9, 10]. TLR3 is mainly expressed in the endosome, where it recognizes the double-stranded RNA [11] or polyinosinic:polycytidylic acid (poly(I:C)), a synthetic dsRNA analog. Several studies reported TLR3 expression in cells of HNSCCs [12, 13].

The influence of TLR3 agonists- especially poly(I:C)- on malignant epithelial cells remains controversial. Some studies reported the pro-apoptotic effects of TLR3 agonists but these effects were generally obtained using very high concentrations of agonists which were not compatible with clinical use [14-17]. In contrast, other studies dealing with low concentrations of agonists showed evidence of their contribution to carcinogenesis and promotion of tumor growth and invasiveness [18-20]. We have previously shown that these pro-tumorigenic effects result at least in part from the overexpression of cellular Inhibitor of Apoptosis Protein 2 (c-IAP2). Combining TLR3 agonists with agents inhibiting c-IAP2, not only blocks their pro-tumorigenic effects but also unmasks their strong pro-apoptotic effects [21, 22]. Because c-IAP2 inhibitors are not yet in clinical use, we investigated whether it was possible to create the same type of synergy with other anti-neoplastic agents like cis-platinum.

In our current study we show that the combination of poly(I:C) and cisplatin increases the sensitivity of malignant cells to irradiation. We further investigated the mechanism underlying this radiosensitization.

2. Materials and methods

2.1. Cells and reagents

Human head and neck cancer cell lines (SQ20B, FaDu and Detroit 562) were maintained in Dulbecco's modified Eagle medium (Life technologies, Gaithersburg, USA) supplemented with 2mM L-glutamine, and 10% fetal calf serum in a humidified chamber at 37ºC in 5% CO_2. Detroit 562 cell line was obtained from EACC. The Fadu and SQ20B lines were provided by Pr Eric Deutsch (Gustave Roussy, Villejuif, France). Poly(I:C) and polyadenylic–polyuridylic acid (poly(A:U) was obtained from InvivoGen (San Diego, USA). Doxycyclin was purchased from Sigma Aldrich. Details on RMT5265 have been decribed previously [14]. The compound was kindly provided by Xiaodong Wang, Dallas. Subclones of SQ20B and FaDu cell lines were established by transfection with plasmid carrying shRNA directed against TLR3 and inducible by doxycycline (TET-on system) thus allowing conditional knock-down of TLR3 as described previously [15].

2.2. Proliferation assay

The proliferation experiments were carried out by MTT Cell Proliferation Assay which is a colorimetric assay system that measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. The absorbency (OD, optical density) was measured at 570 nm using a microplate reader. The absorbency is directly proportional to cell viability. Cells were plated at 1×104 cells/well in 96-well microtiter plate and treated with poly(I:C) (10 µg/mL), poly(A:U) (10 µg/mL) and cisplatin (1µM) for 45 minutes followed by irradiation. MTT test was performed after 72 hours. Each test point was performed in quadruplicate in three individual experiments.

2.3. Irradiation

For gamma irradiation experiments cells were exposed to ^{60}Co γ-irradiation panoramic source (20mGy/s, Rudjer Boskovic Institute, Radiation Chemistry and Dosimetry Laboratory) to a total of 2.5 Gy or 7.5 Gy.

2.4. RNA isolation and Real Time PCR

Cells were treated with poly(I:C) and cisplatin for 45 minutes and irradiated. RNA was isolated after 24 hours with RNeasy Mini Kit (Qiagen, Hilden, Germany) and 0.1 μg of RNA was used as a template for cDNA synthesis reaction by TaqMan ® Reverse Transcription Reagens Kit (Applied Biosystems, Branchburg, USA). The PCR reactions were performed with Sybr® Green PCR Master Mix (Applied Biosystems, Warrington, UK) on a CFX96™ Real-Time PCR Detection System (BioRad, USA). Primer sequences are shown as supplementary data. Following amplification program was used: 95ºC for 10 min (initial denaturation), 95ºC for 30s (denaturation), 62ºC for 30s and 60ºC for 30s (annealing and elongation) and we normalized data to 28S rRNA gene. All data were analyzed using the 2-ΔΔCt (relative quantitation) method, after ensuring that all primer sets amplified their respective sequence targets with similar efficiencies and without primer-dimers.

2.5. Measurement of caspase 3/7 enzyme activity

Caspase 3/7 enzyme activity was determined using the Caspase-Glo 3/7 assay (Promega, Madison, USA) as previously described [16]. Briefly, SQ20B cells at a concentration of 1.5×10^4 cells/well were plated in a white-walled 96-well plate in 100 μl culture medium and treated with 10 μg/ml poly(I:C) or 1μM cisplatin for 45 minutes, irradiated and caspase 3/7 activity was determined after 24 hours. 80 μl of Caspase- Glo 3/7 reagent was added on to each well and the plates were incubated at room temperature for 1 hour. Finally, the luminescence of each sample was measured with luminometer (Fluoroscan ascent FL, Thermo Sientific, Germany).

2.6. Western blot analysis

Cells were seeded in 6 well plates at a density of $0.4x10^6$/well, treated with poly(I:C) and cisplatin for 45 minutes and irradiated. Proteins were isolated after 24 hours by resuspension of the cells in RIPA buffer containing protease inhibitor cocktail (Complete Mini EDTA-free, Roche.) and the cells were sonicated. 4× Laemmly buffer (0.25 M Tris-HCl pH 6.8, 20% DTT, 8% Na dodecil sulfate, 40% glicerol, 0.008% bromphenol blue) was added to obtain 1× mixture and the samples were heated to 95˚C. For Western blot analysis equal amounts of proteins (40 μg) were separated on 10% or 12% SDS-PAGE and transferred onto 0.2 μm nitrocellulose membrane (BioRad). The membranes were blocked with 5% nonfat dry milk and were stained with primary antibodies: IAP Family Antibody Sampler Kit (#9770, Cell Signaling Technology)), PARP (Calbiochem) and actin (Sigma-Aldrich). Afterwards, the membranes were stained with peroxidase conjugated secondary antibody (NA934V, Amersham, in a concentration 1:3000) and visualized with the chemiluminescent system (Perkin Elmer). Densitometric calculations of the band intensities corresponding to specific protein were performed using Uvidoc Cambridge Chemiluminescence Imaging system (Uvitec Cambridge) and UVIband software (Uvitec Cambridge).

2.7. Measurement of intracellular ROS production

Cells were seeded at a density of 2×10^4 cells/well in a 96-well white-walled plate. After washing with Hanks Balanced Salt Solution (HBSS), cells were incubated for 30 minutes with a nonfluorescent probe for intracellular ROS detection 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Fluka). This cell-permeable dye remains non-fluorescent inside the cell until the acetate groups are removed by intracellular esterases and oxidized by intracellular ROS to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) which can be detected as a measure for the sensitive and rapid quantitative determination of intracellular ROS in response to oxidative stress. Following incubation with DCFH-DA, cells were treated with 1μM cisplatin and 10μg/mL of poly(I:C), but in HBSS instead of culture medium, and after 2 hours, fluorescence intensity was read with Infinite® 200 PRO microplate reader (Tecan) with excitation at 500 nm and emission detection at 529 nm.

2.8. Cell cycle analysis

Detroit 562 cells were seeded in 12 well dishes and treated with 10 μg/mL of poly(I:C) and 1 μM cisplatin for 45 minutes and irradiated. After 24 or 72 hours, cells were harvested, washed, fixed in ice-cold 70% ethanol, treated with RNAse and stained with propidium iodide (Sigma-Aldrich) to evaluate cell cycle distributions. 15.000 events were counted for each experimental setup with routine flow cytometric analysis (FACSCalibur™, Becton-Dickinson, Heidelberg, Germany). Histograms were created and analyzed using ModFit software (Verity Software House, Topsham, ME, USA). Each experiment was repeated at least three times on different days for validation.

2.9. Statistics

Statistical significance was assessed with two-tailed Student's *t* test, and results are given as the mean ±SD.

3. Results

3.1. Poly(I:C) and cisplatin in combination with irradiation reduce cell survival

We have firstly determined that poly(I:C) in combination with cisplatin may increase radiosensitivity in cancer cells, especially in SQ20B cells which are known to be radioresistant [26]. In Detroit 562 cells poly(I:C) alone induced cell death up to 90%, which was further increased to 100% when cells were irradiated after the pre-treatment with poly(I:C) (Fig. 1A). poly(I:C) and cisplatin combination pre-treatment showed similar results as poly(I:C) alone. Poly(I:C) treatment of SQ20B cells reduced cell survival to 30%, which was slightly reduced by addition of cisplatin. Irradiation of SQ20B cells with 2.5 Gy after poly(I:C) treatment did not show the change, however, irradiation with 7.5 Gy dose after poly(I:C) pre-treatment showed statistically significant reduction in cell survival (Fig. 1B). Combinational pre-treatment with poly(I:C) and cisplatin showed statistically significant

reduction in cell survival only after irradiation with 7.5 Gy in comparison to poly(I:C) pre-treatment alone. All of the poly(I:C)-related pre-treatments were abolished in shTLR3 cells induced by doxycycline and were statistically significant in all experiments except in poly(I:C) and cisplatin without the irradiation experiment but even there the trend is noticeable. This demonstrates that the observed effects are TLR3-dependent. FaDu cells were less sensitive to 10 μg/mL of poly(I:C) than Detroit 562 and SQ20B with more than 50% of the cell survival after this treatment (Fig. 1C). Additionally, irradiation did not affect cell survival, even at 7.5 Gy dose.

3.2. Poly(I:C) and cisplatin downregulate c-IAP2 and reduce cell survival

We have firstly showed that poly(I:C) shows similar increase of c-IAP2 expression in Detroit 562 cell line which is abolished by the addition of cisplatin (Fig. 2A). Other chemotherapeutics, hydroxyurea and methotrexate, did not show this phenomenon. Cleaved PARP expression was also the highest in samples treated with poly(I:C) and cisplatin. We have also treated the cells with different concentrations of IAP inhibitor and showed that even the lowest concentration of IAP inhibitor 10 nM is enough to decrease Detroit 562 cell survival in combination with 10 μg/mL of poly(I:C) (Fig. 2B). Poly(A:U), which is a more selective TLR3 ligand, did not induce statistically significant cell death, however, when combining poly(A:U) with higher concentration of IAP inhibitor (200nM) reduced cell survival for 40%. This means other dsRNA receptors, such as MDA5 and RIG-I are involved in poly(I:C) cell death induction. Proliferation assay of Detroit 562 cells treated with different chemotherapeutics, poly(I:C) and IAP inhibitor showed that treatment with cisplatin in combination with poly(I:C) showed the lowest cell survival which was slightly increased by the addition of IAP inhibitor, suggesting other anti-apoptotic proteins might be involved (Fig. 2C).

3.3. The effect of poly(I:C), cisplatin and irradiation on pro-apoptotic and anti-apoptotic gene expression

Since cisplatin and IAP inhibitor showed similar effect in Detroit 562 cells, concerning c-IAP2 expression and the reduction of cell survival, we sought to further explore this phenomenon in different head and neck cancer cell lines, especially SQ20B because it is radioresistant. In our subsequent experiment we used cisplatin, as it is already included into clinical practice. Additionally, since FaDu cells did not show a significant response to poly(I:C) and cisplatin treatment followed by irradiation, we excluded them from the following experiments. We first aimed to determine whether poly(I:C) and cisplatin treatment induces pro-apoptotic or anti-apoptotic genes and whether the gene expression signature is changed after irradiation. *BAD*, which is a pro-apoptotic gene, is not significantly over-expressed after poly(I:C) or poly(I:C) and cisplatin treatment. However, poly(I:C) and cisplatin treatment in combination with irradiation doubles its expression in SQ20B cells. Interestingly, poly(I:C), cisplatin and irradiation in SQ20B cells in which TLR3 is inhibited increases its expression up to 5 times (Fig. 3A). Poly(I:C) treatment alone or in combination with cisplatin decreases the expression of another pro-apoptotic gene *PUMA* in SQ20B cells. Similar is observed in cells pre-treated with poly(I:C) followed by irradiation. Anti-apoptotic gene survivin expression was increased after poly(I:C) and poly(I:C) and cisplatin treatment and after poly(I:C) treatment followed by irradiation. In case of poly(I:C) treatment and irradiation, its reduction by TLR3 inhibition was statistically significant. *DIABLO*, a pro-apoptotic gene, was increased in a statistically significant manner only after pre-treatment with poly(I:C) followed by irradiation of SQ20B cells. Interestingly, inhibition of TLR3 in combination with poly(I:C), cisplatin and irradiation increased *DIABLO* expression to 4 times. However, irradiation alone increased *DIABLO* in cells where TLR3 is inhibited so this might be a consequence of irradiation. We have also tested *BAD*, *BAX* and *BCL-XL* genes but neither of them showed any statistically significant change (data not shown). Interestingly, in Cal27 cells we have shown different results. *PUMA* gene was over-expressed in a statistically significant manner in all cases: after poly(I:C) alone, poly(I:C) and cisplatin, poly(I:C) with irradiation and poly(I:C) and cisplatin followed by irradiation where, in latter case, its expression was the highest. The expression of survivin and *DIABLO* was decreased after poly(I:C) alone with irradiation and cisplatin alone followed by irradiation treatment (Fig. 3B). In Detroit 562 cells *PUMA* expression was similar to the one in Cal27 cells. Survivin expression was also downregulated after poly(I:C) stimulation (Fig. 3C).

3.4. The expression of IAP family proteins after poly(I:C), cisplatin and irradiation treatment

Survivin expression was increased after poly(I:C) stimulation of SQ20B and decreased after the addition of cisplatin (Fig. 4A), however this was not TLR3 dependent. Detroit 562 cells showed increased expression of

cIAP2 and survivin after poly(I:C) stimulation which was both abolished by the addition of cisplatin (Fig. 4B). We have also determined that increased survivin expression in Detroit 562 cells is MEK dependent (Fig. 4C).

3.5. Poly(I:C) and cisplatin treatment increases apoptosis and ROS formation

To explore the mechanism of the observed cytotoxic and radiosensitizing effect of poly(I:C) and cisplatin in radioresistant SQ20B cells, we have measured caspase 3/7 activity and shown that poly(I:C) alone is enough to increase the caspase 3/7 activity of SQ20B cells for almost two fold and the addition of cisplatin increases the apoptosis to exactly 200% in comparison to the untreated cells (Fig. 5A). The inhibition of TLR3 reduces the apoptosis for 40- 60%. However, irradiation did not increase the observed effect thus the results with irradiated cells were similar to those of non-irradiated cells. We have determined ROS formation after poly(I:C) and cisplatin treatment to see whether this is the mechanism by which cancer cell lines can become radiosensitive. ROS formation was increased for 30% after poly(I:C) treatment and poly(I:C) and cisplatin combination showed only few percent more of ROS formation products (Fig. 5B). Poly(I:C) induced ROS formation was TLR3-dependent while poly(I:C) and cisplatin combination was not, but this is probably due to increased ROS formation in cisplatin alone treatment.

3.6. Poly(I:C) and cisplatin treatment cause G2/M arrest of Detroit 562 cells

By studying the cells cycle changes after poly(I:C) and cisplatin treatment of Detroit 562 cells we have established that the combination of poly(I:C) and cisplatin treatment induced G2/M cell arrest (Fig. 6). After 72 hours there were 24% of control cells and 45% of cisplatin and poly(I:C)-treated cells in G2/M phase. This was further increased by irradiation with 2.5 Gy when 50% of poly(I:C) and cisplatin treated cells were in G2/M phase. Moreover, after 72 hours there were 25% of untreated cells in S phase and only 15% of poly(I:C) and cisplatin treated cells. Poly(I:C) alone in combination with irradiation after 24 hours also increased the number of cells in G2/M phase.

4. Discussion

Poly(I:C) is an interesting candidate for the development of novel therapies against cancer, especially in combinations with other drugs that may improve the direct apoptotic effects of TLR3 on cancer cells [23]. We have shown in this study that poly(I:C) in combination with cisplatin might be a reasonable strategy for cancer treatment as it can, besides synergistically inducing cell death, inhibit the expression of IAP family proteins, precisely cIAP2 and survivin. This is a pre-clinical evidence based on cell line experiments and should also be tested in animals for definitive confirmation.

The combined use of TLR3 activation with poly(I:C) and cisplatin in cancer has been previously investigated. We and the others have shown that poly(I:C) and cisplatin act synergistically to induce cell death [16, 21, 22]. The results of a very recent study confirmed our hypothesis and showed that pre-treatment with poly(I:C) increased the amount of cisplatin in the cytoplasm, and greatly increased the low-dose cisplatin-induced cell death in TLR3- and caspase-3–dependent manner [21].

Nevertheless, we show here another mechanism by which poly(I:C) and cisplatin may reduce cancer cell survival: by reducing survivin and cIAP2 expression. Survivin belongs to IAP (inhibitor of apoptosis) family which is known to inhibit apoptosis mainly via inhibition of caspases-3 and -7 [24] and is normally expressed during fetal development. While survivin is rarely expressed in healthy tissues of adults, it was found to be expressed in numerous cancers [25, 26], implying its role in tumor development. One study showed that poly(I:C) induced apoptosis of TLR3-expressing HNSCC cells and that the expression of survivin was down-regulated during apoptosis [27]. Contrary to that, we have shown here that poly(I:C) induced survivin expression which was reduced by cisplatin. It might be that the poly(I:C), as it can activate TLR3, MDA5 and RIG-I receptors, has a diverse function depending on the activated receptor.

We have also tested other pro-apoptotic gene expression after the treatments and irradiation and showed that their expression is cell-dependent (*DIABLO* and *PUMA*). All cell lines investigated here have *TP53* mutations, but SQ20B which has a mild intron mutation with no known effect on the protein level had one pattern of expression, unlike Detroit 562 and Cal27, which have a non-functional p53 bearing R175H and H193L mutations respectively (http://p53.iarc.fr/). p53 upregulated modulator of apoptosis (*PUMA*) gene expression is regulatated by p53 and it inhibits anti-apoptotic proteins (Mcl-1, Bcl-2, Bcl-XL, Bcl-W and A1) and directly triggers apoptosis mediated by pro-apoptotic proteins Bax/Bak. It was shown that loss of *PUMA* alleles is connected with

radioresistance [28] and since SQ20B cells are radioresistant [29], this might explain the downregulation of *PUMA* after poly(I:C) stimulation and generally different pattern of expression compared to Detroit 562 and Cal27 cells. Cal27 and Detroit 562 show increased *PUMA* expression. Detroit 562 cells are also more sensitive to irradiation. Diablo (Smac) is an endogenous inhibitor of IAP proteins [30]. We have shown here that poly(I:C) stimulation of Detroit 562 cells induced c-IAP2 protein. Additionally, poly(I:C) stimulation downregulated *DIABLO* while poly(I:C) and cisplatin treatment abrogated that effect. This might explain c-IAP2 overexpression after poly(I:C) stimulation and establish once again the similarity in the effect of Smac mimetics and poly(I:C) and cisplatin combinational treatment. Moreover, the different results in survivin expression in Detroit 562 cells on RNA and protein level once more demonstrate the importance of protein expression assessment.

Although we have found that apoptosis and ROS formation are increased by poly(I:C) alone and slightly more after poly(I:C) and cisplatin combination, our cell cycle results show that the combination of poly(I:C) and cisplatin might be a more potent therapeutic regimen. We have shown that poly(I:C) and cisplatin treatment induced G2/M cell cycle arrest of Detroit 562 cells after 72 hours. There were 50% more cells in G2/M phase after the treatment with poly(I:C) and cisplatin than the untreated cells. It is well known that G2/M phase is the most radiosensitive phase while S phase is the least radiosensitive phase. We have shown that, besides poly(I:C) and cisplatin induced G2/M arrest, at the same time only 15% of the cells stay in the S phase. This clearly shows that the combined pre-treatment with poly(I:C) and cisplatin makes the cancer cells sensitive to irradiation.

Radiation therapy also has immunomodulatory effects. On one hand, radiation therapy leads to the increased Fas expression (a trigger of programmed cell death) and the major histocompatibility complex - MHC I, which is responsible for antigen presentation to cytotoxic CD8 + lymphocytes [31, 32]. It was also shown that radiation therapy alone can activate CD8+ lymphocytes through the induction of type I interferons [33]. On the other hand, through induction of cell death, especially necrosis, radiation may lead to the release of Damage-Associated Molecular Patterns (DAMPs), which also have a dual function. They either may induce immunogenic tumor cell death or might contribute to cancer cell survival through chronic inflammation and immunosuppression [34]. The idea of combining the activation of TLR3 ligands and radiation therapy is based on the premise that TLR3 activation may induce apoptosis of cancer cells and irradiation can stimulate the immune system response in addition to inducing cancer cell death through DNA breaks. For TLR9 and TLR7 agonists, studies have already shown the benefits of their combination with radiotherapy [35-37]. Kang *et al* have demonstrated that activation of TLR3 and -7 enhanced autophagy-induced cell death in radiotherapy of breast cancer [38]. Additionally, there are currently two ongoing clinical trials phase I and I/II which are investigating the potential co-administration of

poly ICLC with radiotherapy in patients with lymphoma (NCT02061449, NCT01976585) [10]. Moreover, aside from radiation therapy-induced direct tumor cell death and enhanced tumor specific immunity, it has been observed that irradiation may upregulate programmed death-ligand 1 (PD-L1). The PD-L1/PD-1 axis has been characterized as a potent inhibitor of immune activation through inhibition of effector T cell function. Therefore, an approach to combine radiotherapy with antibodies against PD1/PDL1 axis is another strategy for head and neck cancer therapy [39, 40]. Additionally, another publication also showed that combination of poly(I:C) with PD-L1 mAb inhibited tumor development in several mouse models of cancer. This combinatorial therapy also induced a long-lasting protection against tumor re-challenges [41]. Also, Takeda *et al* showed in a very recent paper that a TLR3 specific ligand ARNAX, which elicits less cytokine production than poly(I:C), in combination with anti-PD-L1 Ab and tumor antigen may overcome PD-1 blockade unresponsiveness and lead to tumor regression without inflammation [42]. Therefore, the utilization of TLR3 agonist in combination with radiotherapy or PD1/PD-L1 inhibitors may induce multiple beneficial effects during cancer treatment.

To conclude, the data presented in this paper suggest that the use of poly(I:C), cisplatin and radiotherapy could be the right strategy for head and neck cancer treatment. The main evidence, besides reduced cell survival, includes reduced survivin and c-IAP expression, increased *PUMA* and *DIABLO* gene expression (depending on the cell lines used), increased ROS and increased G2/M cell cycle arrest.

5. Conflict of interest

The authors declare that they have no conflict of interest.

6. References

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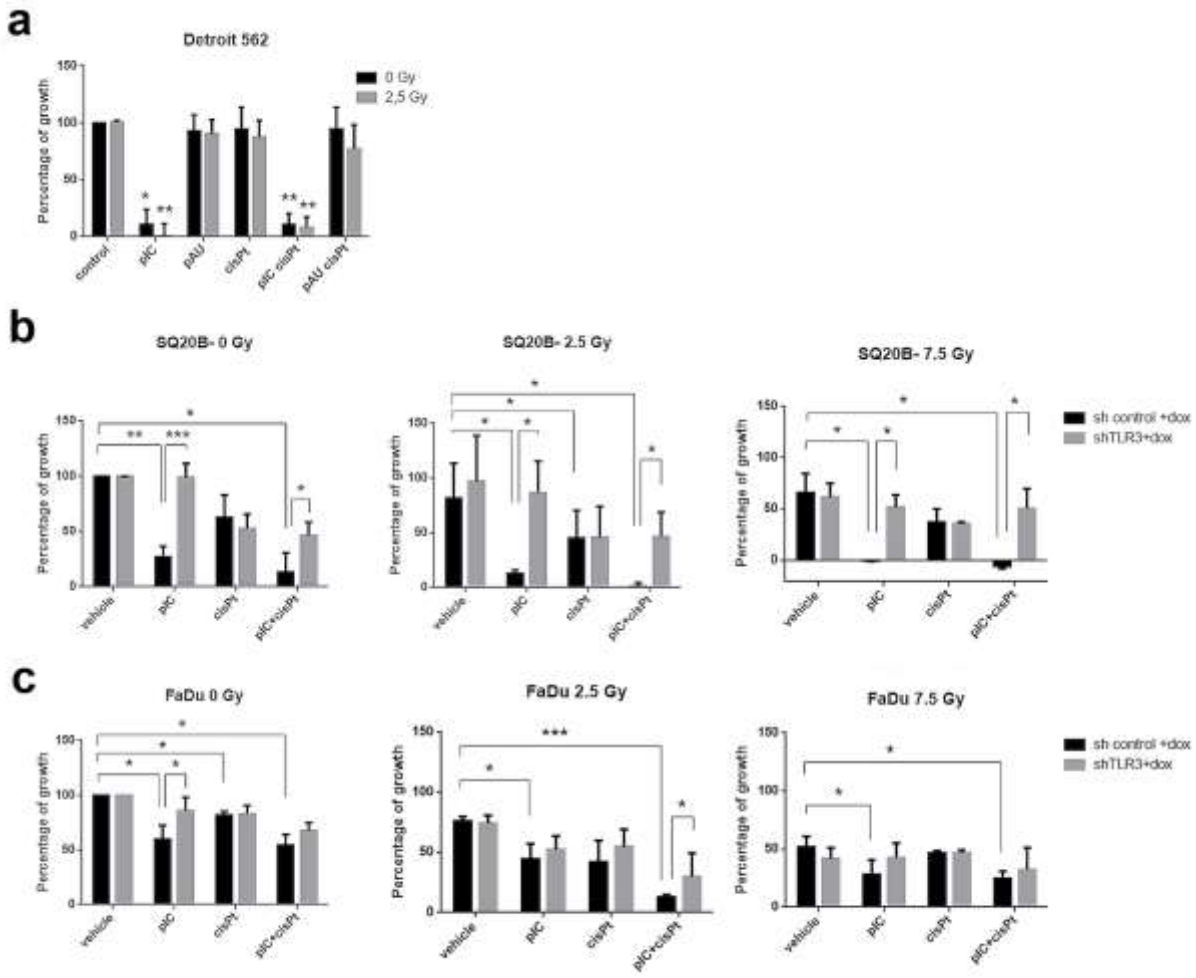

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465 Fig1

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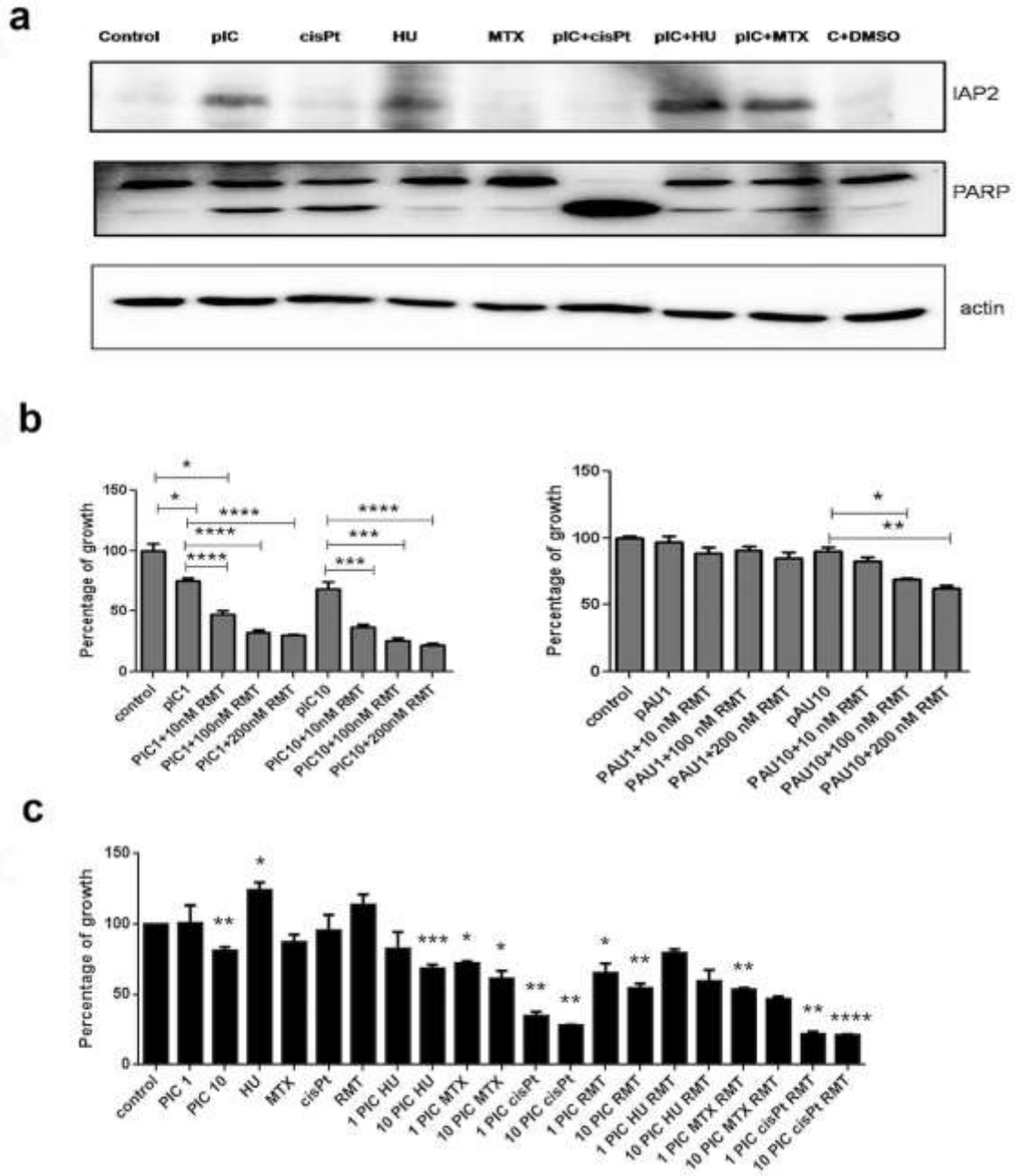
a
Control
pIC
cisPt
HU
MTX
pIC+cisPt
pIC+HU
pIC+MTX
C+DMSO
IAP2
PARP
actin
b
Percentage of growth
control
pIC1
PIC1+10nM RMT
PIC1+100nM RMT
PIC1+200nM RMT
pIC10
PIC10+10nM RMT
PIC10+100nM RMT
PIC10+200nM RMT
pAU1
PAU1+10 nM RMT
PAU1+100 nM RMT
PAU1+200 nM RMT
pAU10
PAU10+10 nM RMT
PAU10+100 nM RMT
PAU10+200 nM RMT
c
Percentage of growth
control
PIC 1
PIC 10
HU
MTX
cisPt
RMT
1 PIC HU
10 PIC HU
1 PIC MTX
10 PIC MTX
1 PIC cisPt
10 PIC cisPt
1 PIC RMT
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Fig2

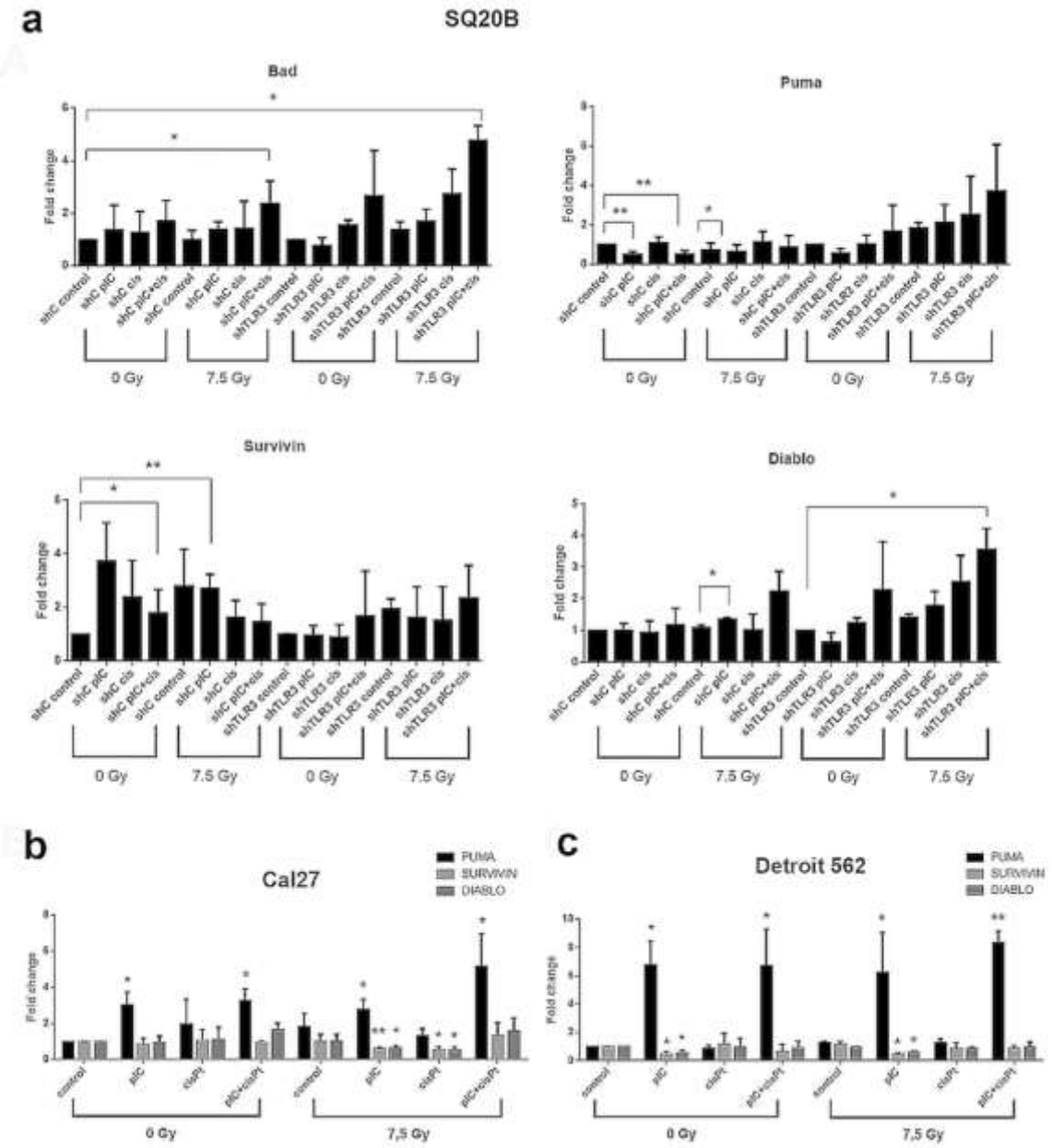
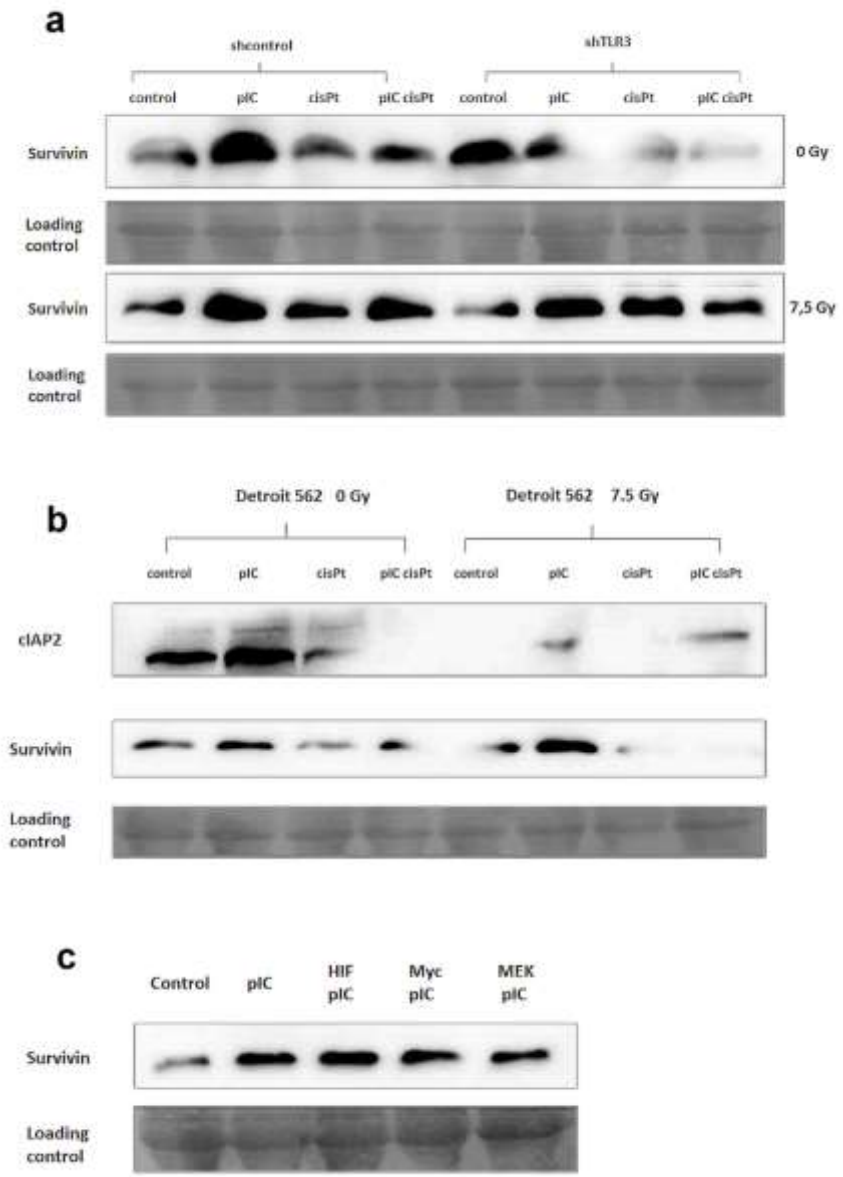

a
SQ20B
Bad
Puma
Survivin
Diablo
Fold change
shC control
shC pIC
shC cis
shC pIC+cis
shTLR3 control
shTLR3 pIC
shTLR3 cis
shTLR3 pIC+cis
0 Gy
7.5 Gy
b
Cal27
c
Detroit 562
PUMA
SURVIVIN
DIABLO
control
pIC
cisPt
pIC+cisPt
0 Gy
7,5 Gy

Fig3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control
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cisPt
pIC cisPt
control
pIC
cisPt
pIC cisPt
Survivin
0 Gy
Loading control
Survivin
7,5 Gy
Loading control
b
Detroit 562 0 Gy
Detroit 562 7.5 Gy
control
pIC
cisPt
pIC cisPt
control
pIC
cisPt
pIC cisPt
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c
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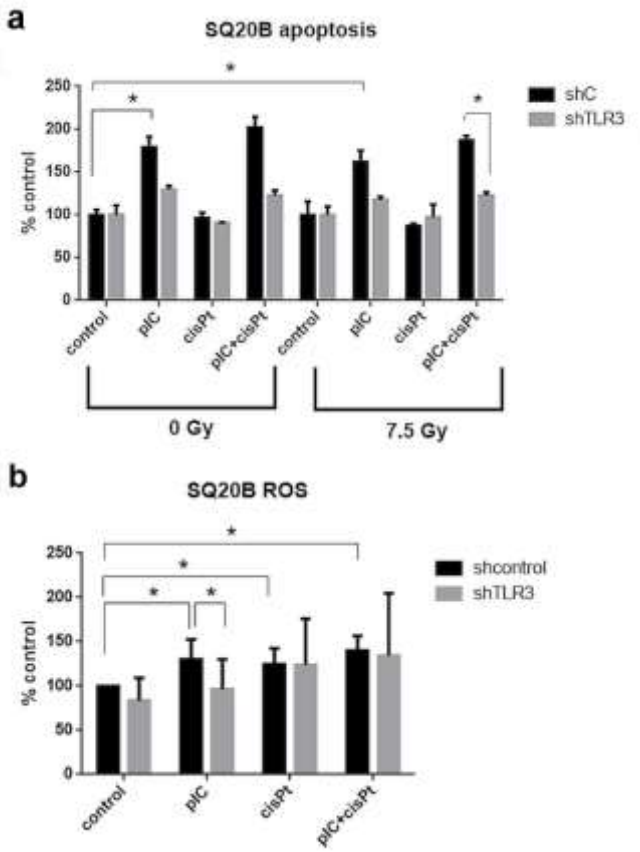
474

475 Fig 4



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477 Fig 5

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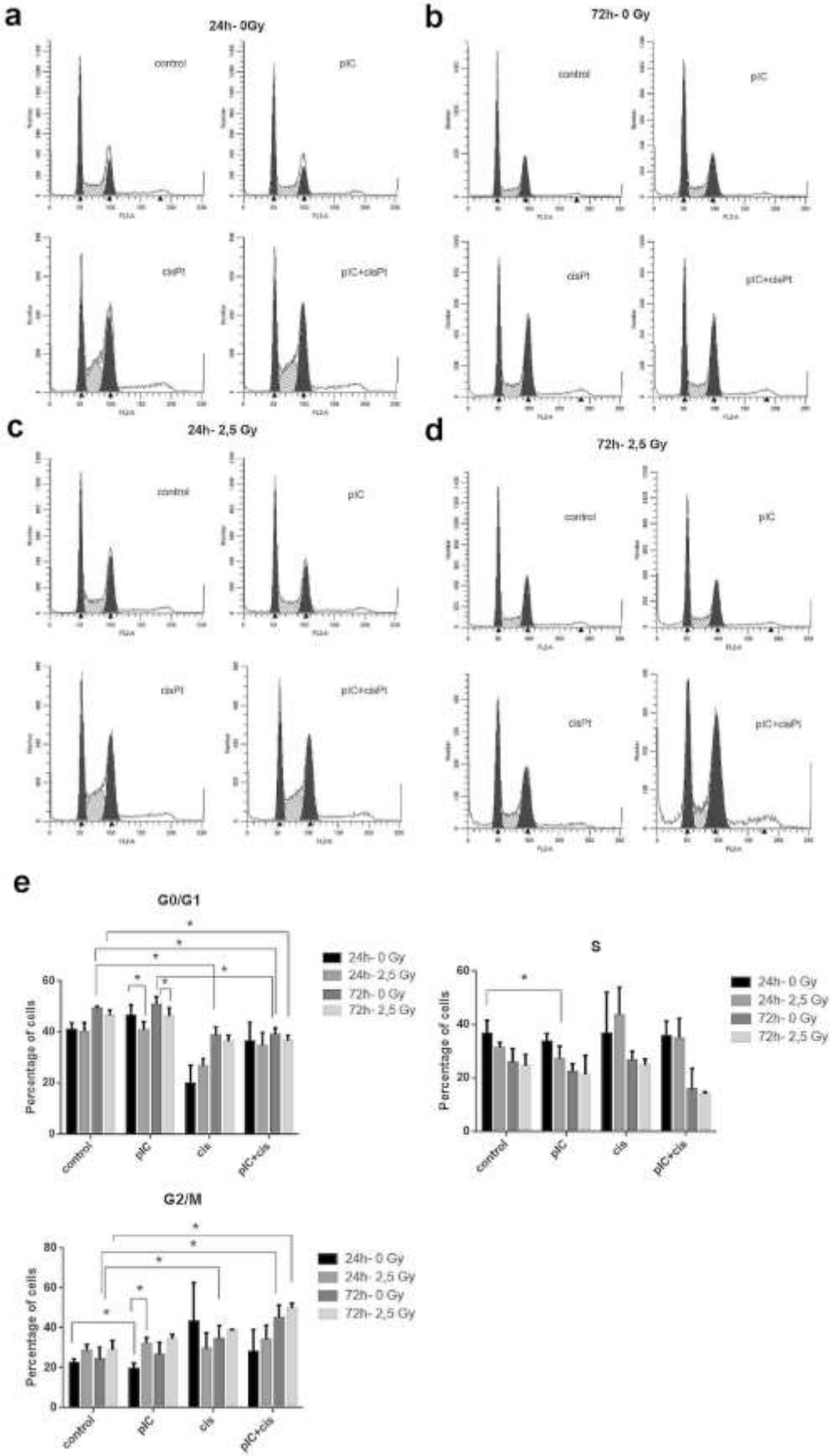
a
24h- 0Gy
control
pIC
cisPt
pIC+cisPt
b
72h- 0 Gy
control
pIC
cisPt
pIC+cisPt
c
24h- 2,5 Gy
control
pIC
cisPt
pIC+cisPt
d
72h- 2,5 Gy
control
pIC
cisPt
pIC+cisPt
e
G0/G1
Percentage of cells
24h- 0 Gy
24h- 2,5 Gy
72h- 0 Gy
72h- 2,5 Gy
control
pIC
cis
pIC+cis
S
Percentage of cells
24h- 0 Gy
24h- 2,5 Gy
72h- 0 Gy
72h- 2,5 Gy
control
pIC
cis
pIC+cis
G2/M
Percentage of cells
24h- 0 Gy
24h- 2,5 Gy
72h- 0 Gy
72h- 2,5 Gy
control
pIC
cis
pIC+cis

479

480 Fig 6

Figure legends

Fig. 1 The survival of head and neck cancer cell lines after the treatment with poly(I:C) (10 μg/mL), poly(A:U) (10 μg/mL) and cisplatin (1μM) for 45 minutes followed by irradiation: Detroit 562 (a), SQ20B (b) and FaDu (c) cells. pIC- poly(I:C), pAU- poly(A:U), cisPt- cisplatin, sh control+dox- cells transfected with control plasmid and induced by doxycyline, shTLR3 dox- cells transfected with TLR3 plasmid and induced by doxycycline. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001

Fig. 2 The effect of poly(I:C), poly(A:U), chemotherapeutics and cIAP inhibitor RMT on Detroit 562 cells. (a) The expression of cIAP2 (70kDa) and PARP (89/116 kDa) after the treatment with poly(I:C) (1 or 10 μg/mL) and chemotherapeutics (HU- 500 μM, MTX- 5μM, cisPt- 5μM). (b) Cell survival after the treatment with poly(I:C), poly(A:U) and RMT 5625. (c) Cell survival after the treatment with poly(I:C), poly(A:U), RMT 5625 (10nM) and chemotherapeutics. pIC- poly(I:C), pAU- poly(A:U), HU- hydroxyurea, MTX- methotrexate, cisPt- cisplatin, DMSO- dimethyl sulfoxide. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001

Fig. 3 The expression of pro- and anti-apoptotic genes after the treatment of head and neck cancer cells. (a) SQ20B cells treated with poly(I:C) (10 μg/mL) and cisplatin (1μM) followed by irradiation. shC- cells trasfected with control plasmid, shTLR3- cells transfected with TLR3 plasmid. (b) Cal27 cells treated with poly(I:C) and cisplatin followed by irradiation. (c) Detroit 562 cells treated with poly(I:C) and cisplatin followed by irradiation. pIC- poly(I:C), cis- cisplatin. *p<0.05, **p<0.01

Fig. 4 The expression of survivin and cIAP2 in: (a) SQ20B cells after poly(I:C) (10 μg/mL) and cisplatin (1μM) treatment followed by irradiation. (b) Detroit 562 cels after poly(I:C) and cisplatin treatment followed by irradiation. (c) Detroit 562 cells pre-treated with HIF, Myc and MEK inhibitors, treated with poly(I:C) and cisplatin followed by irradiation. pIC- poly(I:C), cisPt- cisplatin, HIF-HIF inhibitor, Myc-Myc inhibitor, MEK- MEK inhibitor. Loading control- membrane dyed with amido black and scanned.

Fig. 5 The effect of poly(I:C) (10 μg/mL) and cisplatin (1μM) treatment on SQ20B cells apoptosis induction (a) and ROS formation (b). shcontrol- cells trasfected with control plasmid, shTLR3- cells transfected with TLR3 plasmid, pIC- poly(I:C), cisPt- cisplatin. *p<0.05

Fig. 6 Cell cycle analysis. Histograms present cell cycle distribution of Detroit 562 cells after poly(I:C) (10 μg/mL) and cisplatin (1μM) treatment for 24h (a) and 72h (b) followed by irradiation (c and d). (e) The flow cytometric

histograms are representative of 3 separate experiments and the statistical analysis of each cell cycle phase arrest is presented in section e. *$p<0.05$