# MECHANISM OF APOPTOSIS INHIBITION TO SQUAMOUS CELL CARCINOMA OF ORAL CANCER IN CISPLATIN TREATMENT

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## ABSTRAK

*Penelitian ini bertujuan membuktikan efek pemberian cisplatin pada peningkatansekresi Hsp 70, DNA damage, dan inhibitor of apoptosis protein terhadap apoptosis sel kanker rongga mulut dan mengetahui mekanisme patologi molekulernya. Penelitian ini adalah eksperimental laboratorik in vitro, menggunakan Rancangan Acak Kelompok (Randomized Block Design). Kultur sel kanker rongga mulut dilakukan dari sel kanker yang resisten cisplatin dan sel kanker yang belum pernah mendapat cisplatin. Kedua kelompok sel kanker tersebut diberi cisplatin. Sekresi Hsp 70, DNA damage, Inhibitor of apoptosis protein, dan apoptosis index diperiksa. Dari penelitian ini didapatkan kelompok sel kanker yang resisten cisplatin menunjukkan apoptosis yang rendah dibandingkan dengan sel kanker yang belum pernah mendapat cisplatin. Sekresi Hsp 70 meningkat pada kelompok yang mendapat terapi cisplatin (p= 0.000, b=0.881). Sekresi DNA damage rendah pada sel kanker resisten cisplatin dan pada sel resisten cisplatin tidak terjadi apoptosis. Pada analisa jalur regresi, cisplatin signifikan melalui jalur IAP (p=0.000, b=0.726) untuk mencapai apoptosis. Kultur sel resisten atau sel yang belum pernah terpapar cisplatin menunjukkan signifikan melalui jalur IAP (p=0.000, b=0.496) menuju apoptosis. Sekresi IAP yamg meningkat berpengaruh terhadap terjadinya suatu apoptosis (b= 1.000). Sebagai simpulan, cisplatin menggunakan jalur IAP untuk mencapai apoptosis. Jenis kultur sel juga mempengaruhi IAP dalam proses menuju apoptosis. Jenis kultur sel yang resisten cisplatin akan memberikan pengaruh lebih kuat kepada IAP sehingga hambatan apoptosis akan meningkat.* ***(FMI 2017;53:1-6)***

***Kata kunci:*** *cisplatin, in vitro, kultur sel, Hsp 70, kerusakan DNA, IAP, apoptosis*

## ABSTRACT

*This study was to approve the increased secretion of Hsp 70, DNA damage, and inhibitor apoptosis protein in cisplatin therapy which influence apoptosis of oral cancer cell and to know mechanism of molecular pathology. This study was an in vitro experimental laboratory using Randomized Block Design. Cell culture of oral cancer divided from cisplatin resistance cancer cell and cancer cell never induce cisplatin. Two group of cancer cell would be given cisplatin therapy. Secretion of Hsp 70, DNA damage, Inhibitor of apoptosis protein, and apoptosis index would be examined. Cisplatin resistance cancer cell group showed lower apoptosis than never induce cisplatin cancer cell. Elevated secretion of Hsp 70 in cisplatin therapy group (p= 0.000, b=0.881). Lower secretion of DNA damage protein in cisplatin resistance cancer cell and it was not going apoptosis. In path regression analysis, cisplatin was significans through IAP pathway (p=0.000, b=0.726) to apoptosis. All type of cell cultures were also significans through IAP pathway (p=0.000, b=0.496) to apoptosis. Elevated IAP secretion influenced apoptosis (b= 1.000). In conclusion, cisplatin used IAP pathway to apoptosis. All type of cell cultures also used IAP pathway to apoptosis. Cisplatin resistance cell culture had stronger effect to IAP and IAP increased inhibition to apoptosis.* ***(FMI 2017;53:1-6)***

***Keywords:*** *Cisplatin, in vitro, cell culture, Hsp 70, DNA damage, IAP, apoptosis.*

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## INTRODUCTION

Oral cancer includes malignancy of the lips, gums, tongue anterior 2/3, floor of the mouth, the hard palate to the retromolar triangle. Most oral cavity cancer is a type of squamous cell carcinoma, which reached 90% (Gosselin, 2003). Therapeutic options such as surgery, adjuvant chemoradiation therapy, it is still possible recurrent tumor or metastases. In advanced stages KSSRM recurrent figure in regional locomotive reached 30- 40% and 20-30% of distant metastases (Forastiere et al., 2003).

In the Department of Surgery Soetomo Hospital, Surabaya, in 2007-2011 found 153 cases of cancer of the mouth, with tongue cancer the most, with the result of 88.2% squamous cell carcinoma. In 2009- 2011, T3 as many as 10 patients of 64 patients, T4 many as 36 patients of 64 patients with carcinoma of the oral cavity and the patient receiving chemotherapy as many as 25

patients out of 64 patients, a partial response to chemotherapy and no response by 40%. This response led to the failure of chemotherapy such as chemotherapy. The failure can be caused resistance to chemotherapy, chemotherapy treatment is not perfect/withdrawal, and resistance against oral cancer patients and the cancer has reached an advanced stage. One solution to achieve therapeutic success is to know the cause of chemotherapy resistance. The incidence of recurrent tumor can be reduced. For the activation pathway of cancer cells and inhibition of cancer cell needs to be known. Each protein compounds have a role in facilitating or inhibiting apoptosis. In normal circumstances there is a balance of cell growth. There are roles facilitate and inhibit the role, so that the process of apoptosis could walk normally. Disruption of apoptosis becomes one of the causes of cancer cell growth, including cancer of the oral cavity.

In the intrinsic pathway, Apaf 1 and cytochrome c activates caspase 9 and caspase 3 continued occurrence of apoptosis. Extrinsic pathway of apoptosis is the activation of the Fas death receptor that interacts with Fas ligand or tumor necrosis factor alpha (TNF-alpha) continued activation of caspase 8, caspase 10, and caspase 3 (Gober et al., 2005). Heat shock protein 70 inhibits apoptosis by interfering with the formation of apoptosom by inhibiting the Apaf 1 in the intrinsic pathway, and prevents the activation of caspase 9 and caspase 3 process does not continue (Mosser and Morimoto, 2005). Inhibitor of apoptosis protein (IAP) that shares a common mechanism for inhibiting apoptosis. The expression of XIAP, c-IAP1, c-IAP2, naip, or Survivin showed suppression of apoptosis induced by various stimuli, such as tumor necrosis factor (TNF) and Fas (Duckett et al., 1996; Liston et al., 1996; Ambrosini G, 1997; Li et al., 1998).

IAP proteins and DNA damage has a major role in the growth of cancer cells. In an effort to successful treatment of the cancer cells need to be inhibition of the function of Hsp 70, DNA repair and IAP cut line of work. The growth rate of cancer cells can be measured by the levels of Hsp 70, DNA damage and IAP proteins that cause cell apoptosis disorder. Hsp 70, DNA damage and the IAP proteins have an influence on the response to chemotherapy cisplatin or cisplatin resistance.

Selection of the chemotherapy drug cisplatin as a treatment option for cancer of the oral cavity or as standard therapy protocols oral cancer refers to how the platinum base bind to cancer cell DNA, causing DNA damage and apoptosis (Kelland, 2007; Siddik, 2003; Cepeda et al., 2007). Cisplatin inhibit the action of DNA polymerase, an enzyme required for DNA replication. DNA replication will automatically stop. Cells aware of the existence of DNA damage through damage recognition protein (Siddik, 2003). As a factor of the failure of apoptosis KSSRM, resistance to cisplatin causes of this need to be investigated. The research reveals the role of Hsp 70 control, DNA damage and IAP proteins so as to improve workability and prevent the occurrence of cisplatin resistance.

## MATERIALS AND METHODS

This study was a laboratory experimental study with a randomized block design with research stages, namely: 1. Culture of oral cancer cells (squamous cell carcinoma) who had never received cisplatin therapy and oral cavity cancer cells resistant to cisplatin of tumor biopsies. 2. Results of the cancer cell culture and cancer cells resistant to cisplatin treated with cisplatin to see the response of these cells compared to controls. Cisplatin at a dose of 1ug/ml is given and the response of the cells was observed. Secretion of Hsp 70, IAP proteins and DNA damage was evaluated against apoptosis index.

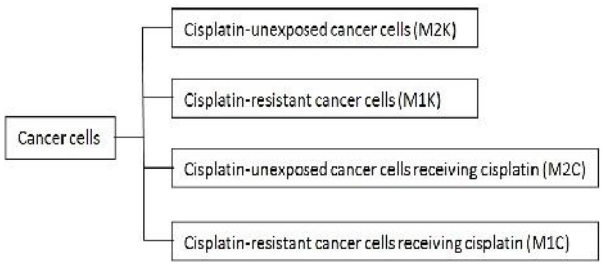


Fig. 1. Cancer cell groups studied in this research

Captions 1. M1 is a group of cells resistant to cisplatin, K is the control, M2 is a group of cells not exposed to cisplatin, C is treated with cisplatin.

Based on Federer formula, the minimum sample size can be calculated. Federer formula: (n-1) (t-1)> 15, n = number of samples per treatment group, t = the number of the treatment group n = 6. Mechanical randomization of samples in this study was based on a random sample of a number of oral cancer cells. The research was conducted at the Institute of Tropical Disease, Airlangga University Surabaya.

Secretion Hsp 70 is the number of cells that secrete the protein Hsp 70 on the surface of the cell membrane. Oral cancer examined by indirect ELISA techniques from cell culture. The secretion of proteins is DNA damage DNA damage was secreted from cancer cells after treatment were detected with kit 8-Hydroxy-2-deoxy Guanosine by indirect ELISA. Secretion Inhibitor

of apoptosis protein (IAP) enzyme was detected by indirect competitive ELISA Human Inhibitor of Apoptosis Protein Like Protein 2 (IPLP-2). The IAP is a protein that inhibits apoptosis. Apoptosis is the sum of oral cancer cells undergoing apoptosis, was examined by the method of indirect Elisa kit Related Apoptosis Human genes (args). Cisplatin is a chemotherapy drug that is given to standard oral cancer cases at a dose of 1ug/ml. Cisplatin-resistant cancer cells is proved by immunocytochemistry COX2 (cyclooxygenase 2). Cyclooxygenase enzyme isoforms induced to convert arachidonic acid to prostanoid. Cox2 role synthesize prostaglandin E2, stimulate Bcl2 and may inhibit apoptosis. Cox2 conjugated with cisplatin. Oral cavity cancer cells resistant culture cisplatin also performed by the same procedure with oral cavity cancer cells.

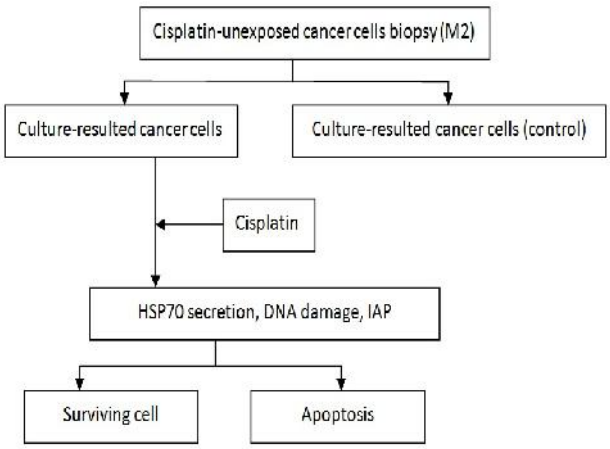


Fig. 2. Research flowchart in cancer cell groups never exposed to cisplatin

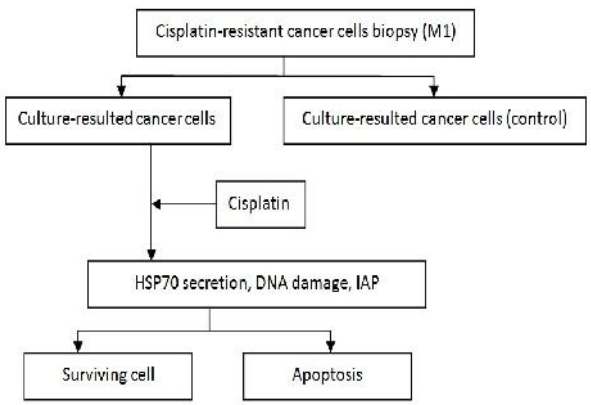


Fig. 3. Research flowchart in cisplatin-resistant cancer cell groups

## RESULTS

ELISA test results were analyzed with descriptive statistics to see the mean (average value), significant if p <0.05, not significant if p> = 0:05. Each group of cells cultured with the measurement of Hsp 70, IPLP2 (IAP), the DNA is damaged proteins and apoptosis were assessed for mean, median, standard deviation, the value of the minimum and maximum values results

Table 1. Results of the treatment and control in all cancer cell cultures

| Groups | | Mean | Median | SD | Min | Max |
|---|---|---|---|---|---|---|
| Hsp 70 | M1K | 0.089 | 0.089 | 0.00303 | 0.08 | 0.09 |
| | M1C | 0.0972 | 0.0975 | 0.00286 | 0.09 | 0.1 |
| | M2K | 0.0893 | 0.0895 | 0.00175 | 0.09 | 0.09 |
| | M2C | 0.0998 | 0.0995 | 0.00232 | 0.1 | 0.1 |
| IPLP2 | M1K | 0.196 | 0.198 | 0.00522 | 0.19 | 0.2 |
| | M1C | 0.2102 | 0.21 | 0.00714 | 0.19 | 0.2 |
| | M2K | 0.2037 | 0.2025 | 0.00398 | 0.2 | 0.21 |
| | M2C | 0.2305 | 0.2295 | 0.00869 | 0.22 | 0.24 |
| DNA damage | M2K | 0.149 | 0.1465 | 0.00645 | 0.14 | 0.16 |
| protein | M2C | 0.1465 | 0.1455 | 0.00362 | 0.14 | 0.15 |
| Apoptosis | M1K | 0.196 | 0.198 | 0.00522 | 0.19 | 0.2 |
| | M1C | 0.2102 | 0.21 | 0.00714 | 0.2 | 0.22 |
| | M2K | 0.2037 | 0.2025 | 0.00398 | 0.2 | 0.21 |
| | M2C | 0.2305 | 0.2295 | 0.00869 | 0.22 | 0.24 |

Path Regression analysis performed on Hsp 70, DNA damage proteins, and the IAP to apoptosis. Cisplatin effect on Hsp 70 with 0,000 significant results (p <0.05). In cell culture against Hsp 70 showed the number p = 0.165. Resistant cell types and cell cultures that had not received cisplatin does not affect changes in the secretion of Hsp 70, (p> 0.05). Hsp 70 protein did not affect DNA damage (p = 0527, p> 0.05).

Cisplatin affects IAP significantly (p = 0.000), and cell culture/cell resistant IAP affect significantly (p = 0.000). IAP does not affect Hsp 70 with a value of p = 0502. IAP induce apoptosis by significant coefficient b = 1.000. b is the value of contributions, meaning IAP affect the occurrence of cell apoptosis. Cisplatin did not give direct effect to apoptosis (coefficient b.000). Cell culture/cell resistant not give effect to apoptosis (coefficient b.000) and Hsp 70 does not give effect to apoptosis (coefficient b.000).

## DISCUSSION

Cisplatin is a standard chemotherapeutic treatment protocols, especially malignancies in the oral cavity are still a primary option. Cisplatin has the characteristics of cancer cells. The workings of cisplatin in cancer cell death primarily to thwart or inhibit cell growth. The

platinum electrodes binds to the DNA of cancer cells, leading to failure of the replication and transcription of DNA resulting in apoptosis (Lippard 1987). In this study focused on the mechanism of action of cisplatin cisplatin with DNA binding to DNA damage occurs. The cause of cell death mostly caused DNA damage permanently so that eventually the cells cannot survive and apoptosis. DNA damage can be detected and, if permanent damage occurs, then there was apoptosis. Damage to the DNA after administration of cisplatin followed by the process leading to apoptosis or an improvement/repair of DNA so that DNA persisted and apoptosis.

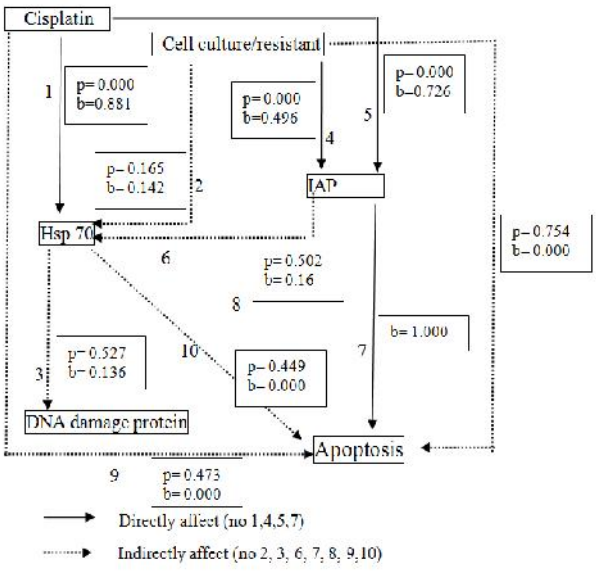


Fig. 4. Results of path regression analysis

This study only observed the end result of DNA damage, do not observe whether the cancer cells undergo DNA repair process or the cancer cells have not been damaged DNA. The test results showed no significant DNA damage in cisplatin as the DNA repair process has been successfully repair the DNA so that DNA damage is not detected. In cell culture studies that are resistant not detect DNA damage because the cells are resistant to cisplatin already have defenses to prevent DNA damage. This type of cell that is resistant to a chemotherapeutic drug cisplatin can be caused have received previous chemotherapy drug cisplatin exposure so that the cancer cells have a memory of the type of the chemotherapy drugs. Cancer cells that still survive have to cisplatin memory so that when getting re-cisplatin exposure will remain. This suggests that patients undergoing chemotherapy treatment and withdrawal would be likely to experience drug cisplatin resistance. Patients who received cisplatin underdose will also potentially undergo cisplatin resistance. This is evidenced in cultured cancer cells when the cancer cells that were resistant to cisplatin can be cultured. With low concentrations of cisplatin IC 50 (the half maximal inhibitory concentration) of the cell will die. (Sunters, 2013)

In this study, cisplatin affect Hsp 70 becomes active. This is shown in the secretion of Hsp 70 was significant in control cells compared to cells that received cisplatin, whether the cells are resistant or not resistant to cisplatin. When compared between the resistant cancer cells and cancer cells that have not been received cisplatin, then the increased secretion of Hsp 70 more tingga in cancer cells that have not received cisplatin. Resistant cancer cells have shown an ability to withstand exposure to cisplatin so that secretion of Hsp 70 has not increased much since resistant cancer cells are able to survive against cisplatin. Secretion of Hsp 70 will increase due to cisplatin, but Hsp 70 does not directly affect the occurrence of apoptosis, due to the increased secretion of Hsp 70 is still passing through a long process to inhibit apoptosis.

Human IAP (XIAP, c-IAP1, and c-IAP2) suppress apoptosis by inhibiting caspases directly). XIAP, c-IAP1, and c-IAP2 bind and inhibit caspases 3, 7 and 9 (Deveraux et al., 1997; Roy et al., 1997). IAP secretion effect on apoptosis when IAP get exposure to cisplatin. Type of cell culture will also influence the IAP in the occurrence of apoptosis. Cancer occurs because of an imbalance between the phases of proliferation and tumor suppressor genes (genes apoptosis) including IAP genes. Resistance to cisplatin occurs through suppressor pathway genes (IAP). In this study, IAP showed significant numbers in cancer cells that received cisplatin compared with controls. In the cisplatin-resistant cancer cells, the results of IAP lower than cancer cells that have never received cisplatin. In this case the cisplatin-resistant cell cultures will affect the IAP to prevent apoptosis.

The theoretical benefits are obtained to determine the role IAP inhibiting apoptosis in cancer cells of the oral cavity. IAP work on approaching the process of apoptosis, preventing apoptosis, prolonging the time of occurrence of apoptosis, and provide an opportunity for the occurrence of DNA repair. Successful repair DNA damage will prevent cell apoptosis.

Type cisplatin-resistant cell cultures show the effect of cisplatin on the secretion Hsp 70 is not significant. This indicates that the cisplatin-resistant cells, when treated or gain exposure to cisplatin, do not cause interference to the cell. Cisplatin-resistant cell culture type of DNA

damage is not significant. This can be explained that cisplatin does not affect DNA damage significantly. The cells have a strong defense to prevent cisplatin damage DNA.

Path regression analysis Hsp 70, DNA damage proteins, and the IAP to the onset of apoptosis were in accordance with the conceptual framework. The role of cisplatin against Hsp 70 is significant, which cisplatin affect the increased secretion of Hsp 70. In theory, Hsp 70 is a response of cells to maintain cell survival.

Cisplatin into the cell affect the IAP, which responds directly IAP inhibition via caspase pathway leading to apoptosis. On this track the cell's defense mechanisms are reinforced to prevent apoptosis. On this kind of cisplatin-resistant cell cultures or who have never received cisplatin also significantly affect the IAP. This indicates that cell trying to survive through the IAP, preventing the cell apoptosis.

IAP does not affect Hsp 70. IAP has a different way of preventing apoptosis. IAP significantly induce apoptosis, which indicates the success of the defense cells against apoptosis is affected IAP. Increased levels of IAP due to cisplatin will inhibit caspase process, return to normal DNA repair and apoptosis inhibition.

Cisplatin did not give a direct influence apoptosis significantly. Cisplatin process towards apoptosis was via cellular defense mechanisms. If cisplatin managed to penetrate the defenses of these cells, then cisplatin will lead to cell apoptosis. When cisplatin failed to break the resistance of these cells, then the cells will survive.

The tumor microenvironment has major effect on survival of tumor. In this study, cancer cells can survive. In this case the tumor microenvironment is not manipulated, so that the cancer cells of the tumor microenvironment assisted.

New findings from the study are inhibitors of apoptosis proteins (IAP) as factors that influenced the occurrence of cell apoptosis. Track to achieve apoptosis is cisplatin with through IAP towards apoptosis. Cisplatin give effect to the IAP and the decline Inhibitor of Apoptosis Protein (IAP) will induce apoptosis directly. Resistance to cisplatin occurs through suppressor pathway genes (IAP).

## CONCLUSION

Cisplatin therapy affects the secretion of IAP as proteins that control the occurrence of oral cancer cell apoptosis. IAP secretion affects the apoptosis. IAP responded to a cisplatin into the cell by inhibiting apoptosis. In the cisplatin-resistant cell cultures that will give more leverage to the IAP, apoptosis barriers will increase. Types of cell cultures who have never received cisplatin also affect the IAP in the control of apoptosis. Clinical benefits that can be applied to cisplatin chemotherapy is cisplatin therapy if the first did not respond, it is necessary to check the IAP. When the results of IAP increases, it is most likely that cancer cells are potentially resistant to cisplatin. Further research is needed on the effect of IAP in the process of apoptosis inhibition. There are many other types of IAP in the process of apoptosis inhibition. The tumor microenvironment is one of the factors the survival of cancer cells alive. Further studies changes in the tumor microenvironment that cancer cells cannot survive.

## REFERENCES

Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat. Med. 1997;3:917–921.

Cepeda, V., et al., 2007. Biochemical mechanisms of cisplatin cytotoxicity.Anticancer Agents in Medicinal Chemistry, 7(1), pp.3-18.

Deveraux, Q.L. & Reed, J.C., 1999. IAP family proteins-suppressors of apoptosis. Genes and Development, 13, pp.239-52.

Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM, Thompson CB. A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J. 1996;15:2685–2694.

Forastiere AA, Goepfert H, Maor M, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003; 349:2091.

Gober, M.D., Wales, S.Q. & Aurelian L., 2005.Herpes simplex virus type 2 encodes a heat shock proteinhomologue with apoptosis regulatory functions. Frontiers in Bioscience, 10, pp.2788-803.

Gosselin, N.H., Brunet, R.C. & Carrier, G. (2003) Comparative occupational exposures to formaldehyde released from inhaled wood product dusts versus that in vapor form. Appl. Occup. Environ Hyg., 18, 384–393

Kelland, L., 2007. The resurgence of paltinum based cancer chemotherapy. Nature reviews. Cancer, vol 7, pp.573-82

Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature. 1998;396:580–584.

Lippard SJ. Chemistry and molecular biology of platinum anticancer drugs. Pure & Appl. Chem. 1987;59:731–42.

Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, Farahani R, McLean M, Ikeda J-E, MacKenzie A, Korneluk RG. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature. 1996;379:349–353.

Mosser, D.D. & Morimoto, R.I., 2004.Molecular chaperones and the stress of oncogenesis. Oncogene, 23(16), pp.2907-18.

Roy, M.D., 2007. Approach for assessing total cellular DNA damage. Bio Techniques, 42(4) pp.425- 35.

Siddik, Z.H., 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene, 22, pp.7265-79.

Sunters A., 2013. How can I create a resistant cell line? https://www.researchgate.net/post/How_can_I_create_a_resistant_cell_line.