Original Article

Comparative Effects of Atorvastatin and its Liposomal Preparation in Impeding the Migratory Potential of Colorectal Cancer Cell Line HT-29

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ABSTRACT

Objective: The objective of my study is to evaluate the effects of Atorvastatin and its liposomal conjugation (lipo-ATO) as an anti-migratory potential in colorectal cancer HT-29 cell line.

Study Design: Experimental Study

Place and Duration of Study: This study was conducted at the Clifton campus of Ziauddin University, Karachi from 15 October, 2022 to July, 2023.

Materials and Methods: After revival, cells were plated in 6 well plates with complete media, a vertical scratch was made with 10µl pipette and different drugs were added in each group. Images of the cells were observed at interval of 24, 48, 72, and 96 hrs. Under an inverted microscope, to determine the difference in wound healing.

Results: The result of our study showed that liposomal atorvastatin significantly delayed wound healing as compared to free atorvastatin and 5-FU P-value(>0.001).However, free atorvastatin produced cytotoxicity at 72hrs.

Conclusion: Liposomal conjugated atorvastatin showed pronounced anti-migratory effects in HT-29 cell line by delaying wound healing. Comprehensive studies are warranted to confirm the findings and to appraise underlying molecular mechanisms.

Key Words: Liposomal Atorvastatin, Cell migration, HT-29 CRC cell line, wound healing.

Citation of article: Shaikh S, Ismail K, Usman S, Ismail MO, Gul J, Shah R. Comparative Effects of Atorvastatin and its Liposomal Preparation in Impeding the Migratory Potential of Colorectal Cancer Cell Line HT-29. Med Forum 2023;34(9):136-140. doi:10.60110/medforum.340932.

INTRODUCTION

The development of a new drug is an expensive and time-consuming practice typically costing 2.6 billion USD and taking around a decade on average[(1)]. To overcome this limitation of drug discovery, researchers are focusing on repurposing of existing drugs that show cytotoxic activity against serious diseases like cancer[(2)]. Recently many researches have taken intensive efforts to repurpose drugs like the antiviral agent remdesivir for serious diseases that was previously used for Ebola virus has been repurposed to treat COVID. The antibiotic levofloxacin has been recently suggested as a potential drug for Alzheimer's disease [(3)]. As with other diseases drug repurposing in the field of oncology attracted researchers since the last few decades. In this regard, metformin an anti-diabetic drug has been evaluated for non-small cell lung cancer[(4)] Statins, the competitive inhibitors of HMG-CoA reductase enzyme are commonly utilized to treat dyslipidemia by inhibiting the cholesterol synthesizing "mevalonate pathway"[(5)].

Statins classification is based on physiochemical properties as lipophilic or hydrophilic; the former group includes fluvastatin, pitavastatin, lovastatin, simvastatin, atorvastatin, and cerivastatin while the later includes pravastatin and rosuvastatin[(6)]. Lipophilic statins can cross cell membranes and are widely distributed and this property attracted researchers to use them as alternate therapy against different diseases independent of lipid-lowering properties. Along with that statins also show marked pleiotropic effects like anti-platelet, anti-inflammatory and antioxidant[(7)]. Many studies have shown that statins can also enhance the efficacy associated with conventional cancer treatment, considering same in context with combination therapy though these trials are still under investigation like in one study, co-treatment with

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Received: August, 2023
Accepted: August, 2023
Printed: September, 2023

simvastatin with Tamoxifen shows a higher survival rate of breast cancer patients compared with Tamoxifen alone[8].

Another widely used strategy in the field of cancer is nanotechnology which shows several promising applications in the diagnosis and treatment of cancer including drug delivery, gene, and targeted therapy[9].Among Nano-carriers, liposomal nanoparticles show greater efficacy and powerful platform for drug delivery to target sites with better safety profile ov]er silver or gold-nano forms. Several liposomal formulated anti-cancer drugs like AmBisome, Doxil, are clinically available to treat cancer[10].

Due to its early metastasis and poor prognosis, colorectal cancer which ranks third among gastrointestinal cancers, is the second most common cancer-related cause of death globally[11].Among all types of CRC, 95% of cases are of the type of Adenocarcinomas[12]. Westernization is the major risk factor behind high mortality rate[13]. CRC is quite notorious for metastasis and resistance by chemotherapy and over all 5 year survival rate is only 10 percent despite in advancement of targeted therapy, immunotherapy and chemotherapy [14].

Keeping in view the treatment related glitches of CRC and anticipated potential of statins, this study was planned to see the anti-metastatic potential of atorvastatin and liposomal-conjugated atorvastatin through wound healing assay in HT-29 colorectal carcinoma cell line.

MATERIALS AND METHODS

5-FU was purchased from sigma pharma. Atorvastatin was purchased by Enaltec Ltd Pvt. The liposomal conjugation of atorvastatin was offered by (HEJRIC).Colorectal cell line HT-29 was gifted by the Director, Biological and Biomedical Sciences, Agha Khan University, Karachi.

Ht-29 Cell Line Revival And Sub-Culturing: The cell line was stored in liquid nitrogen at a temperature of -196°C was transferred to a falcon tube filled with (DMEM) sterilized with 70% ethanol and thawed in warm water at 37°C. After centrifugation, it was seeded in a tissue culture-treated T75 flask with 9 ml of complete media and kept at incubator for cell proliferation after that sub-culturing was done, media was aspirated and 4ml of 10X trypsin was given to cells to deattact it from plate surface.To stop the action of trypsin, media was added and the suspended cells were then transferred to a 15 ml falcon tube, centrifuge at 1000 rpm for 8 minutes. The supernatant was discarded once the cell pellet appeared and aspirated, after which seeded into two distinct T75 tissue culture flasks, each containing 9ml of media.

Preparation Of Serially Diluted Drugs: The serial dilutions of atorvastatin and 5-FU were formulated, which later were used to constitute different concentrations of drugs.

Grouping Of The Treated Cells: The IC50 of drugs at which wound healing assay was performed were: 51.168µg/ml for Atorvastatin, 32.54008µg/ml for Liposomal atorvastatin, 234.72µg/ml for 5-FU.

Following treatment groups were analyzed:

- Group 1: Untreated HT-292 cells (control group);
- Group 2: HT-29 cells treated with atorvastatin (atorvastatin group);
- Group 3: HT-29 cells treated with liposomal conjugated atorvastatin (liposomal-atorvastatin group).
- Group 4: HT-29 cells treated with 5-fluorouracil (5FU group);

Cell Counting: Cell counting was done by Neubauer chamber under inverted microscope

Scratch assay: To assess the anti-migratory potential of the control and treated groups, the scratch assay was conducted where cells were seeded in a 6-well plate; once the cells were completely confluent within 24hrs a vertical scratch was made using a sterile 10µL tip in each plate. The media was then withdrawn, and the drugs (at IC50 concentrations) were added along with media. After that, imaging was performed at 0, 6, 24, 48, 72, and 96 hours. Image J software was used to examine cells migration and wound closure. The formula for wound closure is as follows:

$$\% \text{ of wound closure} = [(At = 0h - At = h) / At = 0h] \times 100\%$$

Where:

0h (the area of the wound measured).

Δh (the area of the wound measured at 0, 24, 48, 72 and 96 hrs).

Stastical Analysis: Data was analyzed through SPSS program (Version 24). Numerical data was calculated as mean ± SE of the mean (SEM), generated by applying ANOVA (analysis of variance) followed by Tukey's post hoc tests to observe the comparison among the groups. The significant difference between different groups were considered significant at P-value < 0.05.

RESULTS

Table:The Migratory Potential of control and Treated Groups on the HT -29 CRC Cell Line at Different Time Interval.

The scratch assay was followed over 96 hrs in control group and liposomal atorvastatin groups, while in 5FU and free atorvastatin group it was checked up to 72 hrs because of appearance of cytotoxicity in these groups.

Wound healing was significantly delayed at treated groupas compared to untreated cells. P- value(>0.001)(fig:1)(fig:5).On comparison of free atorvastatin and liposomal atorvastatin we have found significant difference between these two groups at 24 and 48 hrs. Liposomal atorvastatin delayed wound closure more

significantly than free atorvastatin depicting liposomal atorvastatin was far better than free atorvastatin p-value(>0.001)(fig:2)(fig:3).As 5-FU is well known anti-cancer drug it significantly inhibited cell migration up to 48 hrs. Then produce cytotoxicity afterwards.(fig:4), (fig:5). However, liposomal atorvastatin was better than 5-FU in delaying the wound closure with p-value (>0.05)(fig:5.)

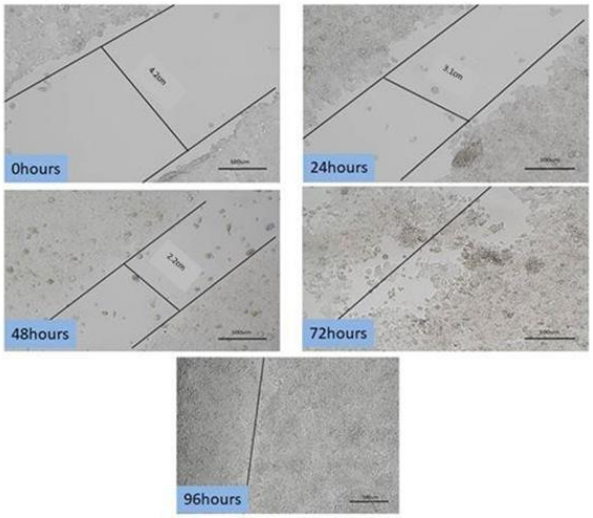

Figure No. 1: Wound healing responses of colorectal cancer cell line HT-29 in untreated (control) group (Images 0, 24, 48, 72 and 96 hours after induction of scratch). 24, 48, 72 and 96 hours after induction of scratch)

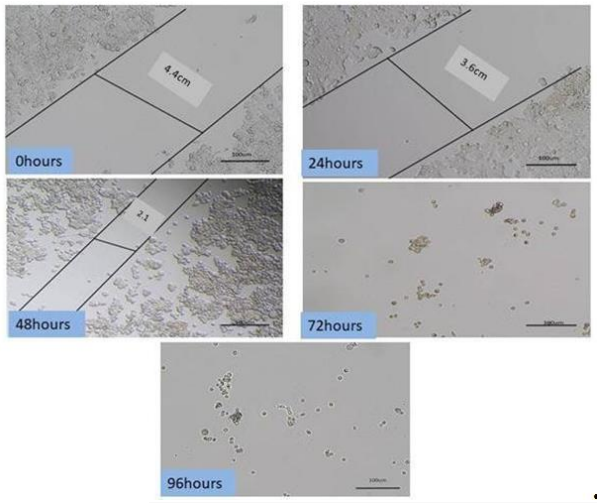

Figure No. 2: Wound healing responses of colorectal cancer cell line HT-29 in atorvastatin (Images taken at 0, 24, 48 and 72 hours after induction of scratch).

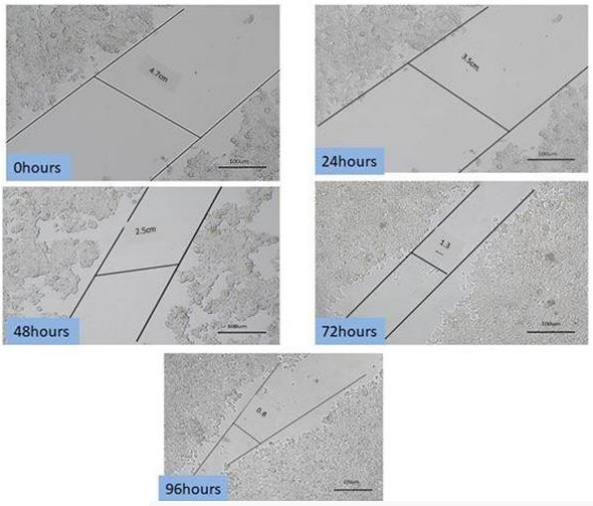

Figure No. 3: Wound healing responses of colorectal cancer cell line HT-29 in liposomal atorvastatin (Images taken at 0, 24, 48 and 72 hours after induction of scratch).

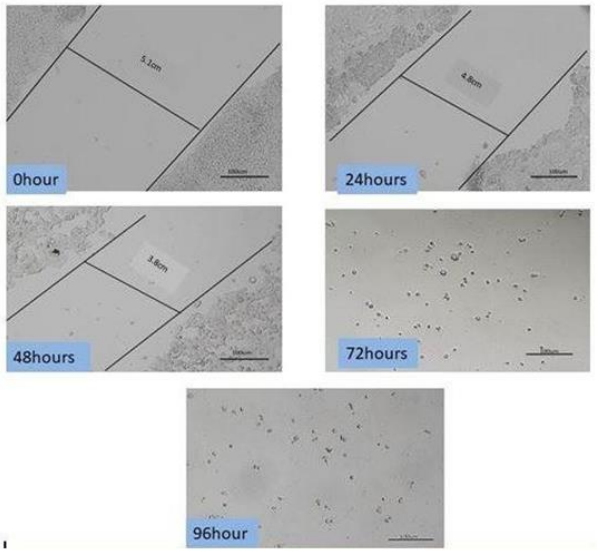

Figure No. 4: Wound healing response of HT-29 in 5FU group (Images taken at 0, 24, 48, 72 and 96 hours after induction of scratch).

Table No. 1: The Migratory Potential of control and Treated Groups on the HT -29 CRC Cell Line at Different Time Interval

COMPONENT	CONTROL	ATORVA	LIPO-ATO	5-FU
% HEALING	43%	22%	16%	20.80%
P-VALUE	(<0.001).	(<0.001).	(<0.001).	(<0.001).
		AT 24HOURS		
% HEALING	63%	42%	28%	38%
P-VALUE	(<0.001).	(<0.001).	(<0.001).	(<0.001).
		AT48 HOURS		

% HEALING	100%	0	34%	0
P-VALUE	(<0.001).	(<0.001).	(<0.001).	(<0.001).
		AT 72HOURS		
% HEALING	100%	0	62%	0
P-VALUE	(<0.001).	(<0.001).	(<0.001).	(<0.001).
		AT 96HOURS		

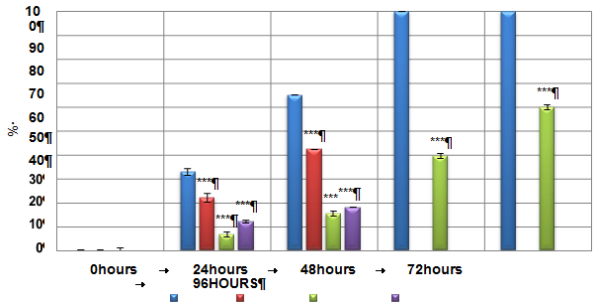

Figure No. 5: Graphical representation of % wound healing in different treated groups.

(Experiments were run in triplicates, data represented as mean ± SEM)

DISCUSSION

Amongst important factors responsible for CRC prognosis, the utmost is tumor invasion from original to distant site. The adhesiveness and migratory potential of cancer cells, and their micro- environment is responsible for invasion and progression of this tumor. Among existing procedures intended to observe these changes, scratch assay is a simple and economical technique and is frequently utilized to analyze cell migration in in-vitro pharmacological studies.

The basic principle is the obliteration of a confluent cell monolayer, generating a cell-free region, which mimics a wound, where cell migration and repair are reported through scratch analysis. In this 2-D wound healing assay, cells essentially proliferate and migrate in the wound bed, where the migration phase is the rate-limiting event in wound healing. Therefore, scratch assays can effectively evaluate cell migration in in-vitro studies [(15)].

We have performed wound healing assay at IC50 concentration of different compounds including atorvastatin, 5-fluorouracil, and liposomal atorvastatin (though drug concentration below IC50 can also be utilized to check the anti-migratory effects on various time intervals 0, 24, 48, and 72 and 96 hrs. In our study we found that atorvastatin has potential to delay wound closure in CRC. Supporting our study, Zanfardino M et al.,checked the effects of simvastatin on anti- migratory effects on melanoma cancer cells with intervals of 24 and 48 hours and they observed that cells treated with simvastatin showed inhibition in cell growth and migration up to 48 hours compared to control group[(16)]. Another in vitro study conducted on various breast cancer cell lines (MDA-MB-231 and MCF-7 cell lines) with different statins like mevastatin, pitavastatin simvastatin and lovastatin reported that among all statins, simvastatin showed the highest anti-migratory potential in MCF-7 breast cancer cell line compared to other statins[(17)]. Moreover, atorvastatin also significantly decreased the cell migration of breast cancer MDA-MB-231 and MCF-7 cell lines.

Our liposomal results demonstrated that liposomal atorvastatin inhibit wound healing more significantly compare to free atorvastatin. In accordance with our study , one study was done on glioblastoma tumor cells to see the effects of atorvastatin alone at 5 and 10 μM- and exosome-conjugated atorvastatin AtoEXOs at 5 and 10 μM concentration on wound healing, and it was observed that AtoEXOs at 10 μM concentration shows a significant anti - migratory effects compared to atorvastatin alone, along with suppressing VEGF, an important determinant of tumor invasion and metastasis[(8)] this superiority of liposomal preparations are also evident with conventional drugs like liposomal conjugated 5-FU shows better anti-migratory effects on CoLo-205 and CaCo-2 colorectal cell lines than free 5-FU.[(19)]

Various factors involved in proliferation and metastasis pathway namely VEGF, PDGF, TNF, MMPS particularly 7 and 9 primarily secreted by primary CRC tumor and involved in metastasis thereby enhances tumor cell invasion.[(20)]Though, in our study, we have not check the effects of atorvastatin on inhibition of growth factors like VEGF and MMPS but recent studies illustrate that rosuvastatin inhibits VEGF and MMP9 in human prostate cancer[(11)]Studies have also showed that anti-proliferative activity of atorvastatin in the ovarian cancer cell line SKOV3 was associated with apoptosis and cell cycle arrest by down regulating AKT/mTOR pathway.[(21)]

CONCLUSION

Liposomal conjugated atorvastatin causes a higher rate of cell growth inhibition asevident by reduced percentage healing compared to free atorvastatin. Moreover, atorvastatin slow down the wound healing up-to 48hrs only, after which it showed cell death. Further studies are warranted to validate our finding and pathways involved in anti-migratory potential of our repurposed drug.

Author's Contribution:

Concept & Design of Study:	Seema Shaikh
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Revisiting Critically:	Seema Shaikh, Kauser Ismail
Final Approval of version:	Seema Shaikh

Conflict of Interest: The study has no conflict of interest to declare by any author.

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