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EVALUATION OF THE COMBINED EFFECTS OF PIPERINE AND 5-FLUOROURACIL ON MG-63 OSTEOSARCOMA CELL LINES AN IN – VITRO STUDY

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ABSTRACT

Background and Aim: Osteosarcoma is a malignant cancer that effect bone and metastasising to many vital organs like lungs. There are many available drugs to treat the disease including Doxorubicin, 5-Fluorouracil (5-FU), Methotrexate (MTX), cisplatin., etc which have their own side effects and hurdles to become drugs of choice for the disease. On the other hand introduction of herbal drugs as chemotherapeutic agents opened up new arena to potentiate the existing treatment by exhibiting synergy. Thus this study was designed to investigate the synergistic inhibitory potential of Piperine (PPN) and 5-FU on the MG63 osteosarcoma cell lines invitro.

Materials and Methods: The cell lines were cultured on DMEM medium and investigated for cytotoxicity of the drugs using MTT assay at 570nm in UV spectroscopy. Three groups of cell lines administered with PPN, 5-FU and PPN+5-FU (1:1) in various concentrations of (7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 g/mL) and IC50 values were calculated based on the % cell viability graphs.

Results: Results showed that the IC50 of PPN was 14.49, 5-FU was 17.76 and PPN+5-FU were 10.79 proving the significant synergistic cytotoxic effect of Piperine and 5-FU in inhibiting the proliferation of MG63 cell lines. Microscopic morphological studies indicated that the cytotoxicity induced apoptosis in the MG63 cell lines leading to bulging of cell membranes and cell organelles.

Conclusion: Further research needs to be conducted in this field to elucidate the synergistic pathways in which Piperine has shown a better anti-osteosarcoma effect when combined with methotrexate.

Keywords*: Osteosarcoma, MG63 cell lines, Piperine, 5-Fluorouracil, Synergism, MTT assay.*

INTRODUCTION

Osteosarcoma is commonest of the malignant bone cancers that occupies almost 30% of the share in the mortality and the medical treatment cost. It usually occurs in adolescent age where the growth of the bones is rapid and is starts in the metaphyses part of the bone. This metastatic disease aggressively differentiates in the fibrous tissues, cartilage tissue and bone (He et al., 2014). The prevalence of osteosarcoma is high during puberty between 10-14 years and its second wave is seen in geriatric patients where the bones tend to get weak due to various factors. The incidence of osteosarcoma is most in advance countries like the US where over 400 cases were newly diagnosed every year (Cotterill et al., 2004).

There are numerous anti-cancer drugs that are available for treating osteosarcoma. Doxorubicin is the drug of choice but its use is limited due to its cardiotoxicity. Cisplatin is also used alongside with most chemotherapy regimens to treat osteosarcoma. On the other hand, methotrexate in high doses is also used in combination with leucovorin commonly.

Recently, addition Ifosfamide to the existing regimens is widely accepted. In several parts of the world, treatment of osteosarcoma gave positive results with these combinations but few patients showed no response to the treatment significantly (Meyers et al., 2005).

As a result, increasing the chemotherapy dosage is necessary to adequately treat osteosarcoma but is constrained by mortality and toxicity. Population growth and an increase in osteosarcoma cases in recent years have created a demand for chemotherapeutic drugs that cannot be met. Usually these drugs have proven common side effects like nausea, vomiting, alopecia etc. However there are other serious side effects due to toxicity of chemotherapeutic drugs that are tolerated in young patients but adversely affect elderly patients. Methotrexate causes problems in renal function which results in alkanisation and hyper hydration. Leucovorine counters these effects to a certain extent (Carrle and Bielack, 2006). Also there are other cytotoxicity related side effects involving heart resulting in cardiac failure due to long term chemotherapy (Goldsby et al., 2008).

There isn't yet a conventional chemotherapeutic treatment plan for osteosarcoma that returns after receiving multimodal first-line therapy. The prognosis is bleak, with less than 20% of patients experiencing long-term post-relapse survival. Even while a minor advantage was seen in certain retrospective data, the adjuvant impact of second-line chemotherapy in patients with a second complete surgical remission is not as evident (Kempf-Bielack et al., 2005).

Additionally, the use of synthetic medications in larger doses is restricted by drug resistance (Austin et al., 1999). Thus to overcome this issue 5-fluorouracil (5-FU) in combination with cisplatin on the proliferation of human osteosarcoma cells in vitro in some studies. Results suggested that the combination of drugs exerted an inhibitory effect on the proliferation and invasion of human osteosarcoma cells in vitro, and promoted cell apoptosis (Jiang et al., 2019). There are few other studies that proved the antiproliferative effects of a combination between inorganic phosphate (Pi) and doxorubicin, Taxol and 5-fluorouracil (5-FU) against human osteosarcoma cells (Spina et al., 2013). Piperine is one of those constituents isolated from *Piper nigrum* with amide group was reported to fight lung metastasis and also inhibited many types of cancers in various animal models [Pradeep and Kuttan, 2002; Sunila and Kuttan, 2004; Bzerra et al., 2006]. Piperine also exhibited potent activity against breast cancer in combination with thymoquine and sulforaphane [Aumeeruddy et al., 2019]. The cytotoxicity of piperine on hepatocellular carcinoma by inducing apoptosis via free radical mediated pathways in mitochondria was reported [Gunasekaran et al., 2017].

In light of the aforementioned information from earlier studies, piperine (PPN) and 5-Fluorouracil (5-FU) were shown to have antitumor activities. Numerous investigations on the anticancer potential of these medications have produced convincing data. On the other hand, there hasn't been much study in this area looking at the combination of anticancer medications. According to research, using chemotherapeutic drugs in combination improves the treatment of osteosarcoma. Consequently, a combination of these medications may be an effective and safer therapy choice for osteosarcoma. In order to improve the disease's treatment profile, the current study was created to compare the individual anti-cancer effects of PPN and 5-FU to their combined effects on MG3 osteosarcoma cell lines.

MATERIALS AND METHODS

Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS) and other chemicals were procured from Sigma-Aldrich India, Bangalore. The reagents used in the work were of analytical grade.

Cell lines

National centre for cell sciences (NCCS), Pune, Indiana, provided human osteosarcoma cells (MG63 cell lines). The cell lines were maintained in a humidified compartment with (DMEM) supplemented with 10% FBS, antibiotics (penicillin-100U/ml and streptomycin-100g/ml), at 5% CO_2 at 37^{o}C.

In Vitro Anticancer activity

MG63 osteosarcoma Cell lines in mother cultures were adjusted to $1x10^5$ cells/ml of media and cell lines were grown for exponential growth. Different quantities of PPN, 5-FU, and PPN+5-FU (1:1) (7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 g/mL) were applied to cell cultures, and they were then incubated for 24 hours (Chang et al., 2015). The cells were seeded onto 24 well plates (Mosmann, 1983). Following a PBS and DMEM without serum wash, the cells were examined under a microscope to check for morphological alterations. All of the wells received volume of 100 µL/well (5 mg/ml) of 0.5% MTT reagent, which was then incubated for 4 hours under the same conditions. Each well received 100 µL of the MTT stop solution at the conclusion of incubation. The optical density of the 96-well plates was then measured using a spectrophotometer (SpectraMax M3, Molecular Devices) at a wavelength of 540 nm. A standard curve illustrating the linear connection between various cell densities and optical densities was used to calculate cell density. The percentage of cells that were viable in each sample was determined, and the graphs of cell viability were used to determine the respective

IC_{50} values. The ratio of the cell density in each sample to the cell density in the control sample was used to indicate cell viability.

RESULTS

Morphological changes in MG63 osteosarcoma cell lines due to PPN+5-FU

The normal group cell lines that are untreated with any drug showed a regular spindle shaped tail like protrusions of MG63 osteosarcoma cell lines. They showed a transparent to translucent cytoplasm of cells. Post incubation of the cell lines with drugs separately or in combination showed shortening of tails and their multiplication also slowed down as shown in figure 1. The rise in treatment concentration of drugs resulted in the darkening and rounding of the cells. At the highest dose of drugs, 1000 µg/ml, cells showed a shrinkage of tails and overall size too. Bulging of cell membranes resulting in the swollen cells indicates the effect of apoptotic mediators. The cells exhibited the dose dependant apoptosis with the exposure to drugs. It is clearly noticed in figure 1 that rounding of cells and swelling of the cells and intracellular organelles as thick and dark dots inside the cells. This indicates that starting of apoptosis due to administration of Piperine (PPN) and 5-Fluorouracil (5-FU) which suggest cytotoxicity in MG63 cell lines.

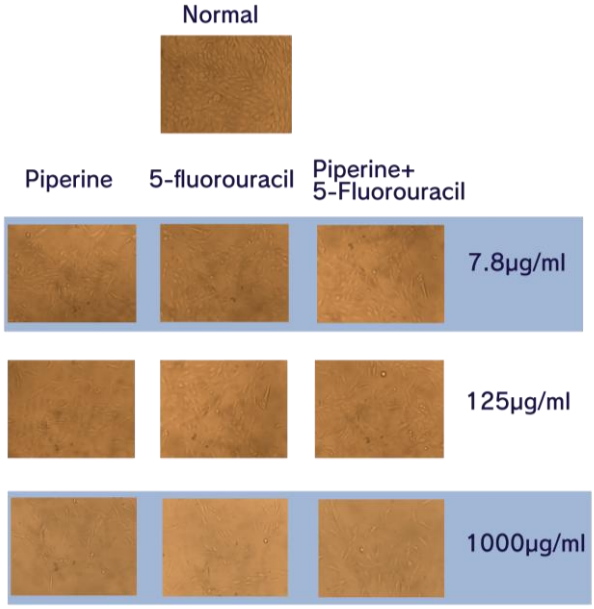

Figure 1: Morphological changes in MG63 cell lines with the treatment of Piperine and 5- Fluorouracil

IC_{50} values of PPN+5-FU on MG63 cell lines

The determination of cytotoxicity of the drugs in terms of the IC_{50} using MTT assay method is often reliable for anti-cancer drugs. This method uses spectrometric principle by reading the developed colour of the stain that indicates the growth and progression of cell line. In current research the % cell viability of PPN, 5-FU and PPN+5-FU combination were administered on MG63 osteosarcoma cell lines at various doses from higher to lower concentrations (7.8, 15.6, 31.2, 62.5, 125, 500 and 1000 µg/ml) and their respective IC_{50} was calculated. Table 1 shows the relation between the % cell viability to the dose concentration of the drugs. There was significant cytotoxicity exhibited by the 5-FU in combination with PPN where the IC_{50} was 10.79 µg/ml compared to that of the single drug 5-FU of 17.76 µg/ml. this was drastically lowered by co-administration with PPN. Individually PPN IC_{50} was 14.49 µg/ml. this anti-cancer activity of PPN might have helped to increase the toxicity of 5-FU even at the same dose when used as single drug. At 1000 µg/ml dose the cell viability of MG63 cell lines with PPN showed cell viability of 11.38% and 5-FU showed 14 % but when used in combination they showed 8.51% which clearly indicates the synergistic activity of both the drugs.

Table 1: Effect of Piperine and 5-Fluorouracil on cell viability of MG63 cell line

S.No	Concentration (µg/ml)	Cell viability (%)		
		PPN	5-Fluorouracil	PPN+ 5-Fluorouracil
1	1000	11.34	14.00	08.51
2	500	17.46	19.94	14.77
3	250	23.58	25.97	20.93
4	125	26.69	31.91	27.20
5	62.5	35.90	37.85	33.46

6	31.2	42.02	43.88	39.82
7	15.6	48.13	49.82	46.18
8	7.8	54.25	55.67	52.44
IC_{50} (µg/ml)		14.49	17.76	10.79

DISCUSSION

The fluoropyrimidine-based drug 5-FU is an antimetabolite and thymidylate synthase inhibitor that primarily inhibits DNA synthesis during the S phase of the cell cycle (Longley et al., 2003). 5-FU is internally converted to several active metabolites, such as fluoro(deoxy)uridine monophosphates [F(d)UMP], through the enzymatic activity of uridine phosphorylase, orotate phosphoribosyltransferase, and thymidine kinase. These metabolites all interfere with RNA and DNA homeostasis to cause cell cycle arrest in the G1/S phase and ultimately lead to apoptosis.

According to some research, immunomodulatory activity of PPN, which is attributed to the induction of cellular and humoral responses, which might be the reason to it its anticancer potential (Sunil and Kuttan, 2004). The low IC_{50} of PPN found in this research shows that there is no proportionate or direct relationship between the antiproliferative property and anticancer activity, despite substantially lesser cytotoxicity of PPN compared to other natural amides-containing drugs. According to several studies, PPN did not significantly slow the rate of proliferation compared to Piplartine in Sarcoma-180 cells when compared to Ki67 positive cell lines (Bezerra et al., 2006). This further confirms our research findings that PPN is superior to many synthetic medications and other natural drugs in its family in terms of its potency to act as a cytotoxic agent and an inhibitor of cell proliferation. Other studies suggest that PPN inhibits growth in metastasing osteosarcoma cells by suppressing the MMP-2 and MMP-9 expression which supports the current research for the potent activity of PPN (Zhang et al., 2015).

The clinical outcomes of osteosarcoma therapies remains depressing despite rigorous advances because of its strong metastatic potential and the frequent development of chemotherapy resistance. Thus, the urgent need for innovative treatment strategies (Hattinger et al., 2010). When anticancer medications are used in clinical settings, cancer cells eventually acquire resistance and stop responding to treatment, requiring greater dosages of synthetic pharmaceuticals and increasing the risk of adverse effects (Autin et al., 1999). Even though chemotherapy is a straightforward method of treating cancer, research have shown that it often fails due to developed resistance to the medications (Hu et al., 2018). As a result, several research focused on this topic to look at how to understand chemotherapeutic resistance (Incles et al., 2003). Combinatorial and Synergistic chemotherapy is getting more attention in an effort to find substances that might raise the therapeutic index of anticancer medications used in clinical trials. It is often anticipated that this combination of two or more chemotherapy medicines may decrease the frequency of chemotherapy while simultaneously improving the cells' responsiveness to treatment (Morinaga et al., 2003). In this context, naturally occurring compounds with antitumor action and minimal toxicity to healthy tissues have been proposed as potential candidates for research into the synergistic effectiveness of these molecules in conjunction with antineoplastic medicines.

The current study's findings shown that, when compared to the synthetic medicine methotrexate, Piperine (PPN) and 5-fluorouracil (5-FU) have a strong anti-osteosarcoma action against the MG63 cell lines. According to National Cancer Institute (NCI) recommendations, PPN's IC_{50} value (14.49 µg/ml) is below the cytotoxicity limits of 20 µg/ml, demonstrating its anti-cancer potential against MG63 osteosarcoma cell lines (Boik, 2001). Our findings also make it clear that 5-FU shown reduced activity compared to the 1:1 combination, which demonstrated activity comparable to PPN. The activity of 5-FU was dramatically increased when PPN and 5-FU were combined 1:1, indicating that PPN and 5-FU had synergistic effects. The IC_{50} value of PPN, which is lower than that of 5-FU and indicates that PPN is more potent than 5-FU as a single medication, makes the potency of PPN obvious. Several studies demonstrate effectiveness of PPN in comparison to 5-flurouracil (5-FU), in producing cytotoxicity in the MDA-MB435 melanoma cell line and the HL-60 promyeloblast cell line (Moringa et al., 2003). Studies revealed that the anti-osteosarcoma activity of PPN is attributable to its ability to block the production of metastatic markers in the illness, which would otherwise promote the growth and metastasis of the condition (Pathak and Khandelwal, 2007).

The evaluation of the anti-osteosarcoma potential of PPN and 5-FU in combination treatment had given good findings, validating our statements of positive impact of both drugs. The selection of MG63 cell lines was also an important role in replicating the activity of osteosarcoma cells. As a result, the action of the medicines on these cell lines may be interpreted to be comparable to that of osteosarcoma. The major limitation of the current study is the assessment of activity in vitro which may be solved by simulations utilization of computer-based software tools and actually introducing the cell lines invivo (preclinical/clinical studies). Proper examination of the pharmacokinetic profile in vivo may also impede drug activity due to the bioavailability and clearance of the medications in the presence of each other, since PPN is recognised as a bioenhancer of other drugs (Mhaske et al., 2018).

Therefore conducted study and prior researches indicate that the synergy may be used as a combination therapy in the treatment of osteosarcoma. The anti-osteosarcoma activity of PPN clearly adds to the cytotoxicity of 5-FU in an unknown manner that may be further explained, however current research credits the increase in activity of the combination to anti-proliferative mechanisms of PPN and 5-FU.

CONCLUSION

5-Fluorouracil (5-FU) and Piperine (PPN) have a synergistic inhibitory action against MG63 cell lines, according to this study. The results indicated that when 5-FU was combined with PPN, its potency increased significantly. Though the mechanism by which PPN increases anti-cancer activity of 5-FU is unknown, this offers up a new avenue of research to investigate the real mechanisms via which PPN shown synergy with 5-FU. This also allows researchers to look at other medicines for synergy in order to develop therapy options for treating osteosarcoma that have a higher therapeutic index and fewer adverse effects.

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Conflicts of interests: The authors declare no conflicts of interest.
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