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Influence of serum and albumin on the *in vitro* anandamide cytotoxicity toward C6 glioma cells assessed by the MTT cell viability assay: implications for the methodology of the MTT tests

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Abstract

Anandamide (AEA), an endogenous ligand of cannabinoid CB1 and CB2 receptors, which also binds transient receptor potential vanilloid type 1 receptor (TRPV1), has been shown to display substantial selective cytotoxicity toward some cancer cell lines in vitro, although the relevant data are not consistent. In the present study, we employed the MTT test to assess short-term cytotoxicity of AEA on C6 rat glioma cell culture. When anandamide was administered to the culture medium with foetal bovine serum (FBS), no cytotoxic effect was observed following 24 h exposure of the glioma cells to micromolar concentrations of AEA. However, if no serum was present in the medium, micro-to-submicromolar concentrations of AEA induced dose-dependent cytotoxicity clearly detectable after 24 h. Control experiments made it possible to exclude significant interference of serum with the MTT test per se. Bovine serum albumin mimicked the effect of FBS. We conclude that the apparent inhibition of short-term cytotoxicity of AEA toward C6 rat glioma cells in vitro is caused by binding AEA to serum proteins such as albumin. Taking into account that blood serum or albumin is practically always present in cell culture media, we discuss implications of binding substances to serum proteins for methodology and interpretation of in vitro cytotoxicity testing.

Key words: *anandamide, glioma C6, serum, albumin, cell culture, cytotoxicity.*

Introduction

The endocannabinoid system consists of cannabinoid (CB) receptors, endogenous lipid signalling molecules generated in the cell membranes from phospholipid precursors termed endocannabinoids and the enzymes that produce and degrade these natural ligands. Two major types of cannabinoid receptors, CB1 and CB2, are G protein-coupled membrane metabotropic receptors expressed predominantly but not exclusively in the central nervous system and peripheral immune system, respectively. The endocannabinoid system is implicated in a variety of physiological and pathological processes such as control of behaviour, control of energy expenditure, inflammation, analgesia, cancer, etc. Two best studied endo-

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cannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [21].

Anandamide is the first identified endocannabinoid [10] which acts on both CB1 and CB2, receptors [14]. Anandamide also binds transient receptor potential vanilloid type 1 receptor (capsaicin receptor, $TRPV_1$), a non-selective cation channel activated by a variety of physical and chemical stimuli [33]. This prototypic substance has been reported to exert substantial antiproliferative and proapoptotic activity toward various cancers [see, for example: 1, 9, 12, 17, 18, 27]. For *in vitro* studies investigators used different cancer cell cultures, employed various methods and protocols to estimate AEA antiproliferative and/or cytotoxic properties, and identified involvement of different receptors in anticancer activity of this compound. For example, Mimeault *et al.* [30] have shown that micromolar concentrations of AEA exert strong inhibitory effects on EGF-stimulated proliferation of prostate carcinoma cells by acting through CB_1 receptors, whereas according to Contassot *et al.* [5], micromolar concentrations of AEA induced apoptosis in the long term and recently established glioma cultures acting on $TRPV_1$, but not CB_1 and/or CB_2 receptors. Diversity of results is explained by complexity of mechanisms through which cannabinoids and their receptors impact proliferation and apoptosis of cancer cells, and by different reactions of various types of cancer cells [16].

One of the more frequently used experimental model systems for malignancy is rat C6 glioma. This rapidly proliferating cell line, originally obtained by exposing the rat to *N,N*'-nitrosomethylurea, presents several features similar to human glioblastomas and enables both *in vitro* and *in vivo* experiments [15]. The effects of AEA on rat C6 glioma cell line have been repeatedly investigated [3,7,23-26] and in several of these studies the MTT cell viability assay was used. Although numerous alternatives have been proposed, the MTT test (introduced by Mosmann in 1983) is still much in use for cellular chemosensitivity assays [34]. Considering its extreme simplicity, it might have been expected that the MTT test should be a robust and dependable tool for assessing viability of cell cultures exposed to AEA. However, even these studies resulted in discordant results.

In the present study we report on our attempts to standardize the MTT cell viability assay and use it to characterise the *in vitro* response of the C6 rat glioma cell line to short-term, 24h exposure to AEA. Results of our experiments suggest some general remarks concerning methodology and interpretation of the *in vitro* cytotoxicity tests.

Material and methods

Cell culture

Rat C6 glioma cells were obtained from the American Type Culture Collection (Manassas, VA) and used over a passage range of 2 to 15. Cells were cultured in Petri's dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 in air and the medium was changed every 3 to 4 days.

MTT assay

To determine the effects of anandamide upon cell viability, we measured the redox-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich) – experimental scheme in Fig. 1. C6 glioma cells were seeded in 96-well flat bottom microplates at a density of 2 × 104 cells per well in DMEM supplemented with 10% FBS and penicillin-streptomycin. After 24 h incubation at 37°C, the medium was removed and a new culture medium containing anandamide (Tocris Bioscience, UK) was added (assay volume 200 µl). The cells were then further incubated at 37°C for 24 h, after which 20 µl (5 mg/mL) solution of MTT dissolved in PBS and in medium with or without FBS was added to each well following Table I and Fig. 1. After 3 h of incubation media were removed

Table I. Five different experimental protocols of cytotoxicity assay (MTT) for anandamide (AEA) administered to C6 glioma cells culture depending on FBS (10%) or albumin (60 µM) presence in medium

Protocol	Cell culture medium	AEA	MTT
1	FBS +	FBS –	FBS –
2	FBS +	FBS –	FBS +
3	FBS +	FBS +	FBS –
4	FBS +	FBS +	FBS +
5	FBS +	FBS –/albumin +	FBS –/albumin +

and MTT metabolic product – formazan was dissolved in 200 µl DMSO and plate was placed on a shaking table for 15 minutes. Absorbance at 570 nm was measured by using Epoch microplate reader (BioTek). Experiments were performed according to five protocols shown in Table I and experimental scheme in Fig. 1.

Statistical analysis

The concentration-dependent effects of AEA upon C6 cell proliferation were analysed for statistical significant differences between each concentration and the corresponding control data using one-way analysis of variance (ANOVA) with post hoc Duncan's test. The influence of the presence of serum or albumin in medium on the cytotoxic effect of AEA was analysed using two-way analysis of variance (ANOVA) with post hoc Duncan's test. All data are presented as the means ± SEM.

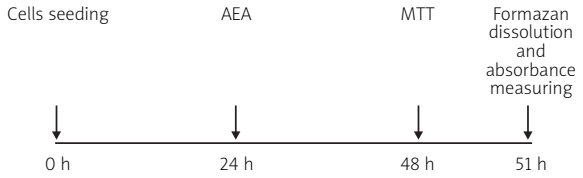

Fig. 1. Experimental scheme: 24 h after cells seeding, different doses of anandamide were administered to C6 glioma cell line. 24 h after AEA challenge, MTT was added and 3 h later, formazan dissolution was given to have redox colour reaction. Then, absorbance was measured.

Results

Protocol I

The activity of anandamide in C6 glioma cells while the drug was administered in DMEM without serum and MTT was added in DMEM without serum has been found cytotoxic in all concentrations in a dose-dependent manner (Fig. 2A).

Protocol II

In the case of anandamide administered in DMEM without serum and MTT added in DMEM with serum, the drug was cytotoxic in all concentrations in a dose-dependent manner similar to the result obtained in Protocol I (Fig. 2B).

Protocol III

Anandamide administration in DMEM with serum and MTT addition in DMEM without serum did not induce any cytotoxic effect (Fig. 3A).

Protocol IV

Anandamide given in DMEM with serum while MTT was administered in DMEM with serum, did evoke no cytotoxic effect, whereas the opposite effect was observed – the greater number of viable cells after AEA treatment at a dose of 3.75 µM (Fig. 3B).

Protocol V

There was no cytotoxic effect following AEA administration in DMEM with albumin (Fig. 4).

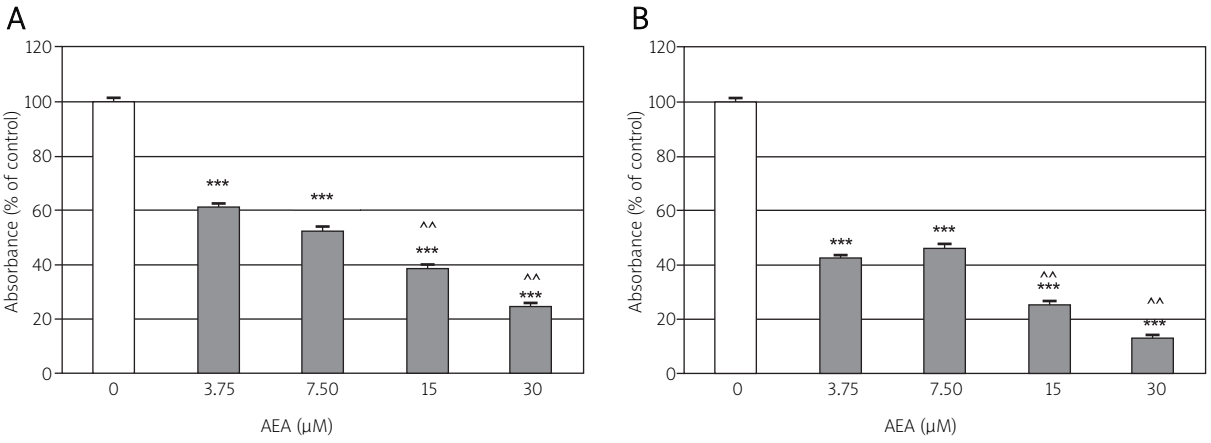

Fig. 2. Dose-dependent cytotoxicity of AEA in glioma C6 cells after 24 h. Cells treated with 3.75-30 µM AEA in DMEM (**A**) without FBS and MTT without FBS or (**B**) without FBS and MTT with FBS. Statistical significance: ***$p < 0.001$ vs. control; $n = 12$. Note the statistically significant differences (^^$p < 0.01$) between particular AEA concentrations excluding insignificant difference between 3.75 and 7.5 µM doses of AEA.

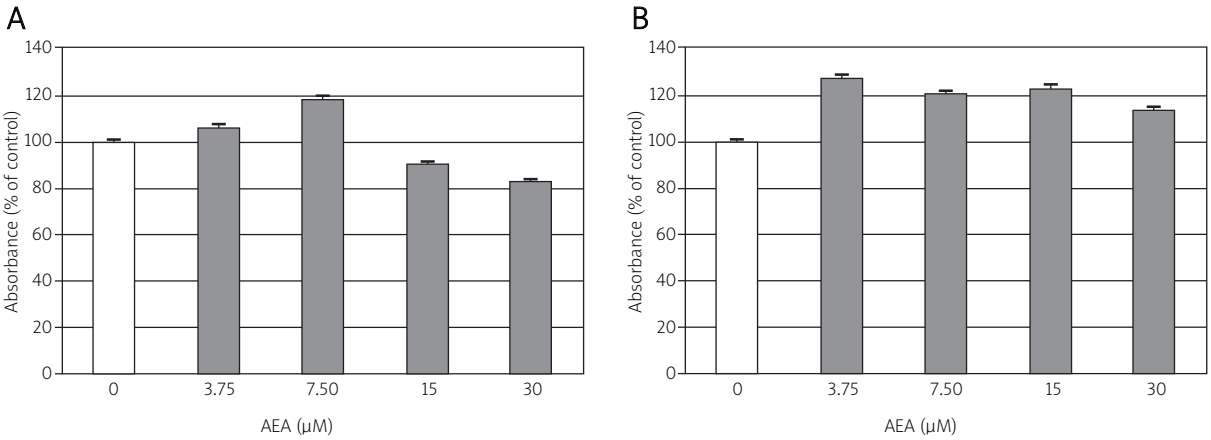

Fig. 3. Non-cytotoxic effect of AEA administration on glioma C6 cells after 24 h. Cells treated with 3.75-30 μM AEA in DMEM (**A**) with FBS and MTT without FBS or (**B**) with FBS and MTT with FBS; n = 12. Note the statistically insignificant trend of an absorbance increase in comparison to control values. It may reflect undisturbed cells proliferation in the culture by elimination of AEA cytotoxicity with binding between AEA and serum components and in this case the cells undergo the normal proliferation process. It cannot be excluded that the reason for the increase in absorbance is the phenomenon of hormesis – dose-response relationships characterised by a reversal of response between low and high doses of chemicals, biological molecules, physical stressors, or other initiators of a response.

Influence of serum or albumin presence in the medium on cytotoxicity of anandamide in C6 glioma cells

The present study showed a significant dose-dependent cytotoxic effect of AEA on C6 glioma cells ($p < 0.001$) in experiments when medium without serum was used. The experiments demonstrated a significant influence of the presence of serum in the medium on the occurrence of cytotoxicity of AEA in C6 glioma cells ($p < 0.001$). Our study indicated a significant interaction between the cytotoxic effect of AEA on C6 glioma cells and the presence or absence of serum ($p < 0.001$) or albumin ($p < 0.001$) in the medium.

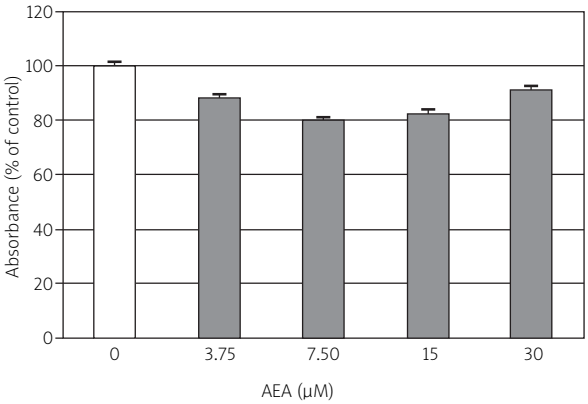

Fig. 4. Effect of AEA administration in glioma C6 cells after 24 h. Cells treated with 3.75-30 μM AEA in DMEM with albumin and MTT with albumin; n = 8. Note the statistically insignificant trend of an absorbance increase in comparison to control values. It may reflect undisturbed cells proliferation in the culture by elimination of AEA cytotoxicity with binding between AEA and albumin.

Discussion

Tetrazolium salts are widely used to assess *in vitro* cytotoxicity of candidate anticancer drugs and other bioactive substances. The popularity of this assay lays in its unsurpassed simplicity and low cost. In our studies we have chosen the MTT test assuming that it would enable us to obtain reproducible results. We have made a choice of a relatively short (24 h) incubation time of the cell culture with AEA, because there is an indication that cytotoxicity of AEA toward the C6 rat glioma is evoked by $TRPV_1$ receptors. Capsaicin, the agonist of $TRPV_1$ receptor, was markedly cytotoxic toward C6 glioma cells [2]. In the other malignant cell model system (Jurkat leukemia line) it has been shown that capsaicin $TRPV_1$-mediated cytotoxicity is the consequence of an increase in intracellular production of free radicals, which is full-blown already 6 h after capsaicin challenge [28].

Our first and a bit surprising finding was that when AEA was added to the culture medium, after 24 h no decrease in the MTT-related optical density was detectable. There was no dose-response relationship, and in fact the readings were in some cases suggestive of an increase in MTT dye due to the addition of AEA to the cell culture. These observations prompted us to critically review the MTT method which we were using.

Several potential sources of errors in the MTT cell viability assay have been identified. For example, Marshall *et al.* [29] pointed out that changes in cell culture environment, such as glucose depletion or change in pH of the medium, may influence the reduction of tetrazolium salts independently of changes in cell viability. However, in our experiment such effect should be minimized by a relatively short incubation time.

Es-Sady *et al.* [11] and Pagliacci *et al.* [32] reported that alterations in cell cycle phase redistribution by compounds under study (ursolic acid and genistein, respectively) significantly influenced the mitochondrial number and/or function, and in this way influenced MTT reduction to formazan independently of the effect on cell viability. A similar mechanism should not be *a priori* excluded in the case of AEA, as it has been shown that nanomolar concentrations of AEA (and some other cannabinoids as well) appeared to promote mitogenic kinase signalling and induce proliferation of model cancer cells, including a glioblastoma and a lung cancer cell line [20].

On the other hand, Zhang and Cox [35] observed a 20% higher MTT signal when viability of airway smooth muscle cells culture were assayed in 10% foetal calf serum (FCS) compared with 5% FCS. Huang *et al.* [22] reported that major contributions to tetrazolium salt reduction by common culture media such as albumin and fatty acids can obscure changes in the cell number during MTT and similar assays. Funk *et al.* [13] found that reductive activity of human serum albumin produced concentration-dependent increase in the MTT signal, although it should be mentioned that such effect did not occur when bovine serum albumin was used.

Considering that interference of blood serum and/or its major constituent albumin appeared to be the most frequently mentioned potential source of erroneous results in the MTT cell viability assay, we conducted experiments aimed at determining whether the presence of FBS influences results of the assay, and if it does, whether interference occurs during incubation of cells with AEA, or during reduction of formazan. The results of our experiments unequivocally indicate that when serum is absent from the medium, 24 h exposure to AEA is dose-dependently cytotoxic toward C6 rat glioma cells in a low-nanomolar concentration range. However, when serum is present, no reproducible cytotoxicity of 24 h exposure to AEA in concentrations up to 10 micromoles is detected. Spectacular inhibition of AEA cytotoxicity by FBS is reproduced by substituting it with an equivalent amount of bovine serum albumin. The effect of serum (or albumin) during 24 h incubation with AEA is of such magnitude that any possible interference of serum during development of MTT signal is of secondary importance.

Blood serum is currently added to virtually every mammalian cell culture [6]. The most widely used serum supplement is foetal bovine serum (FBS), a particularly rich source of hormones, growth factors and trace elements that promote rapid cell growth. Serum has undefined and variable composition, but it is always rich in albumin. What is of importance for the MTT assay, and perhaps for other *in vitro* viability and/or cytotoxicity assays as well, is the fact that albumin reversibly binds numerous endogenous and exogenous compounds. This in particular concerns water-insoluble compounds such as AEA which avidly binds to albumin, similarly to fatty acids [4]. When AEA is added to culture medium containing albumin, the concentration of free AEA able to interact with $TRPV_1$ receptors may decrease significantly, even up to 100 times. In this context particularly interesting is the case of sphingosylphosphorylcholine (SPC). Similarly to AEA, SPC is a lipid metabolite generated enzymatically from cellular membrane constituent (sphingomyelin), which acts as a ligand on some G-protein-coupled receptors. In a marked resemblance of our present results Han *et al.* [19] reported that exposure to SPC induced significant cytotoxicity in Jurkat cells, but both serum and albumin, by binding SPC, inhibited this cytotoxicity.

De Petrocellis *et al.* [8] demonstrated that the potency of anandamide at $TRPV_1$ receptor is markedly reduced in the presence of bovine serum albumin. The authors suggested that BSA prevents the uptake of anandamide, interfering with the carrier-mediated internalisation of this compound, and the subsequent activation of receptor, which they assumed occurs intracellularly. There is, however, little support to the idea that $TRPV_1$, membrane-spanning protein and ionic channel, is located intracellularly. It is worth mentioning that

experiments, in which stimulation of cancer growth by nanomolar concentrations of AEA was observed [20], were performed on serum-starved cells, therefore effective concentration of AEA at the receptor site should be comparable to 100 times higher concentration in experiments of others, in which serum was present in culture media.

Our finding that the presence of albumin may interfere with the MTT viability assay is important not only in the context of AEA cytotoxicity towards cancers, but it also have a bearing on the general methodology and interpretation of *in vitro* cytotoxicity testing. Interference of serum with viability and cytotoxicity assays has been alluded to in the case of LDH assay, considered another robust and cost-effective means of assessing cytotoxicity. Its susceptibility to background signal from LDH found in supplemented growth media has been indicated as possible explanation, but no reference to drug-serum proteins interaction was mentioned [31]. On the basis of our results we would recommend that for cell viability and cytotoxicity testing with the use of MTT and other *in vitro* tests, serum-devoid cell cultures and short times of exposure to tested compounds should be employed.

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