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## **Enhancement of Cisplatin Sensitivity by Microwave Radiation in Ovarian Cancer Cells**

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## Abstract

**Background:** Nowadays, ovarian cancer is the most lethal gynecological cancer worldwide. Tumor debulking surgery followed by Cisplatin-based chemotherapy is the first line of ovarian cancer therapy. However, many patients experience a relapse of the disease due to chemotherapy resistance. Accordingly, this study aims to investigate the ability of microwave (MW) radiation to increase the susceptibility of ovarian cancer cells toward Cisplatin (Cis).

**Methods:** Firstly we designed a hand-made electromagnetic field exposure system and CO<sub>2</sub> incubator to irradiate cells with a frequency equal to 2450±30 MHz and a power density of 2.47 mW/cm<sup>2</sup> at a distance of 30 cm from the antenna. Two ovarian cancer cell lines A2780 (Cisplatin-sensitive) and A2780CP (Cisplatin-resistant) were subjected to either Cis, MW alone or Cisplatin + microwave radiation (Cis+MW). Cell viability, apoptosis, and P53 gene expression were assessed following drug/radiation exposure.

**Results:** After 48 hours of treatment the combination of Cis and MW radiation has significantly inhibited the growth of the A2780 and A2780CP cell lines in comparison with Cis-control groups. The percentages of early apoptosis induced by Cis+MW was significantly increased in comparison with Cis alone. P53 expression was significantly upregulated after treatment with Cis+MW.

**Conclusion:** It can be concluded that MW radiation has been able to decrease the resistance of ovarian cancer cells to Cis and it may improve the chemotherapy protocol for ovarian cancer treatment.

**Keywords:** -Microwave radiation

-Drug resistance

-Ovarian cancer

-Apoptosis

-Cisplatin

## Introduction

Ovarian cancer is the seventh common diagnosed cancer among women.<sup>1</sup> It is one of the fatal gynecological cancers as the disease is diagnosed at an advanced stage.<sup>2,3</sup> Of note, ovarian cancer mortality makes up to 5% of all cancer-related deaths.<sup>3-5</sup> The malignant cells can be originated from epithelial, stromal, and ovum-producing cells, although the epithelial form is more common.<sup>4</sup> Platinum-based chemotherapeutic drugs, such as Cisplatin (Cis), have been proved to be effective against several human tumors, including testicular, bladder, head and neck cancers, and ovarian cancer.<sup>6-8</sup> However, the mean survival rate of ovarian cancer is still low,<sup>3,5</sup> which is partly due to the presence of drug-resistant cells against Cis.<sup>8-11</sup> Therefore, the development of new approaches seems urgent for a more effective outcome. In recent decades, combination therapy is strongly suggested to cancer therapy and drug resistance reduction.<sup>12-15</sup> The MW deposits energy in tissues and cells and its non-thermal effect creates a torque on the magnetic dipoles of biological systems based on Fröhlich's hypothesis and some research has shown that MWs affect the expression of genes involved in replication, repair and cell death.<sup>16, 17</sup> Recent studies have shown that MW radiation induces apoptosis in the cells<sup>18-23</sup> and inhibits tumor cell proliferation *in vitro*.<sup>24</sup> Moreover, it is noted that exposure to MW radiation in frequency about 2450MHz can change the viability of human cells.<sup>19, 21, 25-27</sup> It can also trigger cell apoptosis by inducing DNA oxidative damage and upregulating the expression of the P53 gene.<sup>28-30</sup> The P53 gene repairs or kills damaged cells by inhibiting proliferation or inducing apoptosis. Apoptosis as programmed cell death is an essential the mechanism for tumor suppression because it eradicates cells with damaged DNA that have carcinogenic potential.<sup>23, 31</sup>

Regarding the above-mentioned facts, this study was aimed to evaluate the combined effect of non-ionizing radiation and Cis on ovarian cancer cells to investigate whether the MW can overcome chemo-resistance. To our knowledge, this is the first report showing the synergism of non-ionizing radiation and Cis to induce the apoptosis pathway in ovarian cancer cells.

## **Materials and methods**

### ***The Electromagnetic field exposure system***

A hand-made electromagnetic field exposure system was designed and manufactured in the author's research group. As illustrated in Fig. 1, the system as consisting of three parts: A- An electromagnetic wave generator with an adjustable frequency range from 100 to 4400MHz. B- An antenna with a 14dBi gain waveguide. C- A CO<sub>2</sub> incubator made of PVC with dimensions of 50×50×50cm<sup>3</sup>. This incubator has an electronic board including a temperature sensor (to supply 37 ±0.3 °C), a humidity sensor (95% acceptable level), and a CO<sub>2</sub> sensor to keep the CO<sub>2</sub> level constant at %5. The sidewall of the incubator contains a clear plexiglass window to view the flask in the incubator. It can be penetrated by electromagnetic waves. The frequency and spectrum of the antenna output were calibrated by the Aaronia Spectrum Analyzer model Spectran HF 60105 (Germany).

### ***Cell lines and culture condition***

Ovarian cancer cell lines A2780 (Cisplatin sensitive) and A2780 CP (Cisplatin-resistant) were purchased from the National Cell Bank of the Pasteur Institute, Tehran, Iran. Both A2780 and A2780CP cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 10 µ/mL penicillin, and 10 mg/mL streptomycin (Life Technologies). All cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in the PVC incubator with the plexiglass window to avoid the reflection and attenuation of MW radiation. The cells were trypsinized and sub-cultured every 3 days.

### ***Cell viability assay***

Cell viability assay was performed using methyl thiazole tetrazolium (MTT) reagent (Sigma-Aldrich) according to the user's manual and by the protocol provided in the previous article.<sup>32</sup> In

summary, the A2780 and A2780CP cells were seeded at a density of  $0.8 \times 10^4$  and  $1 \times 10^4$  cells/well in 96-well plates and then incubated at 37°C overnight (24 and 48 hours in the absence and presence of different concentrations of Cis) to obtain the IC<sub>50</sub> of cells. In an independent experiment, the cells were exposed to Cis, MW alone or a combination of Cis and MW radiation (Cis+MW) 24 and 48 hours. Cis concentrations were 29 and 56  $\mu$ M when exposed for 24 hours, while this amount was 4.5 and 14  $\mu$ M when exposed for 48 hours, in both A2780 and A2780CP cell lines respectively. After that, the media was removed completely, and the cells were washed twice with PBS to eliminate any treatment agents. Then 20  $\mu$ l of MTT (5g/l of PBS) was added to each well and incubated at 37°C. Four hours later, the medium was removed and 200  $\mu$ l of dimethyl sulfoxide was added to each well, and formazan crystals were dissolved by pipetting. The proportional colourimetric change was measured at 570 nm using a BioTek ELx808 plate reader. All treated results were compared with their controls to calculate the cell viability percentage of treated cells.

### ***Flow-cytometry***

Apoptosis was detected by staining the cells with Annexin-V-FLUOS and PI staining kit (Biolegend, USA) following the manufacturer's instruction and our previous work.<sup>32</sup> In brief, A2780 and A2780CP cells were plated at a density of  $3 \times 10^5$  cells/well onto six-well plates and incubated overnight to adhere and then treated with desired concentrations of Cis and MW radiation for 48 hours. After trypsinization, cells were rinsed twice with PBS and centrifuged at 200g for 5 minutes. The cells pellet was then resuspended in 100  $\mu$ L of buffer (10  $\mu$ L Annexin-V-FLUOS and 5  $\mu$ L PI labelling solution) and incubated for 15 minutes at 15°C–20°C and immediately analyzed by FACSCalibur flow cytometer. Results were interpreted with the FlowJo V-10 software and the percentage in the lower right quadrant indicated early apoptotic cells.

### ***Gene expression assay***

Total RNA was isolated from control and treated cells using RNX-Plus reagent (Sinaclon Iran) and purified using DNase I digestion (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized with 2 micrograms of total RNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) as recommended by the manufacturer's protocol. Real-time PCR was performed using specific primers in Table 1, RealQ Plus 2x Master Mix Green (Ampliqon) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control. According to the instructions of the manufacturer, the reverse and forward primers, Master Mix, cDNA and PCR-grade H<sub>2</sub>O were mixed in total vol. of 20 µl in nuclease-free microtubes. The PCR program consists of; 10 min. 95 °C for activation of the TEMPase hot-start enzyme and 40 repeats of 2 steps of 95 °C for 15 S and 60 °C for 60 S. Real-time PCR was performed using the Rotor-Gene Q system. The relative difference in gene expression level was calculated with the  $2^{-\Delta\Delta C_t}$  method. This is a common method for measuring relative changes in gene expression known as the comparative Ct method which is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample.<sup>32, 33</sup>

### ***Statistical analysis***

Results were expressed as mean±SD values from three independent experiments. Statistical analysis was performed with ANOVA and Student's paired *t*-test using the statistical software Graphpad prism 8 and SPSS 26. Statistically, a significant difference was considered at  $P<0.05$  for all analyses.

## **Results**

### ***The half-maximal inhibitory concentration***

As shown in Fig 2, the proliferation of A2780 and A2780CP cells were significantly inhibited by Cis in a time- and dose-dependent manner. Following the 24 hours of exposure to various concentrations of Cis, the half-maximal inhibitory concentration (IC<sub>50</sub>) values of Cis in A2780 and A2780CP cells were 29.82 and 56.39 $\mu$ M respectively (Fig. 2A). However, these values in 48 hours of exposure reached 4.87 $\mu$ M in A2780 and 14.22 $\mu$ M in A2780CP (Fig. 2B).

### ***Exposure to microwave***

A2780 and A2780CP cells were cultured on plate 96 cells. After one night of incubation, cells were treated with MW or Cis or Cis+MW for 24 and 48 hours and the control group were incubated without any treatment for the same time. Cell growth in any group was compared by the MTT method. According to Figure 3, after treatment with MW, the viability of the cells decreased. But these changes were not statistically significant. The P-value for the sensitive cells at 24 and 48 hours was 0.657 and 0.379, respectively, and for the resistant cells was 0.975 and 0.554, respectively.

### ***Combination of Cisplatin and microwave***

Fig 4A and B shows the combinatory effect of 29 and 56 $\mu$ M Cis and MW on the A2780 and A2780CP cells during 24h of exposure and Fig 4C and D show this effect during 48h of exposure to 4.5 and 14 $\mu$ M Cis and MW radiation. Cell survival after 24 hours of Cis treatment was 48.92% and 49.78% for A2780 and A2780CP respectively. Whereas, treatment with Cis and MW reduced these values to 46.67% and 47.83% respectively. After 48 hours of exposure to Cis, A2780 and A2780CP cell survival was 48.67% and 50.26% respectively. However, these values reduced to 42.08% and 42.16% after treatment with Cis and MW respectively. As indicated, no statistically significant difference was observed between Cis alone and Cis+MW during 24h of exposure, but the obtained data was significant in 48 hours (P-value= 0.0143 and 0.0286 for A2780 and A2780CP respectively).

### ***Apoptosis detection***

To determine the apoptosis, Annexin-V-FLUOS and PI staining were applied to cells that were exposed to 2450 MHz MW radiation and 14 $\mu$ M Cis on A2780CP and 4.5 $\mu$ M Cis on A2780 cells. As indicated in Fig. 5, there is a significant difference between the 48 hours exposure to Cis and Cis+MW radiation. Percentages of early apoptosis in A2780CP cells following treatment with Cis and Cis+MW radiation was determined as  $12.37 \pm 0.75$  and  $18.47 \pm 1.78$  ( $P=0.0014$ ) and for the A2780 cells were  $16.5 \pm 0.77$  and  $25.19 \pm 1.76$  respectively ( $P=0.0003$ ). The increase in early apoptosis in Cis+MW treated cells was as large as 1.5 times more than cells treated with Cis alone (Table 2).

#### ***Elevated expression of p53 in A2780 and A2780CP cells after treatment***

The effect of Cis and Cis+MW on p53 expression was examined after 36 hours of exposure to MW radiation and 14 $\mu$ M Cis on A2780CP and 4.5 $\mu$ M Cis on A2780 cells. As shown in Fig. 6, an increased expression level of p53 was observed in either Cis exposure or both of RF and Cis. The mean fold change was around  $2.24 \pm 0.31$  in Cis treated ( $P < 0.05$ ) and  $3.78 \pm 0.17$  in cells treated with Cis+MW ( $P < 0.001$ ) in comparison with non-treated control cells. Similarly, the expression level of p53 was also increased in Cis treated cells ( $3.81 \pm 0.16$  fold  $P < 0.001$ ) and Cis+MW treated ( $6.69 \pm 0.26$  fold  $P < 0.001$ ) A2780 cells which were more than A2780CP. Table 2 shows that combined treatment of the cells with both Cis and MW radiation can increase the p53 expression level by approximately 1.7-fold compared with Cis alone ( $P < 0.05$  for A2780CP and  $P < 0.001$  for A2780).

#### **Discussion**

The ability of the cancer cell to become resistant to chemotherapy is one of the biggest challenges in overcoming cancer and ovarian cancer is no exception.<sup>5, 6, 12, 14</sup> Meanwhile, combined therapy is in attention where the cancer cells can be targeted with more than one drugs to, increase the sensitivity of cancer cells to the therapy.<sup>12-14, 34</sup> Combination therapy with Cis and radiotherapy is an effective treatment but the effectiveness of treatment in the sensitive cell is greater than



resistance cells. Another combined therapy is retinoids with Cis. Increased expression of the differentiated phenotype of human melanoma was shown after exposure to retinoids. The other is hyperthermia with Cis. In-vivo applying hyperthermia has complex effects on the drug clearance, diffusion, pH and other factors.<sup>12, 13, 15</sup> A growing body of evidence has indicated that MW radiation is genotoxic and inhibit cell proliferation by induction apoptosis in cancer cells.<sup>18-22, 24-29</sup> Accordingly, the present study was aimed to evaluate the synergism of 2450MHz MW radiation with Cis on ovarian cancer cells. To radiate the non-ionizing wavelength, we have designed an electromagnetic wave generator and incubator capable to expose cells to non-ionizing wavelength. Since non-ionizing radiation is scattered and reflected by metals and the walls of the cell culture incubator, we decided to design a non-metallic wall incubator that passes non-ionizing waves. Plexiglas window of this incubator is capable of passing non-ionizing radiation because Plexiglas materials have been used in non-ionizing radiation experiments.<sup>35</sup>

The obtained data showed that following 48 hours of co-exposure of resistant cells to MW radiation and Cis, cell proliferation was significantly inhibited in comparison with the value of resistant cells treated with Cis alone. Similarly, the induction of apoptosis was also increased in resistant ovarian cells treated with both Cis and MW. Wenhe Zhu et al. showed that exposure to 2450MHz MW can induce apoptosis through the downregulation of Bcl2 and upregulation of Bax.<sup>19</sup> Several studies have indicated that 2450MHz MW radiation can induce genotoxic effects and triggered apoptosis.<sup>27, 36-39</sup> It has been found that the expression level of P53 has increased noticeably in resistant cells receiving combined therapy although this value is still less than sensitive cells. This indicates that MWs can significantly increase the sensitivity of resistant ovarian cancer cells. Previous studies showed that a wide range of genotoxic stress promotes nuclear accumulation of P53 and trigger its ability to activate or even repress several downstream target genes involved in the repair system. Fuqiang Xing reported, increase in caspase3 and p53 expression after exposure to 1800MHz MW radiation.<sup>18</sup> Our result is in agreement with the result

obtained by Luukkonen,<sup>40</sup> Baohong,<sup>41</sup> and Zmyslony.<sup>42</sup> They showed that co-exposure to MW radiation and some chemical mutagens can enhance the cytotoxicity of chemical mutagens. Therefore, the altered expression of P53 was considered in this study to answer the question of non-ionizing radiation can affect the expression level of this gene in response. This indicated that not only ionizing radiation but also low energy wavelengths non-ionizing can also activate cellular repair systems. In other words, non-ionizing radiation therapy with Cis therapy can be promising and increase the effectiveness of cancer therapy at least in the case of resistant cells. As far as we know, the synergistic effect of non-ionizing radiation and Cis to induce the apoptosis pathway in ovarian cancer cells has not yet been reported. It can be used as a novel way to treat cancerous cells. W H-Schmidt et al. reported combination therapy with MW and Cis is an effective method for lung cancer treatment but they used thermal MW effect by ablation.<sup>15</sup> According to our result 24- and 48-hours exposure to 2450 MHz MW radiation in this condition does not significantly affect cell viability. A recent meta-analysis of studies about the biological effects of MWs does not confirm an association between these effects and radiation.<sup>43</sup> Some research stated that non-thermal MW radiation induces apoptosis in the cells<sup>18-23</sup> and inhibits tumor cell proliferation *in vitro*.<sup>24</sup> While other researches are showed that non-thermal MW can induce repairable or no DNA damage and no change in the cell proliferation.<sup>44-46</sup> In a study, Elcin Ozgur et al. concluded the contradictory results in radiofrequency radiation biological effects due to differences in P53 mutation.<sup>23</sup>

## Conclusion

In the end, it is suggested to survey the mechanism of the effect of MW radiation on apoptosis induction. Further research on the effect of radiation intensity and frequency is also recommended. In this article, we have demonstrated the combination of Cis and MW radiation can enhance the efficacy of ovarian cancer therapy which is partly due to the induction of apoptosis by upregulation

of the p53 gene. Therefore, it can be concluded that MW radiation has been able to overcome drug resistance and thus improve the chemotherapy protocol.

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### References

1. Reid BM, Permuth JB, Sellers TA. Epidemiology of ovarian cancer: a review. *Cancer Biol Med*. 2017; 14(1): 9. doi:10.20892/j.issn.2095-3941.2016.0084
2. Vargas AN. Natural history of ovarian cancer. *Ecancermedicalscience*. 2014; 8: 465. doi:10.3332/ecancer.2014.465
3. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, *et al*. Use of proteomic patterns in serum to identify ovarian cancer. *The lancet*. 2002; 359(9306): 572-7. doi:10.1016/S0140-6736(02)07746-2
4. Jochem C, Schlecht I, Leitzmann M. Epidemiologic Relationship Between Obesity and Ovarian Cancer. *Focus on Gynecol Malignancies*: Springer; 2018. p. 21-30.
5. Samuel P, Mulcahy LA, Furlong F, McCarthy HO, Brooks SA, Fabbri M, *et al*. Cisplatin induces the release of extracellular vesicles from ovarian cancer cells that can induce invasiveness and drug resistance in bystander cells. *Phil Trans R Soc B*. 2018; 373(1737): 20170065. doi:10.1098/rstb.2017.0065
6. Maurmann L, Belkacemi L, Adams N, Majmudar P, Moghaddas S, Bose R. A novel cisplatin mediated apoptosis pathway is associated with acid sphingomyelinase and FAS proapoptotic protein activation in ovarian cancer. *Apoptosis*. 2015; 20(7): 960-74. doi:10.1007/s10495-015-1124-2
7. Hunakova L, Gronesova P, Horvathova E, Chalupa I, Cholujoval D, Duraj J, *et al*. Modulation of cisplatin sensitivity in human ovarian carcinoma A2780 and SKOV3 cell lines by sulforaphane. *Toxicol letters*. 2014; 230(3): 479-86. doi:10.1016/j.toxlet.2014.08.018

8. Sorensen BH, Thorsteinsdottir UA, Lambert IH. Acquired cisplatin resistance in human ovarian A2780 cancer cells correlates with shift in taurine homeostasis and ability to volume regulate. *Am J Physiol Cell Physiol*. 2014; 307(12): C1071-C80. doi:10.1152/ajpcell.00274.2014
9. Yang X, Zheng F, Xing H, Gao Q, Wei W, Lu Y, *et al*. Resistance to chemotherapy-induced apoptosis via decreased caspase-3 activity and overexpression of antiapoptotic proteins in ovarian cancer. *J Cancer Res Clin Oncol*. 2004; 130(7): 423-8. doi:10.1007/s00432-004-0556-9
10. Cao Z-T, Chen Z-Y, Sun C-Y, Li H-J, Wang H-X, Cheng Q-Q, *et al*. Overcoming tumor resistance to cisplatin by cationic lipid-assisted prodrug nanoparticles. *Biomaterials*. 2016; 94: 9-19. doi:10.1016/j.biomaterials.2016.04.001
11. Davis A, Tinker AV, Friedlander M. "Platinum resistant" ovarian cancer: what is it, who to treat and how to measure benefit? *Gynecologic oncology*. 2014; 133(3): 624-31. doi:10.1016/j.ygyno.2014.02.038
12. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. *European journal of pharmacology*. 2014; 740: 364-78. doi:10.1016/j.ejphar.2014.07.025
13. Szturz P, Wouters K, Kiyota N, Tahara M, Prabhash K, Noronha V, *et al*. Altered fractionation radiotherapy combined with concurrent low-dose or high-dose cisplatin in head and neck cancer: A systematic review of literature and meta-analysis. *Oral oncology*. 2018; 76: 52-60. doi:10.1016/j.oraloncology.2017.11.025
14. Ramezani T, Nabiani M, Baharara J, Parivar K, Namvar F. Sensitization of Resistance Ovarian Cancer Cells to Cisplatin by Biogenic Synthesized Silver Nanoparticles through p53 Activation. *Iran J Pharm Res: IJPR*. 2019; 18(1): 222. doi:10.22037/IJPR.2019.2358
15. Hohenforst-Schmidt W, Zarogoulidis P, Stopek J, Kosmidis E, Vogl T, Linsmeier B, *et al*. Enhancement of intratumoral chemotherapy with cisplatin with or without microwave ablation and lipiodol. Future concept for local treatment in lung cancer. *J Cancer*. 2015; 6(3): 218. doi:10.7150/jca.10970
16. Roy B, Niture S, Wu MH, Kumar D. Biological effects of low power nonionizing radiation: A Narrative Review. *arXiv preprint arXiv:201015557*. 2020.
17. Geesink HJ, Meijer DK. A predictive model that reveals a causal relation between exposures to non-thermal electromagnetic waves and biological effects (update January 2021). 2021.
18. Xing F, Zhan Q, He Y, Cui J, He S, Wang G. 1800MHz Microwave Induces p53 and p53-Mediated Caspase-3 Activation Leading to Cell Apoptosis In Vitro. *PloS one*. 2016; 11(9): e0163935. doi:10.1371/journal.pone.0163935
19. Zhu W, Zhang W, Wang H, Xu J, Li Y, Lv S. Apoptosis induced by microwave radiation in pancreatic cancer JF305 cells. *Can J Physiol Pharmacol*. 2014; 92(4): 324-9. doi:10.1139/cjpp-2013-0220
20. Zuo H, Lin T, Wang D, Peng R, Wang S, Gao Y, *et al*. Neural cell apoptosis induced by microwave exposure through mitochondria-dependent caspase-3 pathway. *Int J Med Sci*. 2014; 11(5): 426. doi:10.7150/ijms.6540

21. Zuo H, Lin T, Wang D, Peng R, Wang S, Gao Y, *et al.* RKIP regulates neural cell apoptosis induced by exposure to microwave radiation partly through the MEK/ERK/CREB pathway. *Molecular neurobiology*. 2015; 51(3): 1520-9. doi:10.1007/s12035-014-8831-5
22. Esmekaya MA, Seyhan N, Kayhan H, Tuysuz MZ, Kurşun AC, Yağcı M. Investigation of the Effects of 2.1 GHz Microwave Radiation on Mitochondrial Membrane Potential ( $\Delta\Psi$  m), Apoptotic Activity and Cell Viability in Human Breast Fibroblast Cells. *Cell Biochem Biophys*. 2013; 67(3): 1371-8. doi:10.1007/s12013-013-9669-6
23. Ozgur E, Kayhan H, Kismali G, Senturk F, Sensoz M, Ozturk GG, *et al.* Effects of radiofrequency radiation on colorectal cancer cell proliferation and inflammation. *Turk J Biochem*. 2021. doi:10.1515/tjb-2020-0148
24. BENEDUCI A, CHIDICHIMO G, DE ROSE R, FILIPPELLI L, STRAFACE SV, Venuta S. Frequency and irradiation time-dependant antiproliferative effect of low-power millimeter waves on RPMI 7932 human melanoma cell line. *Anticancer research*. 2005; 25(2A): 1023-8.
25. Caraglia M, Marra M, Mancinelli F, D'ambrosio G, Massa R, Giordano A, *et al.* Electromagnetic fields at mobile phone frequency induce apoptosis and inactivation of the multi-chaperone complex in human epidermoid cancer cells. *J CellPhysiol*. 2005; 204(2): 539-48. doi:10.1002/jcp.20327
26. Song X-L, Wang C-H, Hu H-Y, Yu C, Bai C. Microwave induces apoptosis in A549 human lung carcinoma cell line. *Chin Med J*. 2011; 124(8): 1193-8. doi:10.3760/cma.j.issn.0366-6999.2011.08.013
27. Sarkar S, Ali S, Behari J. Effect of low power microwave on the mouse genome: a direct DNA analysis. *Mutation Res /Genetic Toxicol*. 1994; 320(1): 141-7. doi:10.1016/0165-1218(94)90066-3
28. Pacini S, Ruggiero M, Sardi I, Aterini S, Gulisano F, Gulisano M. Exposure to global system for mobile communication (GSM) cellular phone radiofrequency alters gene expression, proliferation, and morphology of human skin fibroblasts. *Oncology Research/Anti-Cancer Drug Design*. 2002; 13(1): 19-24. doi:10.3727/096504002108747926
29. Sahin D, Ozgur E, Guler G, Tomruk A, Unlu I, Sepici-Dinçel A, *et al.* The 2100MHz radiofrequency radiation of a 3G-mobile phone and the DNA oxidative damage in brain. *J chemical neuroanatomy*. 2016; 75(Pt B): 94-8. doi:10.1016/j.jchemneu.2016.01.002
30. Bhartiya P, Mumtaz S, Lim JS, Kaushik N, Lamichhane P, Nguyen LN, *et al.* Pulsed 3.5 GHz high power microwaves irradiation on physiological solution and their biological evaluation on human cell lines. *Scientific reports*. 2021; 11(1): 1-16. doi:10.1038/s41598-021-88078-x
31. Pecorino L. *Molecular biology of cancer: mechanisms, targets, and therapeutics*: Oxford University Press, USA; 2016. p. ISBN:0198717342
32. Mirgani MT, Isacchi B, Sadeghizadeh M, Marra F, Bilia AR, Mowla SJ, *et al.* Dendrosomal curcumin nanoformulation downregulates pluripotency genes via miR-145 activation in U87MG glioblastoma cells. *Int J Nanomedicine*. 2014; 9: 403. doi:10.2147/IJN.S48136

33. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques*. 2005; 39(1): 75-85. doi:10.2144/05391RV01
34. Wang X, Pan L, Mao N, Sun L, Qin X, Yin J. Cell-cycle synchronization reverses Taxol resistance of human ovarian cancer cell lines. *Cancer cell international*. 2013; 13(1): 1-8. doi:10.1186/1475-2867-13-77
35. Zeni O, Scarfi MR. Experimental requirements for in vitro studies aimed to evaluate the biological effects of radiofrequency radiation. *Microw Mater Charact*. 2012: 121-38.
36. Lai H, Singh NP. Acute low-intensity microwave exposure increases DNA single-strand breaks in rat brain cells. *Bioelectromagnetics*. 1995; 16(3): 207-10. doi:10.1002/bem.2250160309
37. Lai HNPS. Single-and double-strand DNA breaks in rat brain cells after acute exposure to radiofrequency electromagnetic radiation. *INT J RADIAT BIOL*. 1996; 69(4): 513-21. doi:10.1080/095530096145814
38. Gupta SK, Mesharam MK, Krishnamurthy S. Electromagnetic radiation 2450 MHz exposure causes cognition deficit with mitochondrial dysfunction and activation of intrinsic pathway of apoptosis in rats. *Journal of biosciences*. 2018; 43(2): 263-76. doi:10.1007/s12038-018-9744-7
39. Gupta SK, Patel SK, Tomar MS, Singh SK, Mesharam MK, Krishnamurthy S. Long-term exposure of 2450 MHz electromagnetic radiation induces stress and anxiety like behavior in rats. *Neurochemistry international*. 2019; 128: 1-13. doi:10.1016/j.neuint.2019.04.001
40. Luukkonen J, Hakulinen P, Mäki-Paakkanen J, Juutilainen J, Naarala J. Enhancement of chemically induced reactive oxygen species production and DNA damage in human SH-SY5Y neuroblastoma cells by 872MHz radiofrequency radiation. *Mutation Res*. 2009; 662(1): 54-8. doi:10.1016/j.mrfmmm.2008.12.005
41. Baohong W, Jiliang H, Lifan J, Deqiang L, Wei Z, Jianlin L, *et al*. Studying the synergistic damage effects induced by 1.8 GHz radiofrequency field radiation (RFR) with four chemical mutagens on human lymphocyte DNA using comet assay in vitro. *Mutation Res*. 2005; 578(1): 149-57. doi:10.1016/j.mrfmmm.2005.05.001
42. Zmysłony M, Politanski P, Rajkowska E, Szymczak W, Jajte J. Acute exposure to 930 MHz CW electromagnetic radiation in vitro affects reactive oxygen species level in rat lymphocytes treated by iron ions. *Bioelectromagnetics*. 2004; 25(5): 324-8. doi:10.1002/bem.10191
43. Wood A, Mate R, Karipidis K. Meta-analysis of in vitro and in vivo studies of the biological effects of low-level millimetre waves. *J exposure sci & environmental epidemiol*. 2021: 1-8. doi:10.1038/s41370-021-00307-7
44. Lixia S, Yao K, Kaijun W, Deqiang L, Huajun H, Xiangwei G, *et al*. Effects of 1.8 GHz radiofrequency field on DNA damage and expression of heat shock protein 70 in human lens epithelial cells. *Mutation Res*. 2006; 602(1): 135-42. doi:10.1016/j.mrfmmm.2006.08.010

45. Scarfi MR, Fresegna AM, Villani P, Pinto R, Marino C, Sarti M, *et al.* Exposure to radiofrequency radiation (900 MHz, GSM signal) does not affect micronucleus frequency and cell proliferation in human peripheral blood lymphocytes: an interlaboratory study. *Radiation res.* 2006; 165(6): 655-63. doi:10.1667/RR3570.1
46. Speit G, Schütz P, Hoffmann H. Genotoxic effects of exposure to radiofrequency electromagnetic fields (RF-EMF) in cultured mammalian cells are not independently reproducible. *Mutation Res.* 2007; 626(1): 42-7. doi:10.1016/j.mrgentox.2006.08.003
47. Huang Y, Boyle K. *Antennas: From Theory to Practice*. first ed. Singapore: John Wiley & Sons Ltd; 2008. p. ISBN:978-0-470-51028-5



## Appendix

### *Antenna and Incubator performance evaluation*

The generator power-adjustable in a range of zero to 33 dBm, which was fixed at 33dBm during irradiation in this study. The exposure condition was selected as the frequency of  $2450 \pm 30$  MHz, and the intensity of  $3.5 \text{ mW/cm}^2$  at a distance of 25 cm from the antenna.

To evaluate our antenna function, the radiated power density was measured with the Spectrum Analyzer at a distance of 25cm from the antenna (inside the incubator, the cell flask location) which was  $3.5 \pm 0.1 \text{ mW/cm}^2$ . Using the Friis transmission formula, the power density in distance R from an antenna with power  $P_t$  and gain  $G_t$  can be calculated as follows <sup>47</sup>:

$$S \left( \text{mW} / \text{cm}^2 \right) = \frac{P_t \left( \text{mW} \right)}{4\pi R^2} G_t \left( \text{dBi} \right) \quad (1)$$
$$P_t \left( \text{dBm} \right) = 10 \log \left( \frac{P_t \left( \text{mW} \right)}{1 \text{ mW}} \right)$$

Where the power density will be  $3.56 \text{ mW/cm}^2$  which is in agreement with measurement.

At First, we should check to make sure our incubator (Hand-made) is working properly. Therefore; two cell lines (A2780, A2780CP) were cultured with different concentrations in 96 well plates and incubated for 24, 48 hours. As can be seen in Fig A-1, the cell metabolic activity was assessed using the MTT test and the optical density was obtained and compared with the commercial CO<sub>2</sub> incubator (Mettler type: INC 108, made in Germany).

Using SPSS software and due to the high sample size (approximately 41 samples in each group), even though in all cases there was no normal distribution, the T-test was used for comparison. The results of the T-test on optical density were compared for both incubators, taking into account the cultured cell category, incubation time and once in general. As can be deduced from Table A-1, the p-value is higher than 0.05 which shows that there is no statistically significant difference between the two incubators.



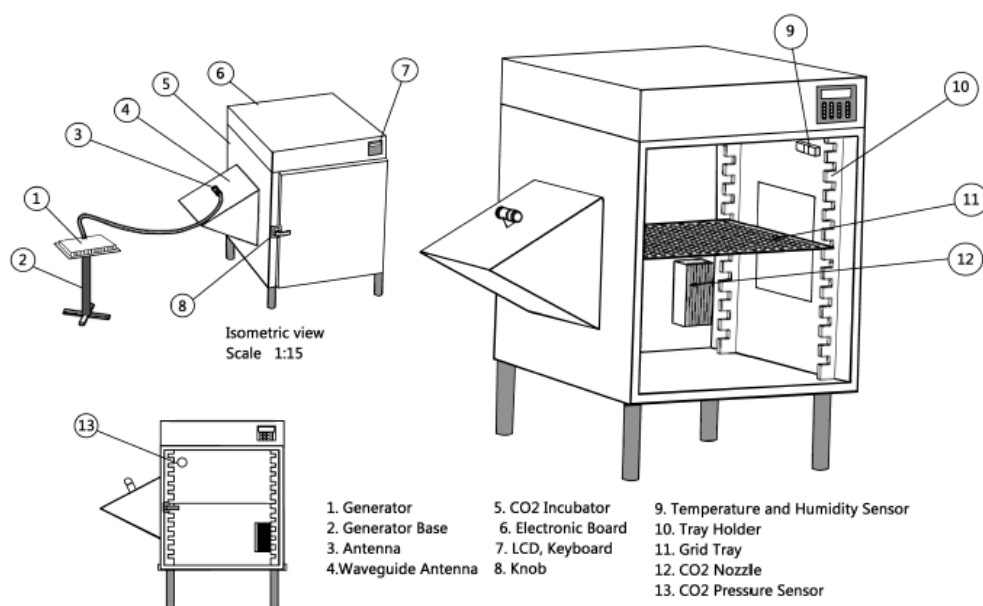


Figure 1. Schematic form of the irradiation and incubation system (upper) and photographic image (lower)

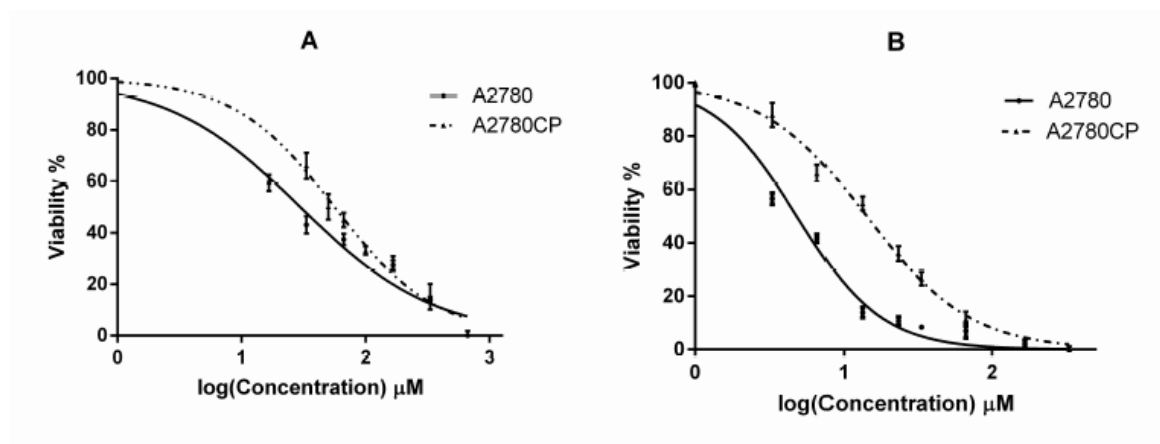


Figure 2. Survival curve of A2780 and A2780CP cells in the presence of different concentrations of Cis by MTT assay. A) 24 hours B) 48 hours

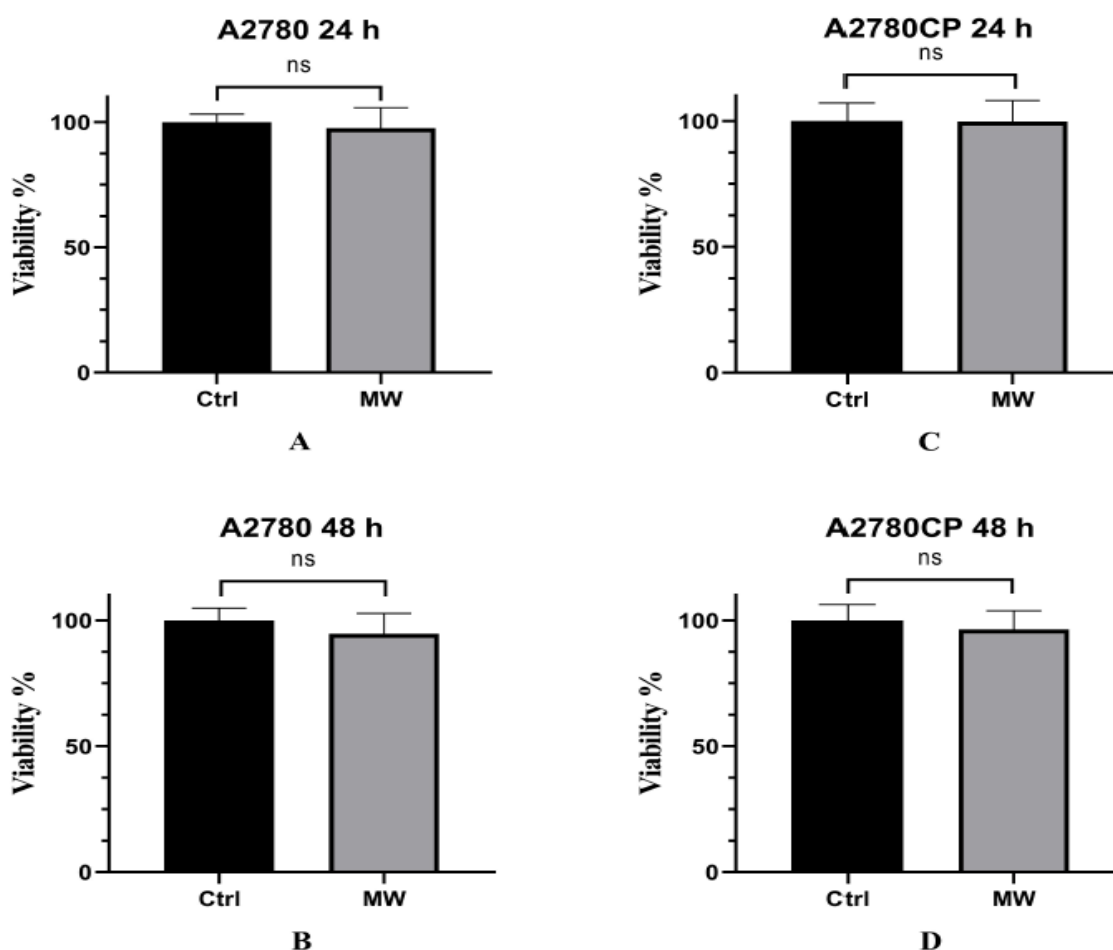


Figure 3. Effects of exposure to 2450 MHz MW radiation on the viability of A2780 and A2780CP cells. A) A2780 24 hours B) A2780CP 24 hours C) A2780 48 hours D) A2780CP 48 hours. The viability was determined by the MTT assay. Results are given as mean $\pm$ S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

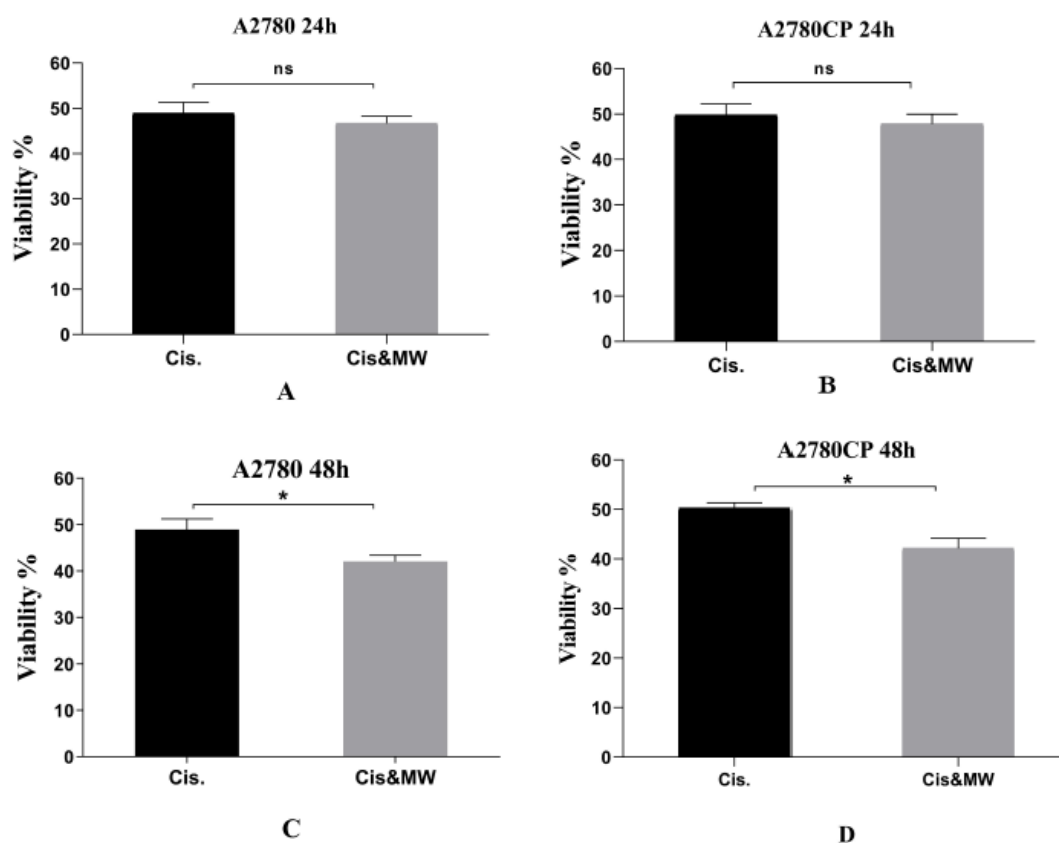


Figure 4. Effects of exposure to Cis and combination of Cis and MW radiation on the survival of A2780 and A2780CP cells. A) A2780, 24 hours B) A2780CP, 24 hours C) A2780, 48 hours D) A2780CP, 48 hours. The viability was determined by the MTT assay. Results are given as mean $\pm$ S.D. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

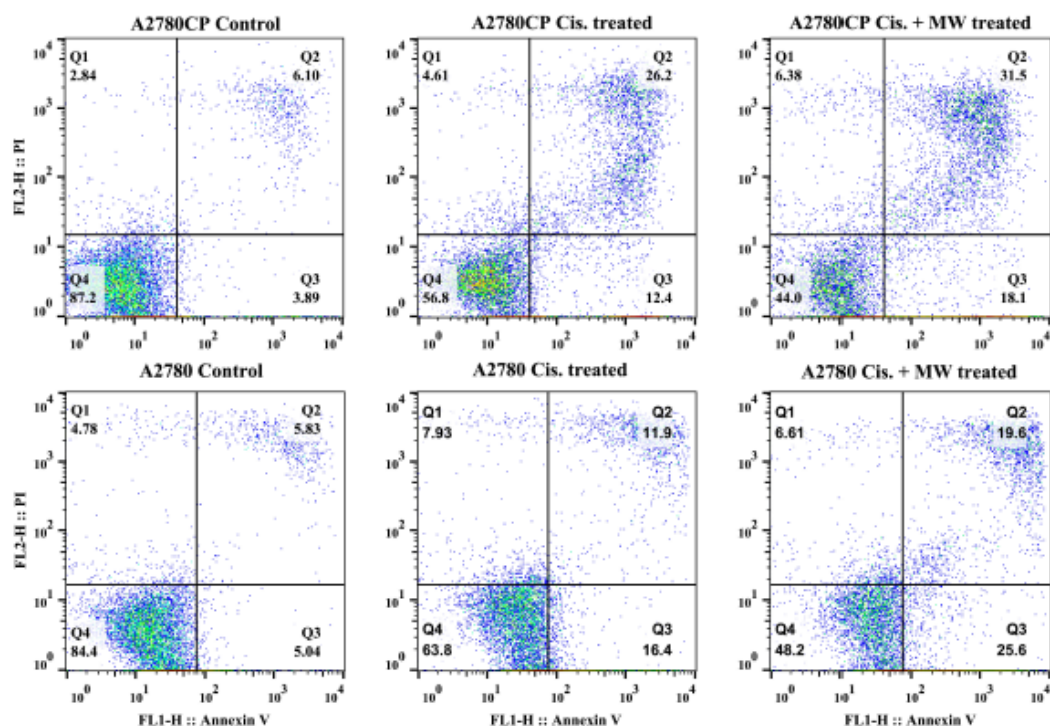


Figure 5. Apoptosis induced by 48 hours exposure to Cis and MW radiation a) Flow cytometry histograms of apoptosis b) comparison between early apoptosis in two groups. Apoptosis was analysed by flow cytometry with Annexin V and PI. Results are given as mean±S.D. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001

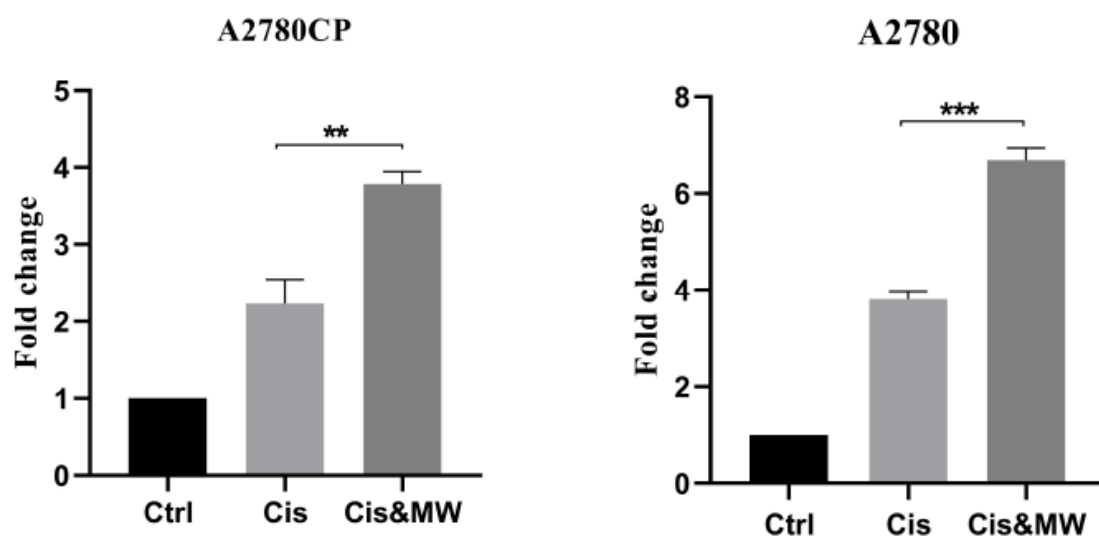


Figure 6. Relative expression P53 gene in absence or presence of Cis and MW radiation after 36 hours. . Relative difference in gene expression level was calculated with the  $2^{-\Delta\Delta C_t}$  method. Results are given as mean $\pm$ S.D. \*  $P<0.05$ , \*\*  $P<0.01$ , \*\*\*  $P<0.001$

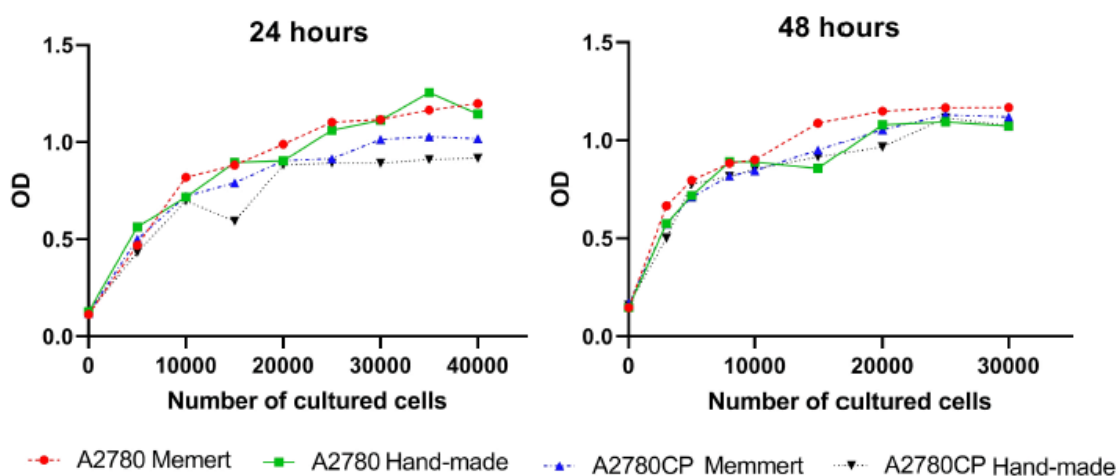


Fig A-1. Comparison between the optical density obtained using MTT test for two CO<sub>2</sub> incubators (Memmert and hand-made) in two incubation times (24,48hr), and two cell lines (Cisplatin-resistant; CP, Sensitive; S).

Table 1. P53 sequence used for PCR study

P53 forward	TGG CCA TCT ACA AGC AGT CA
P53 reverse	GGT ACA GTC AGA GCC AAC CT
GAPDH forward	GTGAACCATGAGAAAGTATGACAAC
GAPDH reverse	CATGAGTCCTTCCACGATACC

Table 2. The ratio of the combination of drug and 48hr MW irradiation to the treated drug alone. The increased apoptosis in Cis+MW treated cells was about 1.5 times more than cells treated with Cis alone. P53 expression was significantly up-regulated after treatment with Cis+MW, and this enhancement was approximately 1.7-fold over chemotherapy alone

5	A2780			A2780CP		
	Cis	cis+MW	(cis+MW)/cis ratio	Cis	cis+MW	(cis+MW)/cis ratio
Cell viability (%)	48.67±1.9	42.1±1.32	0.865	50.26±1.1	42.16±2	0.84
	P-value = 0.0143			P-value = 0.0286		
Early Apoptosis (%)	16.5±0.77	25.19±1.76	1.53	12.37±0.75	18.47±1.78	1.49
	P-value = 0.0003			P-value = 0.0014		
P53 expression Fold change	3.81±0.16	6.69±0.26	1.76	2.24±0.31	3.78±0.17	1.69
	P-value < 0.0001			P-value = 0.0015		

Table A-1. Comparison of cell viability in Memmert and hand-made incubator. Results are based on the incubation time, cell line, and overall (incubation time and cell line) comparison by MTT test.

Cell Line	CO <sub>2</sub> Incubator	Mean OD±SD	P-value
A2780CP	Memmert	0.815±0.30	0.952
	Hand-made	0.744±0.28	
A2780	Memmert	0.886±0.33	0.537
	Hand-made	0.834±0.31	
Incubation time			
24 hours	Memmert	0.850±0.31	0.948
	Hand-made	0.791±0.31	
48 hours	Memmert	0.851±0.32	0.481
	Hand-made	0.787±0.29	
Overall comparison			
	Memmert	0.850±0.32	0.580
	Hand-made	0.789±0.32	