



Assessment of Anticancer Effects of the Investigational Agent MEX2R as K-RAS Oncogene Blocker in Human Colorectal Cancer Cells Model SW480 Cell Line

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ABSTRACT

Objective: To survey K-RAS blocker of choice among a group of inhibitors with characterizing their safety profile in colorectal carcinoma. An experimental model of continuous SW480 cell line and Vero cell line was applied, in vitro assessment by using tissue culture as a standard technique for checking the cytotoxicity of many compounds and in development of new drugs. **Materials and methods:** This study tested the cytotoxicity assay of MEX2R and chemotherapy (methotrexate) as comparative agents in the mechanism of action with the tested drug by Crystal Violet assay (CV). After exposure both SW480 cell line and Vero cell line were treated first to different concentrations of MEX2R compound (15.625, 31.25, 62.5, 125, 250 and 500) µg/ml for 36 hours and secondly to different concentrations of methotrexate (15.625, 31.25, 62.5, 125, 250 and 500) µg/ml for 36 hours. **Results:** The results showed that the investigational agent MEX2R exhibits highly significant cytotoxic effects ($p \leq 0.05$) in SW480 colorectal cancer cells when compared with control (untreated cells), and less cytotoxic influences on normal Vero cell line in a dose-dependent manner. The tested drugs showed anticancer activity with half maximal growth inhibitory concentrations (IC₅₀) for MEX2R 65.738 µg/ml which was more potent than antitumor drugs methotrexate (MTX) that has cell cycle arrest activity like investigational agent's action, IC₅₀ of MTX was about 93.574 µg/ml. The selectivity index of MEX2R compound (3.252) was higher than the selectivity index of MTX (2.65). **Conclusion:** From the overall results, MEX2R has highly significant cytotoxic effects on SW480 colorectal cancer cell line when compared with MTX, also MEX2R agent possessed safe profiles in Vero cell line as normal cells in comparison with standard chemotherapy MTX.

Keywords: Colorectal carcinoma, Oncogene blocker, Investigational agent MEX2R, SW480 cell line

INTRODUCTION

Colorectal cancer (CRC) represents the third most common type of malignancies in the world, the CRC begins as a benign adenomatous polyp in the colon and rectum, and it prevails initially into the colon wall and possibly metastasis into the lymphatic nodes and the other organs [1]. Relative studies in the Iraqi Cancer Registry from 1965-1994 appeared an elevated incidence of CRC from 25% to 50% with an incidence rate of 2.6% and the number of cases with CRC in 2011 was about 1086 per 100,000 with an increase in the incidence rate of about 3.26% [2,3].

The genetic and epigenetic alterations were common in CRC, activation of proto-oncogene K-RAS and inactivation of some tumor suppressor gene as APC and P53 were responsible for genetic instability, in addition to the abnormal promoter of DNA methylation and histone modification as epigenetic instability [4].

The most frequently mutated gene in CRC is K-RAS, it is responsible for about (40%-45%) of CRC [5]. Additionally, it provides resistance to some treatment that was used in CRC management like epidermal growth factor receptor

inhibitors due to the presence of a mutation in K-RAS gene, it stimulates intensive attempt in developing target RAS inhibitors for cancer treatment [6,7]. The K-RAS proteins show the main role in human cancer but have not produced to therapeutic attack. New skills in drug discovery and insights into signaling pathways that K-RAS controls have stimulated improved efforts to develop therapies, either through direct K-RAS targeting, new ways of blocking K-RAS processing, these agents act by inhibiting GTPase activity of K-RAS through binding to distinguishing pocket on K-RAS and have similar strategy to recognize a GDP analog as SML-8-73-1, and its prodrug derivative SML-10-70-1, which are specific. Direct inhibitors covalently bind to G12C mutant K-RAS and they are under investigation or by identifying targets that K-RAS cancers depend on for survival [8]. Those agents targets RAS-membrane interaction and act by suppressing its post-translational modifications like prenyltransferase inhibitors (PTIs); one that inhibits farnesyltransferase (FTase) enzyme which suppresses the proper plasma membrane attachment through inhibition of the prenylation of K-RAS as Farnesylthiosalicylic acid (Salirasib) that mimics the RAS farnesylcysteine and selectively disturb the binding of persistently active RAS proteins with plasma membrane but these agents do not exhibit clinical efficacy as single agents [9]. Another one that inhibits geranylgeranyltransferase (GGTI) as GGTI-2417 and GGTI-2418, has failed to give clinical efficacy as single-agent and when combined with FTIs, they led to toxicities in clinical trials illegal further clinical development [10]. While drugs that block the well-established downstream pathways, RAF-MAPK, and PI3 kinase, are being tested in the clinic and their effects may differ according to the type of tissue and even the specific mutational variant [11,12]. On the other hand, resistance to these downstream inhibitors has been ascribed to negative feedback mechanisms and reflexive activation of other downstream signaling partners of RAS in addition to their severe side effects [13,14]. In this study, MEX2R investigational agent as K-RAS oncogene blocker which inhibits the cell proliferation by targeting the cell cycle, and tested the cytotoxicity effect in human colorectal cells.

Methotrexate

Methotrexate (MTX) is a cytotoxic chemotherapeutic agent, as it acts on cell cycle [15]. We choose it to compare its action with our investigational agent (MEX2) as MEX2 also act on cell cycle. MTX act by limiting the synthesis of thymidine and purine nucleotides and interferes with DNA synthesis via inhibiting the dihydrofolate reductase enzyme [6,16]. This antifolate agent has severe adverse effects on the bone marrow and the intestinal epithelium so there is a risk for spontaneous hemorrhage and life-threatening infection.

MATERIALS AND METHODS

Chemicals and Reagents

MEX2R (investigational agent), methotrexate, penicillin/streptomycin purchased from Sigma-Aldrich (USA), RPMI-1640 media (US Biological, USA), is supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Germany), Crystal violet stain (Sigma-Aldrich, USA). All other agents were of analytical grade and used as received.

Cell Lines and Drugs Treatment

SW480 colorectal cancer cell line and Vero cell line were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured in 95% humidified atmosphere with 5% CO₂ at 37°C. The cells were incubated with drugs for 36 hours and the attached cells were harvested for subsequence analysis.

Crystal Violet Stain Assay

Dose-response curve was made to observe the cytotoxic activities of investigational agent MEX2R and MTX, in cancer SW480 cell line and normal Vero cell line by crystal violet stain assay as following several doses of drugs were prepared in concentrations (15.625, 31.25, 62.5, 125, 250 and 500) µg/ml, and it was used to determine the concentration that inhibits 50% of the cells viability IC₅₀ of the tested drugs in these cell lines.

Crystal violet dye binds to proteins and DNA and stains the living attached cells after treatment with the different concentrations of tested drugs in 96-well plates which was performed at 36 hours. The medium with tested agents was

aspirate. The wells were washed with PBS. Later, 100 µl of staining solution (0.5% crystal violet and 20% methanol) were added per well. Plates were washed 4 times by dipping in tilt way in streaming tap water after incubation for 20 min at room temperature with gentle shaking. To remove the remaining liquid, the plates were inverted on filter paper and tap gently. Plates were air dried, and 200 µl methanol was added to each well and incubated for 30 min at room temperature. The optical density (OD) of each well was measured by a plate reader (HumaReaderHS) at 570 nm. The OD of control cultures was normalized to 100% and compared with treated cells. The experiment was performed thrice and the average OD value of the treated cells was compared with the average of untreated cells [17]. The percentage of inhibition was calculated by using the following formula [18].

$$\text{Cell Growth Inhibition}\% = 100 - \left(\frac{\text{the optical density value of test well}}{\text{the optical density value of control well}} \right) \times 100$$

Selectivity index (SI) is an important parameter in the cytotoxicity studies; it elucidates the selectivity of compounds against cancer cells relative to normal cells [19]. The SI was calculated by the ratio between IC50 of the each tested drugs in normal Vero cell line and IC50 of the same drugs in SW480 colorectal cancer cell line. SI value greater than 2 was considered highly selective to cancerous cells over normal cells [20].

Statistical Analysis

Statistical analysis was performed using one way ANOVA by using IBM SPSS version 20 computer program. Data were expressed as means ± S.E and the p-value less than 0.05 were considered significant. Halves inhibitory concentrations were determined by blotting of inhibition percentages versus the concentration of any compounds used.

RESULTS

In Vitro Cytotoxicity Assay

Effect of MEX2R cytotoxicity on SW480 and Vero cell line by crystal violate assay: There was a significant increase ($p \leq 0.05$) in inhibition of growth of SW480 cells when treated with the concentrations of MEX2R (15.625, 31.25, 62.5, 125, 250 and 500) µg/ml for 36 hours. As compared with control group and the calculated IC50 of MEX2R about 65.738 µg/ml Vero cells show non-significant increase ($p \leq 0.05$) in cells growth inhibition as compared with the control group at the lower concentrations (15.625 µg/ml and 31.25 µg/ml) of MEX2R, while the higher concentrations (62.5, 125, 250 and 500) µg/ml of MEX2R give highly significant rise in growth inhibition percent as compared with the control group after 36 hours, and the IC50 of MEX2R in Vero cell of about 213.785 µg/ml (Table 1 and Figure 1).

Table 1 Cytotoxicity of MEX2R presented by Mean ± SE and GI% values by crystal violate assay

Cell line Conc. µg/ml	SW480			Vero		
	%GI Mean ± SE	p-value (vs. control)	IC50	%GI Mean ± SE	p-value (vs. control)	IC50
Control (untreated) 0.00	0.001 ± 0.57	-	65.738	0.001 ± 0.15	-	213.785
15.625	24.10 ± 1.45	0.001(HS)		8.21 ± 4.39	0.230 (NS)	
31.25	29.51 ± 0.95	0.001 (HS)		10.70 ± 1.71	0.124 (NS)	
62.5	47.80 ± 1.43	0.001(HS)		22.41 ± 5.82	0.004 (HS)	
125	58.89 ± 0.86	0.001 (HS)		47.78 ± 4.31	0.001 (HS)	
250	85.88 ± 0.56	0.001 (HS)		51.72 ± 6.60	0.001 (HS)	
500	91.83 ± 0.52	0.001 (HS)		64.87 ± 5.59	0.001 (HS)	
L.S.D	5.42			14.2		

G.I%: Growth inhibition percent; IC50: Half maximal inhibitory concentration; L.S.D: The least significant difference; NS: Non-significant; HS: High significant

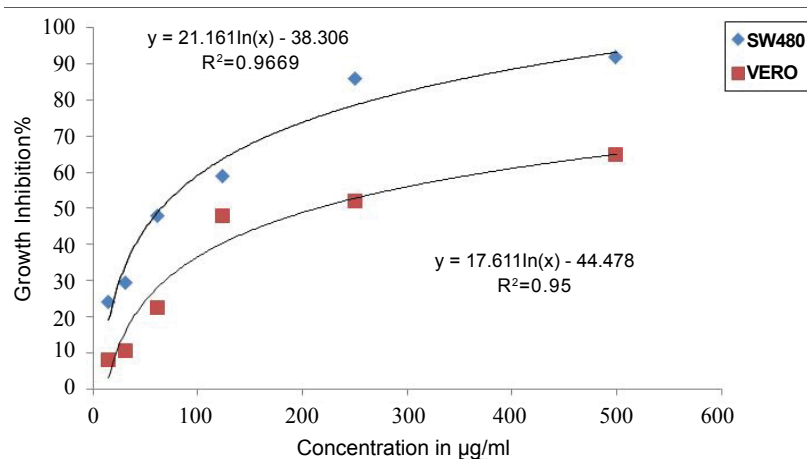


Figure 1 Dose-response curve of growth inhibition of MEX2R on SW480 and Vero cell lines presented by plotting of drug concentration versus Growth inhibition % values

Effect of MTX cytotoxicity on SW480 and Vero cell line by crystal violate assay: The MTX showed high significant difference ($p \leq 0.05$) in SW480 cells growth inhibition also for Vero cells when treated these cells with MTX concentrations (15.625, 31.25, 62.5, 125, 250 and 500) $\mu\text{g/ml}$ in comparative with control untreated cells and the IC50 of MTX were 93.574 $\mu\text{g/ml}$ and 247.872 $\mu\text{g/ml}$ in SW480 and Vero cells respectively (Table 2 and Figure 2)

Table 2 Cytotoxicity of MTX presented by Mean \pm SE and growth inhibition percent (GI %) values by crystal violate assay

Cell line	SW480			Vero			
	Conc. $\mu\text{g/ml}$	%GI Mean \pm SE	p-value (vs. control)	IC50	%GI Mean \pm SE	p-value (vs. control)	IC50
Control (untreated) 0.00		0.001 \pm 0.57		93.574	0.001 \pm 0.77		247.872
15.625	28.37 \pm 4.09	0.001 (HS)	20.35 \pm 1.64		0.001 (HS)		
31.25	44.62 \pm 1.49	0.001 (HS)	26.19 \pm 3.46		0.001 (HS)		
62.5	48.77 \pm 4.34	0.001 (HS)	32.55 \pm 4.26		0.001 (HS)		
125	52.42 \pm 7.46	0.001 (HS)	48.23 \pm 1.86		0.001 (HS)		
250	57.82 \pm 3.74	0.001 (HS)	51.67 \pm 1.95		0.001 (HS)		
500	64.82 \pm 4.58	0.001 (HS)	54.27 \pm 2.02		0.001 (HS)		
L.S.D	13.2				12.2		

G.I%: Growth inhibition percent; IC50: Half maximal inhibitory concentration; L.S.D: The least significant difference; HS: High significant

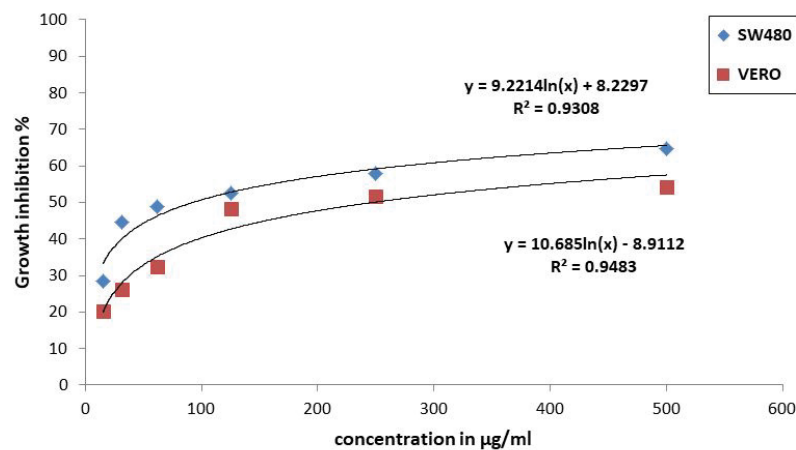


Figure 2 Dose-response curve of growth inhibition of MTX on SW480 and Vero cell lines presented by plotting of drug concentration versus Growth inhibition % values

Selectivity Index

The results in Table 3 showed that MEX2R has a selectivity index about 3.252 (SI>2), while it is 2.65 for MTX.

Table 3 Selectivity index of MEX2R and MTX

Drug	SW480	Vero	SI
	IC50 (µg/ml)	IC50 (µg/ml)	
MEX2R	65.738	213.785	3.252
MTX	93.574	247.870	2.650

IC50: Half maximal inhibitory concentration; SI: Selectivity index

DISCUSSION

The effect of MEX2R and MTX on SW480 and Vero cells is done by crystal violet assay. This procedure has been applied in proliferation and cytotoxicity studies to screen the new drug products potential on cell growth [21].

The results of MEX2R showed for the first time that the high growth inhibition percent of SW480 cells was about 91.83%, when the cells were treated with a higher concentration of 500 µg/ml in a dose-dependent manner and its IC50 was about 65.738 µg/ml when these cells were incubated with different concentrations of MEX2R (15.625, 31.25, 62.5, 125, 250 and 500) µg/ml for 36 hours and the anticancer outcome may be due to its action on mutant K-RAS oncogene by binding to docking site and it blocked its target K-RAS oncogene activity.

Generally, Bahrami, et al., and Porru, et al., mentioned the current strategies of targeting K-RAS in cancer carrying K-RAS oncogene which were useful to reduce the resistance for the used management and improved survival, like, Farnesyl transferase inhibitors were targeting K-RAS protein by suppressing farnesylation and results in cell growth arrest as they targeted the cell cycling and this is similar to the principle of MEX2R action [22-24]. An example of these drugs is Salirasib; it is S-trans-farnesylthiosalicylic acid that blocks RAS activation leading to inhibition of proliferation and induction of apoptosis but it is not effective sufficiently due to the presence of another way for activation of K-RAS which is geranylgeranylation so more efforts are exerted to develop new drugs that are effective in blocking K-RAS oncogene [25].

In this study, the MTX had mild cytotoxicity effect on SW480 cells and the MTX was not effective in producing SW480 complete cell death at the higher concentration used at 500 µg/ml dose after 36 hours of exposure, the growth inhibition percent was about 64.82%, with IC50 it was about 93.574 µg/ml. As this chemotherapy did not have an antitumor effect in colorectal cancer and it is needed to be combined with other agents to increase its antitumor effect *in vitro* as demonstrated by Jo, et al., [26]. Morales, et al., showed that the MTX caused cell growth inhibition in SW480 cells which were one of the colorectal cell lines that were tested in their study by inhibiting the dihydrofolate reductase enzyme [27], but the anti-proliferative effect of MTX may be dependent on time of exposure rather than concentration as founded by Huysentruyt, et al., study who showed that Cisplatin is more effective than MTX on (VM-M3) cultured cells and implanted mice [28]. Remarkably, the inhibition of cells proliferation for normal Vero cells by MEX2R and MTX was the first time examined in this line. MEX2R results may indicate that this compound has minimal toxicity to the normal cells as these cells did not contain K-RAS oncogene on which the test drugs acted when compared with SW480 cancer cells. While MTX results on Vero cells showed not markedly different with the results in SW480 cells with higher IC50 about 247.872 µg/ml. Al-Shammari, et al., mentioned that MTX did not have clear cytotoxic action in Vero cells and it is combined with other therapy to increase its effectiveness. MEX2R and MTX have selectivity indices above 2, but the SI for MEX2R was 3.252 that was higher than MTX which was 2.65, which may be related to the fact that cancer cells replicate faster than normal cells or due to the genetic load which makes cancer cells sensitive to common drugs more than the normal cells [29,30].

The high selectivity MEX2R for cancer cells in the present study increase the prospects that these compounds might be as novel anticancer drugs and further investigations will be required to validate them as a potential candidate to use in human cancer therapy.

CONCLUSION

From these results it can be concluded that MEX2R might be of greater value in the treatment of this type of cancer than MTX as they act on the cell cycle as the tested drugs MEX2R also acted on the cell cycle by targeting one of the

main troubles that interfere with treatment of CRC which is K-RAS gene mutation and inhibit the cell proliferation in addition to it was safer on the normal cells as it has higher selectively index than MTX.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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