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Original Research

The concurrent effect of acyclovir and rosemary on glioblastoma cell culture

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Abstract: Human cytomegalovirus (HCMV) is a beta herpesvirus which large amount of people in world has interacted with. Recent studies indicated that CMV DNA is associated with several cancer types including "Glioblastoma (GBM)" which is the most common and aggressive type of primary brain cancer. In clinical studies it was shown that several antiviral medicines prolonged life span of glioblastoma patients. One of them is Acyclovir (ACV) which is a type of nucleoside analog, used to cure viral infections and might be a potential treatment supplement for Glioblastoma. In this study we aimed to investigate if ACV had cytotoxic effect on glioblastoma cell line U87 MG and also the effect of ACV on healthy cells. Furthermore it was aimed to search the effect of *Rosmarinus Officinalis* also known as rosemary which is an aromatic, perennial plant concurrent with ACV on glioblastoma and healthy cells.

Key words: Human cytomegalovirus (HCMV); Acyclovir (ACV); Glioblastoma multiforme (GBM); Mouse Embryonic Fibroblast (MEF); Rosmarinus Officinalis.

Introduction

Human Cytomegalovirus (HCMV) is a beta-herpesvirus that affect large amount of people in the world (1). Almost nearly all adults in the developing countries and about 70% of adults in developed countries have been infected by HCMV. The HCMV infection may not be lethal for healthy individuals, but in the newborns and immunocompromised individuals HCMV can affect many organs and cause serious diseases that even may cause death (2–6). Besides that, many studies have shown that HCMV infects various cell types such as fibroblasts, hematopoietic, neuronal, smooth muscle, endothelial and epithelial cells and may be related with many serious diseases including cardiovascular diseases and cancers (7–9).

HCMV is a double-stranded DNA virus and has the largest known human virus genomic DNA to be about 230 kb in size (8,10). Several studies have mentioned that the HCMV DNA and viral mRNA could be found in many cancer types including gliomas despite the fact that HCMV isn't known as an oncovirus (11–14). Furthermore recent studies have indicated that HCMV has a role as oncomodulator on both tumor cells and microenvironment to promote immune escape, inflammations and angiogenesis (15, 16). One of the cancer types claimed to be caused by HCMV is Glioblastoma (GBM) which is the most common and aggressive type of primary brain tumor (1,17–19). GBM is highly invasive and have very poor prognosis. Unfortunately the life expectancy of the patients is not more than 12-16 months after the diagnosis (20–22). Furthermore it has been reported that HCMV may promote the GBM pathogenesis and may induce tumor growth and invasion (19, 20). Recent studies have reported that some antiviral medications have positive effects on GBM therapy (23, 24). It is thought that it may be related with the presence of HCMV genome in tumor cells (6, 7). One of the medications which is effective on HCMV is acyclovir (ACV) (25–27). Acyclovir is a type of nucleoside analog and well tolerated, widely used antiviral drug (28–32). For suppression of HCMV infection, acyclovir is effective and can be used in combination with other antiviral drugs to enhance the effectiveness (25–27).

Rosmarinus Officinalis also known as rosemary is an aromatic, evergreen, Mediterranean originated plant that belongs to Lamiaceae mint family (33–35). Rosemary is being used frequently in food and cosmetic industry and also in traditional medicine in the treatments of many diseases such as diabetes, inflammatory diseases, cancer etc. (36–38). Furthermore many studies have been reported that rosemary possesses antimicrobial, antioxidant, anti-inflammatory and anti-cancer properties and also it is effective on various cancer types including glioma (39–48).

The purpose of this study is to investigate the effect of *Rosmarinus Officinalis* concurrent with ACV on tumor and healthy cells. To investigate the cytotoxic effects MTT test was applied. mRNA level of an apoptosis-associated gene, survivin, was evaluated by real-time RT –PCR.

Materials and Methods

Preparation of Acyclovir solution and dose optimization

Acyclovir was supplied as powder for (Zovirax 250 mg, GlaxoSmithKline) and dissolved in apyrogenic wa-

ter. For 250 mg acyclovir, 5 ml apyrogenic water was used.

To be able to determine the optimum dosage of acyclovir on both GBM and MEF cells, on the second day of the culture, except the control group 250, 500 and 1000 µg/ml ACV was given to the cell cultures and to adjust to volume only apyrogenic water was given to the control group. After one day incubation MTT assays were applied. According to test results 500 µg/ml ACV was determined as optimum for the later of the study.

Preparation of the *Rosmarinus Officinalis* infusion and extraction analysis

Rosmarinus Officinalis infusion solution was prepared as described below. For 100 ml boiling double distilled water, 2 gr leaves of Rosmarinus Officinalis was added. It was sterilized by 22 µM pore sized filter. From our previous studies 1/75 (v/v) rosemary has already been determined as optimum for both MEF and GBM cells (48). It is the dose which has minimum cytotoxic effect on the MEF cells and maximum cytotoxic effect on the GBM cells.

To investigate the contents of the infusion solution the liquid-liquid extraction of *Rosmarinus Officinalis* infusion solution with dichloromethane, and gas chromatography–mass spectrometry (GC-MS) analysis was applied by using Agilent 7000 Series Triple Quad GC/MS. The conditions of GC-MS are listed on Table 1.

Cells and culture conditions

Studies were carried out with the Glioblastoma (GBM) U87 MG (ATCC® HTB-14™) and Mouse Embryonic Fibroblast (MEF) cell lines. U87 MG cells were provided by Asst. Prof. Tuba Bağcı Önder (Koç University, Faculty of Medicine, Bağcı Önder Laboratory) and MEF cells were provided by Prof. Devrim Gözüaçık (Sabancı Univetsity, Gözüaçık Laboratory).

For cell viability and gene expression assays non-contaminated cells were used. Their mycoplasma contamination status was checked by Hoechst 33342 nucleic acid stain. After ensuring that the cells were not contaminated, cell viability and gene expression studies were performed.

Cells were seeded into 24 well plates (40000 GBM cells and 10000 MEF cells for each well) and cultured with Dulbecco's Modified Eagle's medium (DMEM, Lonza, Belgium) supplemented with 10% fetal bovine serum (FBS, South America) in a humidified incubator at 37 °C in which the CO_2 level was kept constant at 5%.

Cell viability assay

On the second day of the culture both U87-MG and MEF cells were exposed the 1/75 (v/v) rosemary and 500 µg/ml ACV for 24 hours. After the end of incubation periods cytotoxic effects were measured by MTT cell viability assay. Experiment was repeated 6 times.

The MTT assay was applied to be able to determine the cell viabilities. After incubation periods, growth medium was taken away and 500 µL (0,25 mg/lt) of MTT solutions was transferred to each well. Cells were incubated for nearly 2 hours and after the purple MTT formazan crystals formed the MTT solution was taken away. 1000 µL MTT solubilisation solution (isopropanol) was used to dissolve purple formazan crystals and then waited for 1 hour. After that the absorbance values were measured by spectrophotometer at 570 nm (Shimadzu UV-VIS, UVmini-1240, Japan). Cell viabilities were calculated with respect to control groups in percentage of cell viability.

For statistical analysis p values were calculated by using student's t test and confidence intervals (95%) were determined and indicated in the graphic as error bars.

Expression analysis of survivin gene

For expression analysis of an apoptosis related gene (Survivin); RNA isolation, cDNA synthesis and Real Time PCR was performed.

For RNA isolation U87-MG (GBM) cells were seeded in to 100 x 20 mm petri dishes and cells were exposed to (except the control group), 1/75 (v/v) rosemary, 500 µg/ml ACV, 1/75 (v/v) rosemary & 500 µg/ml ACV combination for 1 day. After 1 day incubation cells were collected and total RNA was isolated by RNA isolation kit (GE Healthcare Illustra RNAspin Mini RNA Isolation Kit cat.no: 25050070)

mRNA samples were converted to cDNA by using High-Capacity RNA-to-cDNA Kit (Applied Biosystems cat.no:4387406)

Beta-Actin and Survivin primers were synthesized

Table 1. The equipment and running conditions of GC-MS used for analysis of *Rosmarinus Officinalis* infusion solution.

	Equipment and running conditions
GC conditions	
Column	HP - 5MS (30 m length x 0,25 mm inner diameter x 0,25 µm film thickness, %5 phenyl methyl poly siloxane)
Column Temperature	50°C-240°C (inc. 3 °C/min)
Injector	Gerstel multipurpose sampler
Injection volume	1µL
Inlet temperature	250 °C
Carrier Gas	Helium (flow rate, 1 mL/min)
Split Flow	40 ml/min
Split Ratio	20
Mass spectrum	
Source tempature	290 °C
Electron Energy	70 eV
Mass Spectra	50-600 m/z

by Sentegen Biotechnology. The sequences of the primers were: 5-GTGGACATCCGCAAAGAC-3 (Beta-actin forward primer), 5- AAAGGGTGTAACGCAACTA-3 (Beta -actin reverse primer), 5-TCCACTGCCCCACTGAGAAC-3 (survivin forward primer), 5 -TGGCTCCCAGCCTTCCA-3 (survivin reverse primer).

For real time PCR the following method was applied;

25 µl PCR mixture for Beta actin amplification included 12,5 µl of Master Mix (Qiagen Cat. No: 204141), 2,5 µl primer mix (final concentration 500 nM for each), and 10 µl cDNA and 25 µl PCR reaction mixture for survivin amplification included 12,5 µl of Master Mix (Qiagen Cat. No:204141), 2,5 µl primer mix (final concentration 500 nM for each), and 10 µl cDNA was prepared.

The PCR cycling parameters were, 1 cycle of 95 °C for 15 minutes (min), 40 cycles of 95 °C for 15 seconds (s) and 60 °C for 60 s.

Results

Rosmarinus Officinalis extraction analysis results

According to GC-MS analysis the chemical composition of Rosmarinus Officinalis extract which is illustrated in Table 2 was revealed. The major components were Levoverbenone (34,88 %), (+)-2-Bornanone (19,56 %), Endo-Borneol (15,35 %) and Eucalyptol (6,88 %), respectively.

Cell viability assay results

MTT assay results indicated that if they were used separately rosemary and 500 µg/ml ACV reduced the viability of U87-MG (GBM) cells to approximately 57,2% and %52,7 respectively. 500 µg/ml ACV & rose-

Table 2. GC-MS analysis of *Rosmarinus Officinalis* extract.

Name	RT (minutes)	Score	Height	Area	Area %
2-Methylenecyclohexanol	5,33153	73,8	424876	1707462	0,24
Cyclopentane, 1,2,3,4,5-pentamethyl-	6,30243	87,2	2281952	5475146	0,76
9-Octadecenoic acid (Z)-, tetradecyl ester/4-Hexen-1-ol, 2-ethenyl-2,5-dimethyl-	6,3669	75,9	596802	1481011	0,21
Cyclohexane, 1,1'-dodecylidenebis(4-methyl-/3-Heptene, 4-propyl-	6,45792	77	536987	1284306	0,18
2,4,4-Trimethyl-1-hexene/Octane, 3-methyl-6-methylene-	7,14438	86,8	3818291	9179933	1,27
m-Menthane, (1S,3R)-(+)-/Bicyclo(3.3.1)nonan-1-ol	7,2354	81,6	945940	2954282	0,41
Octane, 3-methyl-6-methylene-	7,37193	83,6	1261564	3131044	0,43
2,6,6-Trimethyl-bicyclo(3.1.1)hept-3-ylamine	8,27837	79,58	386923	1484354	0,21
Eucalyptol	10,16327	98,01	17841146	49661241	6,88
trans-Sabinene hydrate	11,58548	79,24	746680	2997784	0,42
Fenchone	12,44828	83,66	945711	3794784	0,53
γ-Terpinene	12,856	86,64	553666	1654959	0,23
Linalyl acetate	12,96978	92,85	6047876	19958927	2,77
N,N'-Bis(Carbobenzyloxy)-lysine methyl(ester)/ Bicyclo(8.2.0)dodeca-3,7-diene, 11,11-dimethyl-	13,09872	80,95	440623	1742399	0,24
Chrysanthenone	13,98998	90,95	2182262	7474954	1,04
10,13-Octadecadiynoic acid, methyl ester/Bicyclo(3.1.0) hexan-3-ol, 4-methylene-1-(1-methylethyl)-, acetate	14,5399	83,19	636277	2517976	0,35
Bicyclo(3.1.0)hex-3-en-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-	14,66505	88,81	1635846	5455789	0,76
(+)-2-Bornanone	14,77125	95,83	39567274	141132185	19,56
Isopinocarveol	15,48045	83,13	700220	2311313	0,32
endo-Borneol	15,70422	91,08	29095903	110725886	15,35
3-Pinanone, cis	16,05692	90,44	4939625	15769380	2,19
Terpinen-4-ol	16,21242	85,15	1655880	5733443	0,79
Crypton/1-Phenyl-1-butene	16,5765	87,92	1694699	5965119	0,83
3-Carene	16,80025	90,61	4843407	19963292	2,77
(-)-Myrtenol	17,05691	85,78	628745	2889023	0,40
p-Mentha-1,8-dien-7-ol	17,38432	83,83	848737	3095817	0,43
Levoverbenone	17,59668	97,15	72029891	251649731	34,88
1-Isopropenyl-3-propenylcyclopentane	18,74583	83,14	1026769	4846448	0,67
7-Propylidene-bicyclo(4.1.0)heptane	18,98867	86,24	2075707	11217071	1,55
D-Verbenone	20,27282	75,65	627701	2388203	0,33
Acetic acid, 1,7,7-trimethyl-bicyclo(2.2.1)hept-2-yl ester	20,91518	87,28	914879	3331905	0,46
Cyclododecane	25,40938	83,77	588068	2225742	0,31
1-Octanol, 2-butyl-	56,04972	74	257101	2607151	0,36

mary combination reduced the viability of GBM cells nearly 10% more (Figure 1). Statistically, experimental groups were significantly different from the control group ($p < 0.01$) according to student's t test. The cell culture images are correlated with these results (Figure 2).

Besides this it was observed that if rosemary and ACV were used separately they have no meaningful cytotoxic effect on the MEF cells. But if they were used in combination the results indicated that they have statistically meaningful cell number decreasing effect ($p<0,01$).

Cells were exposed the 1/75 (v/v) rosemary (RM) and 500 µg/ml Acyclovir (ACV) for 24 hours and according to viability assays rosemary increased the effect of acyclovir on GBM cells.

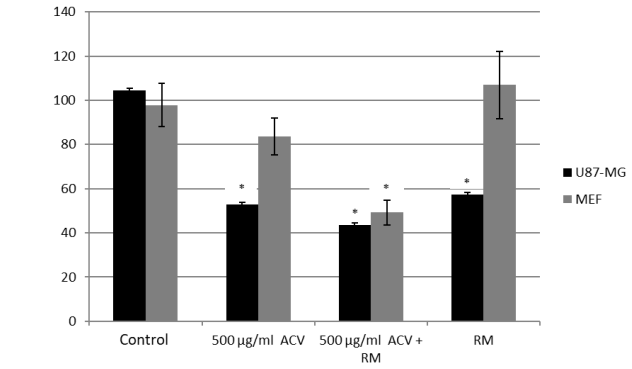

Figure 1. The MTT assay results of rosemary (RM) concurrent with acyclovir (ACV) on U87-MG (GBM) and MEF cells. n=6 per group; *, $p < 0,01$ compared with the control groups.

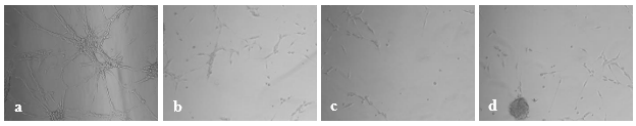

Figure 2. U87-MG (GBM) cell images taken by invert microscope (10X). a)Normal cells (control group), b)500 µg/ml ACV given cells, c) 1/75 (v/v) rosemary and 500 µg/ml ACV given cells, d) 1/75 (v/v) rosemary given cells.

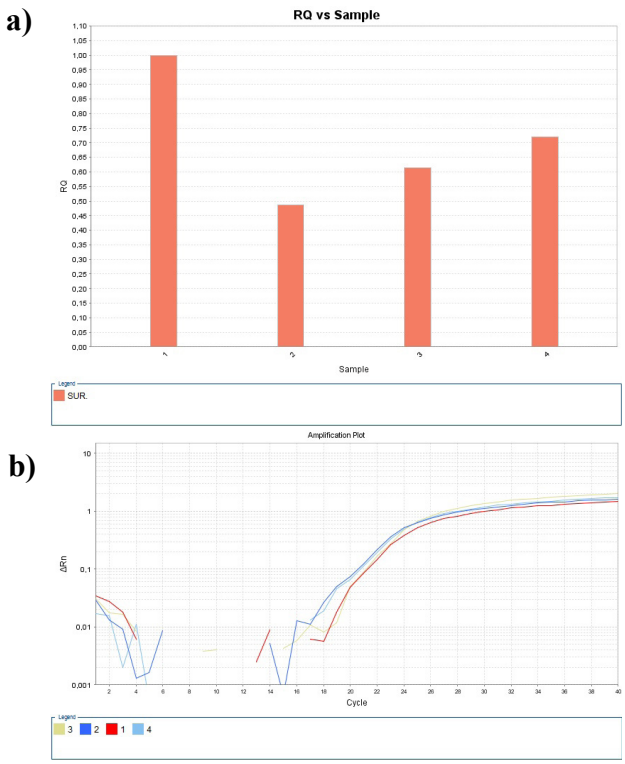

Figure 3. Application of survivin qPCR to rosemary and acyclovir treated U-87 MG cells. Difference in transcriptional activity of only rosemary (2), 1/75 (v/v) rosemary and 500 µg/ml ACV (3) and only 500 µg/ml ACV (4) treated U-87 MG cells, compared to non-treated (1) cells. (a) The relative expression (fold) of differentially expressed genes; (b) qPCR amplification plots. PCR: polymerase chain reaction.

Survivin Real Time PCR results:

Survivin gene expression profile were evaluated comparing Ct values between rosemary and acyclovir treated and non-treated cells. Only rosemary (2), 1/75 (v/v) rosemary and 500 µg/ml ACV (3) and only 500 µg/ml ACV (4) treated cells showed a clear decrease of survivin expression, the highest decrease was observed in the only rosemary treared (2) group (figure 3a and b).

Discussion

According to MTT assay on GBM cells and microscopic investigations of cell culture test groups, it was observed that ACV, rosemary and their combination reduced the cell viabilities (Figure 1, 2) Besides this cytotoxicity study, an apoptosis related gene, survivin, expression level was compared by real time PCR. The results of gene expression analysis indicated that survivin expression was clearly decreased in the rosemary, rosemary & ACV combination and only ACV treated GBM cells, the highest decrease was observed in the only rosemary treated group (Figure 3a and b). These results supported the cell viability test findings. Because survivin is an anti-apoptotic protein and it is a direct inhibitor of caspase 3 and 7 (49, 50) and it is known that down regulation of survivin expression is related to the cytotoxic conditions (51, 52).

According to previous study it was observed that while increased quantities of Rosmarinus Officinalis infusion solution induced MEF cell proliferation, it inhibited the survival of GBM cells in three and five days (48). In this study the same effect of Rosmarinus Officinalis infusion solution was observed in one day.

From literature it is already known that antiviral medications are effective on glioblastoma therapy probably because of the presence of the HCMV DNA in the genome of glioblastoma cells (1, 2, 24). Söderberg et al. reported that the survival rate of patients who received antiviral medications concurrent with their treatment, was increased (24). However the cell line we used in our study isn't infected with HCMV, which was confirmed by qPCR. According to qPCR results the entire HCMV genome isn't present on cells genome. Thus the effectiveness of ACV on this cell line is interesting. Despite the cell line doesn't contain HCMV genome the antiviral medication ACV is effective on these cells. On the other hand, the cell viability assays on the MEF cell culture indicated that ACV reduced the viability of MEF cells but it was lower than the cytotoxic effect on the GBM cells (Figure 1).

The cell viability assay results indicated that rosemary enhanced the effectiveness of ACV on both MEF and GBM cells (Figure 1). Indeed, due to proliferative effect of rosemary on MEF cells (48), rosemary was expected to protect MEF cells from the side effects of ACV. But

no protective effect was observed when it was used with ACV. Rosemary reduced the number of both GBM and MEF cells both. However the cell number decreasing effect on the GBM cells was higher than the effect on the MEF cells.

In conclusion, the obtained results indicated that ACV decreased the viability of GBM cells. In addition to that, when rosemary was used concurrent with ACV, they eliminated the tumor cells more than ACV did alone. But in combination it damages the healthy cells (MEF cells) too (Figure 1). ACV was effective on GBM cells but we know that, from our real time PCR results, this cell line isn't infected with HCMV. It is considered that entire HCMV DNA doesn't exist in GBM cell genome, but its fragments may exist.

From our preliminary data it can be deduced that ACV and rosemary may have a possible role for positive contribution to effectiveness of GBM pharmacotherapy if they are used in different times.

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Interest conflict
The authors declare that they have no conflict of interest.

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