The non-competitive metabotropic glutamate receptor-1 antagonist CPCCOEt inhibits the *in vitro* growth of human melanoma

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Abstract. Five decades ago, the dicarboxylic amino acid glutamate became recognized as the major excitatory neurotransmitter in the central nervous system. In recent years, the expression of glutamate receptors was detected also in peripheral, non-neuronal tissues. Furthermore, it was found that glutamate stimulated the proliferation and migration of several peripheral tumor cells, and that glutamate receptor antagonists limited tumor growth. Most of these studies, however, used broad spectrum compounds and/or group-specific antagonists. Here we report that a selective, non-competitive metabotropic glutamate receptor-1 antagonist, CPCCOEt (7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester), significantly inhibited the proliferation and modified the morphology of two human melanoma cell lines. These effects were independent of the external glutamate level in the culture medium. In addition, CPCCOEt significantly enhanced the tumoricidal effects of cytostatic drugs. Thus, selective non-competitive metabotropic glutamate receptor antagonists may be used alone and/or with the synergistic effects of chemotherapy, thus enhancing existing therapies of melanoma and possibly other malignancies.

Introduction

Glutamate is the predominant excitatory neurotransmitter in the central nervous system and stimulates receptors which are divided into ionotropic (ligand-gated ion channels) and metabotropic (G-protein-coupled) glutamate receptors (1-3). Until recently, glutamate signalling was thought to be restricted to the central nervous system, and glutamate has been implicated in the pathogenesis of human neurological and neurodegenerative psychiatric disorders, as well as traumatic brain injury (4,5). More recently, however, it has become apparent that also various peripheral, non-neuronal cells express different types of glutamate receptors (6). Accordingly, increasing attention has been directed to the role of glutamate signal transduction in these tissues. Triggered by the observation that glutamate regulates proliferation, migration and survival of neuronal progenitors (7,8), Rzeski *et al* (9) reported that glutamate also stimulates the proliferation and migration of tumor cells, and ionotropic glutamate receptor antagonists were found to inhibit the proliferation of several tumor cell lines, such as colon adenocarcinoma, breast carcinoma, thyroid carcinoma, lung carcinoma, astrocytoma and neuroblastoma. Furthermore, this study showed that the same ionotropic glutamate receptor antagonists enhanced the tumoricidal effects of cytostatic drugs (cyclophosphamide, cisplatin, thiotepa, and vinblastin). In line with these data, our group observed that glutamate as well as ionotropic glutamate receptor reactive drugs differentially modulated growth and morphology of human histiocytic lymphoma-derived U937 cells (10). Results from another group indicated that overexpression of the glutamate receptor subunit NMDAR1 (N-methyl-D-aspartate) significantly correlated with tumor size, lymph node metastasis and cancer stage of oral squamous cell carcinoma (11).

Recent microarray data revealed that many G-protein-coupled receptors (GPCRs) including metabotropic glutamate receptors (mGluRs), are implicated in the tumorigenesis and metastasis of human cancers (12). Metabotropic glutamate receptor-4 expression was detected in colorectal carcinoma (68%), but also in 63% of malignant melanoma, and overexpression was associated with recurrence and poor disease-free survival (13). mGluR1 mRNA expression was found in human MG-63 osteoblast-like osteosarcoma cells (14) as well as in Jurkat T cells (15), and upregulation of mGluR1 was detected in metastatic melanoma (12). In addition, duplex RT-PCR analysis indicated that the mGluR1

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subunit was expressed in 7 of 19 melanoma samples, but not in two benign nevi (16). Western blot analysis likewise showed that mGluR1 was expressed in 12 of 18 human melanoma cell lines, but not in normal human melanocytes (16). The same group characterized a mouse melanoma model, which has implicated the ectopic expression of mGluR1 in melanogenesis and metastasis (16,17). In continuation of these experiments Marín *et al* (18) showed in mouse melanoma cell lines that stimulation of mGluR1 results in inositol triphosphate (IP_3) accumulation and extra-cellular-regulated kinases 1 and 2 (ERK 1/2) activation, which both could be inhibited by pretreatment of the tumor cells with a mGluR1 subtype-specific competitive antagonist LY367385.

Based on these findings, we investigated the effect of a selective non-competitive mGluR1 antagonist CPCCOEt (7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester) on the growth and morphology of two human melanoma cell lines (Human Bowes melanoma, HBMC; n15006 melanoma), which have been shown to stably express the mGluR1 subunit [16]. Litschig *et al* (19) were the first to describe this negative allosteric modulator and its specific binding site within the transmembrane heptahelical domain of human mGluR1. In addition, we also addressed the question whether combining this mGluR1 antagonist with established chemotherapeutic drugs (20-22) would enhance the cytostatic effect.

Materials and methods

Reagents. CPCCOEt was obtained from Sigma-Aldrich, Austria. Docetaxel was purchased from Fluka Riedel-de Haën, Switzerland. Cell culture vessels were obtained from Sarstedt (Wiener Neudorf, Austria). Culture media and supplements were obtained from Cambrex Bio Science (Verviers, Belgium) and PAA Laboratories (Linz, Austria).

Cell culture. Human Bowes melanoma cells (HBMC) (ATCC no. CRL-9607) were kindly provided by Dr Barbara Kofler, Department of Pediatrics, General Hospital Salzburg, Austria. The melanoma cell line n15006 was a gift from Dr Suzie Chen, Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, NJ, USA. Human skin fibroblasts were used as non-malignant proliferating cells and obtained by outgrowth technique from a skin explant. Cells were cultured in normal Eagle's minimum essential medium (EMEM) (Cambrex Bio Science), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories), 100 IU penicillin, and 100 μg/ml streptomycin (PAA Laboratories). For the experiments the cells were cultured without antibiotics. The optimal cell density was evaluated in preliminary tests. For glutamate starvation culture conditions, cells were cultivated in EMEM without glutamate, supplemented with 10% FBS. Cells were cultured at an initial cell number of $1x10^5$ cells/ml at 37˚C in a humidified atmosphere containing 5% CO_2. For experiments, cells were transferred in microplates (24 wells, flat bottom) (Sarstedt). After 24 h, medium was changed (day 0) and the cells were exposed either to CPCCOEt (1-200 μM) alone, or

docetaxel (5-10 ng/ml) (10 nM=8.08 ng/ml) (Fluka Riedel-de Haën) alone, and to 200 μM of CPCCOEt together with 5-10 ng/ml docetaxel for 3 days (day 1, day 2, day 3 in Figs.). CPCCOEt was diluted in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Austria). Docetaxel was first diluted in DMSO and then further diluted in Hanks' balanced salt solution (HBSS) (Cambrex Bio Science). Compared to the culture medium, DMSO and HBSS per se did not appreciably modify cell growth. For cell counting the adherent cells were trypsinized with 0.25% trypsin diluted 1:1 in calcium-magnesium-free phosphate-buffered saline (CMF-PBS) (both Cambrex Bio Science) for 6 min. Cell counting was performed by the CASY-1® cell counter and analyser (Schärfe, Reutlingen, Germany).

WST-1 assay. The WST-1 assay is a test for cell viability and proliferation (Roche Diagnostics GmbH, Vienna, Austria) based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenase. Quantification of the formazan dye was performed by OD measurements at 450/650 nm by an ELISA reader (Molecular Devices Corporation, Sunnyvale, CA). The WST-1 assay was routinely performed together with determination of cell counts. Cells were transferred into 96-well microplates (Sarstedt, flat bottom) in a final volume of 100 μl/well, to which cell proliferation reagent WST-1 (10 μl/well) was added. The appropriate incubation time with the cell proliferation reagent WST-1 was determined to be 45 min for both melanoma cell lines. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and shaken thoroughly for 5 min on a shaker before measurement.

Scanning electron microscopy. Cells were cultured as described above and cell pellets were fixed with 3% glutaraldehyde (Sigma-Aldrich, Germany) in cacodylate buffer (Sigma-Aldrich, Germany) (0.1 M, pH 6.8-7.2) for 24 h at 4˚C and processed for scanning electron microscopy by routine methods.

Statistical analysis. Statistical analysis was performed using the one sided Student's t-test. P values ≤0.05 were considered significant. All experimental results were expressed as mean values ± SEM.

Results

Effects of CPCCOEt on growth and morphology of HBMC and n15006 melanoma cells cultured in media with glutamate or under glutamate starvation. Compared to controls, a 3-day treatment with 200 μM of CPCCOEt significantly attenuated cell proliferation of both HBMC and n15006 melanoma cells cultured in normal EMEM (Fig. 1a). This effect started at day 2, was less pronounced at 100 μM of CPCCOEt and declined at 50 μM (data not shown). In the WST-1 assay a decrease of mitochondrial dehydrogenase activity was observed during the whole period of treatment (Fig. 1b and c). Since CPCCOEt exerts its effect via binding within the receptor transmembrane heptahelical domain, which is topo-graphically distinct from the binding site of the endogenous ligand, glutamate, we also examined the effect of CPCCOEt
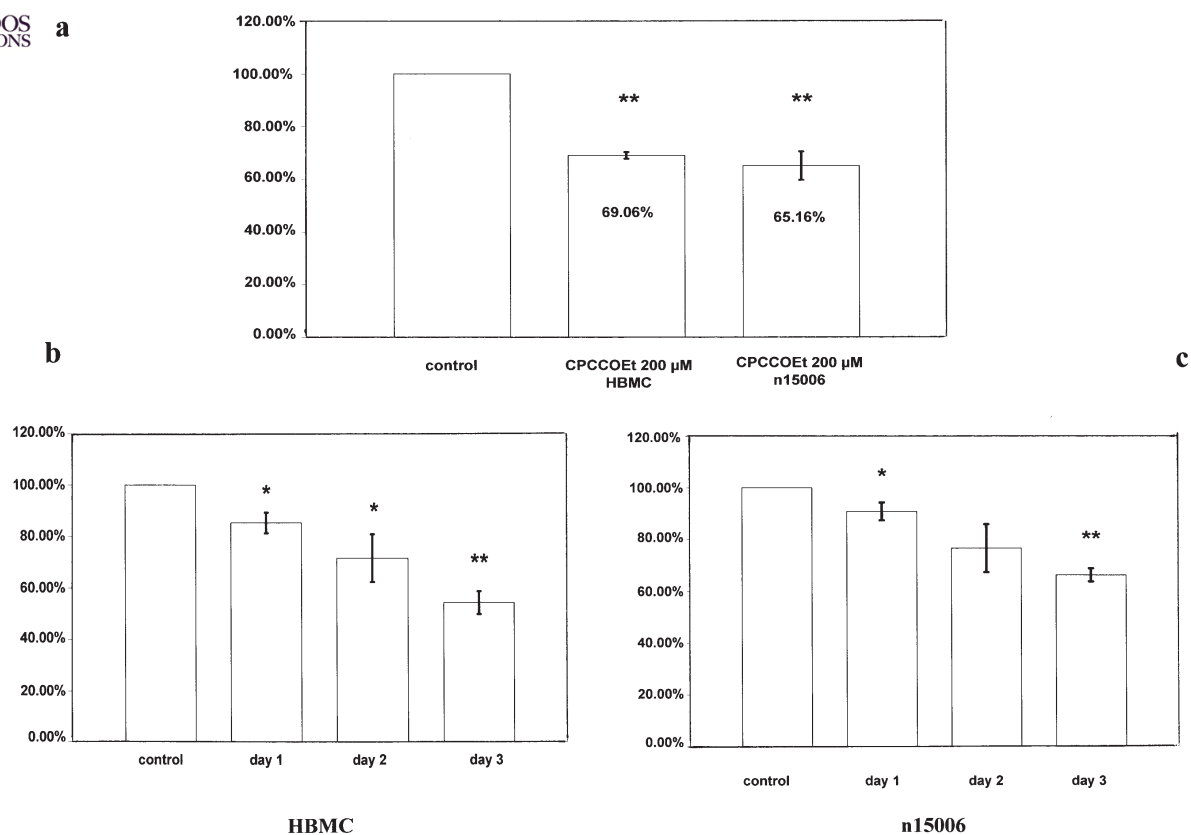
Figure 1. Modulatory effects of a 3-day treatment with CPCCOEt on proliferation and mitochondrial activity of human melanoma cells (HBMC, n15006) cultured in normal EMEM. (a) CPCCOEt (200 μM) significantly decreased cell growth of both cell lines ([**]$p \leq 0.01$). The bar diagrams illustrate overall effects on cell proliferation. (b) CPCCOEt (200 μM) significantly decreased the enzymatic activity of mitochondrial dehydrogenase of HBMC cells in the WST-1 assay ([*]$p < 0.05$; [**]$p \leq 0.01$). Three mean values derived from 6 samples each were compared. (c) CPCCOEt (200 μM) likewise significantly decreased the enzymatic activity of mitochondrial dehydrogenase of n15006 cells in the WST-1 assay ([*]$p < 0.05$; [**]$p \leq 0.01$). Three mean values derived from 6 samples each were compared. Error bars represent SEM. All experiments were performed in triplicate.
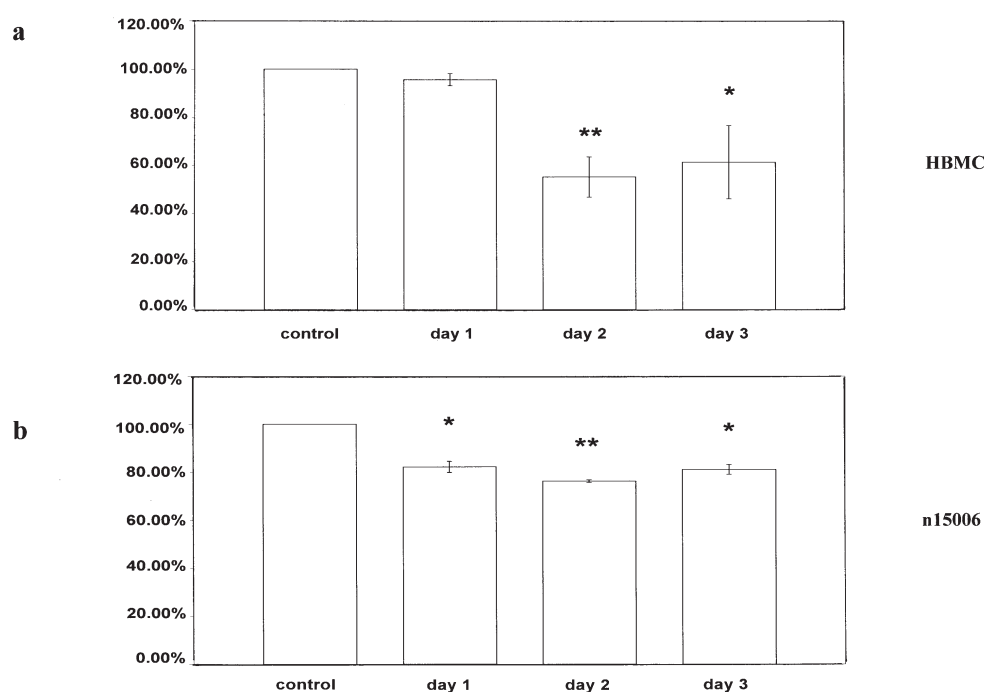

Figure 2. Modulatory effects of a 3-day treatment with CPCCOEt on mitochondrial activity of human melanoma cells (HBMC, n15006) cultured in glutamate-free EMEM supplemented with 10% FBS. (a) CPCCOEt (200 μM) significantly decreased the enzymatic activity of mitochondrial dehydrogenase of HBMC cells in the WST-1 assay ([*]$p < 0.05$; [**]$p \leq 0.01$). Three mean values derived from 6 samples each were compared. (b) CPCCOEt (200 μM) likewise significantly decreased the enzymatic activity of mitochondrial dehydrogenase of n15006 cells in the WST-1 assay ([*]$p < 0.05$; [**]$p < 0.01$). Three mean values derived from 6 samples each were compared. Error bars represent SEM. All experiments were performed in triplicate.
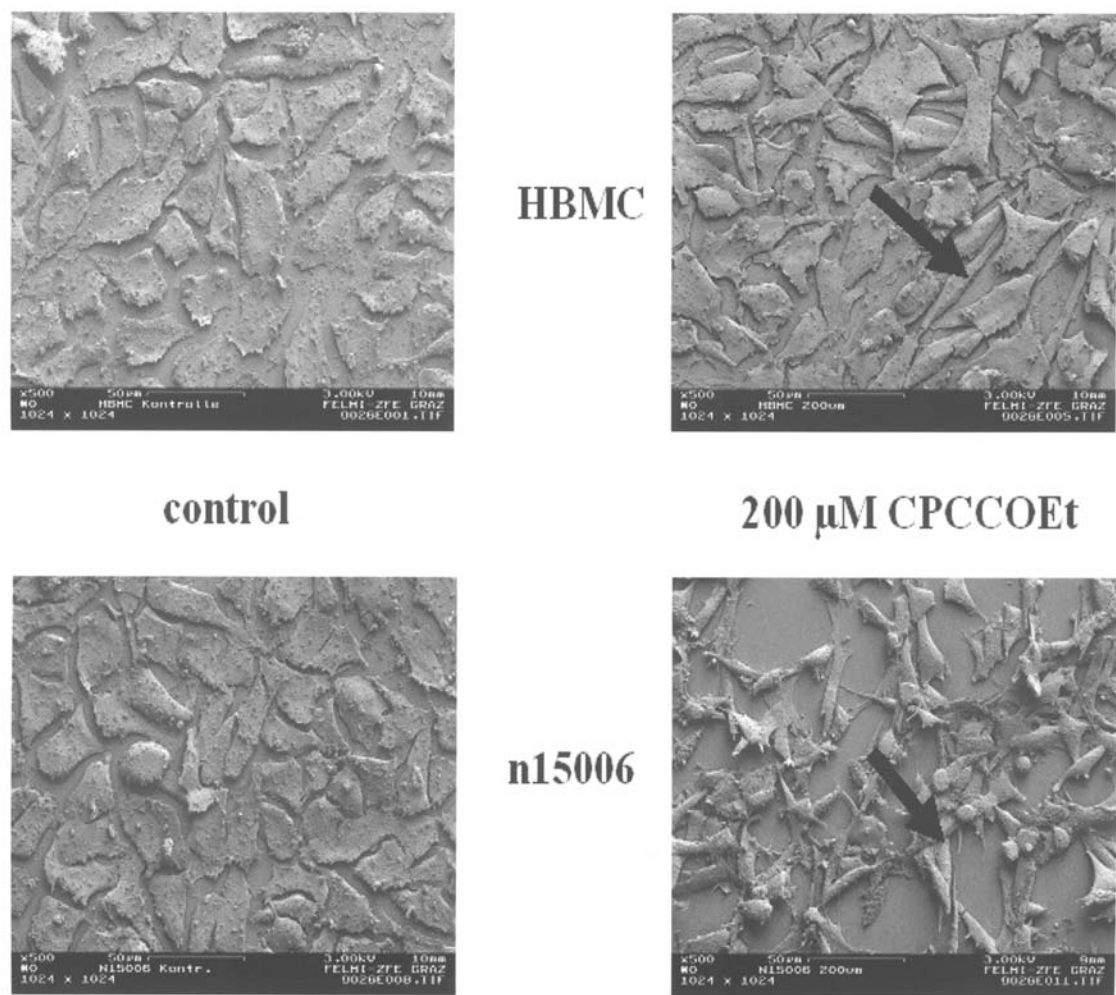
Figure 3. Scanning electron micrographs of HBMC and n15006 cells after treatment with CPCCOEt. Following administration of 200 μM of CPCCOEt, HBMC as well as n15006 melanoma cells appeared to be more spindle-shaped (arrow). Bar, 50 μm.
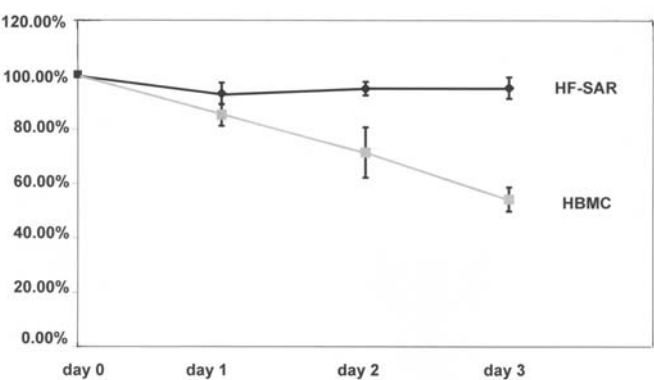

Figure 4. Effect of CPCCOEt on mitochondrial activity of human skin fibroblasts (HF-SAR) as non-malignant proliferating cells. In the WST-1 assay 200 μM of CPCCOEt did not affect the enzymatic activity of mitochondrial dehydrogenase of HF-SAR cells, but again decreased the enzymatic activity of HBMC melanoma cells.

on melanoma cells cultured under glutamate starvation. To minimize the glutamate content, cells were cultured in a glutamate-free medium supplemented with 10% FBS. CPCCOEt likewise decreased cell growth at the same concentrations with the maximal effect observed at 200 μM

(not shown). WST-1 activity was also decreased in both cell lines, whereby the greatest decline was seen at day 2 (Fig. 2a and b). Interestingly, the competitive mGluR1 antagonist (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), as well as the agonists (1S,3R-)1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and glutamate per se did not affect cell proliferation of both cell lines (not shown). To examine possible morphological changes, scanning electron microscopy was performed. HBMC as well as n15006 melanoma cells appeared to be more spindle-shaped after treatment with 200 μM of CPCCOEt, which may indicate a loss of adherence (Fig. 3). Human skin fibroblasts (HF-SAR) as non-malignant proliferating cells served as controls, and WST-1 activity was compared with melanoma (HBMC) cells treated with 200 μM of CPCCOEt. CPCCOEt did not affect mitochondrial activity of HF-SAR cells, but again decreased the enzymatic activity of HBMC melanoma cells (Fig. 4).

CPCCOEt enhanced the tumoricidal effects of the cytostatic drug docetaxel. HBMC melanoma cells were subjected to treatment with either CPCCOEt, docetaxel alone, or CPCCOEt in combination with docetaxel for 3 days. CPCCOEt (200 μM) as well as docetaxel (10 ng/ml) similarly inhibited cell proliferation. This effect was enhanced in cells treated with
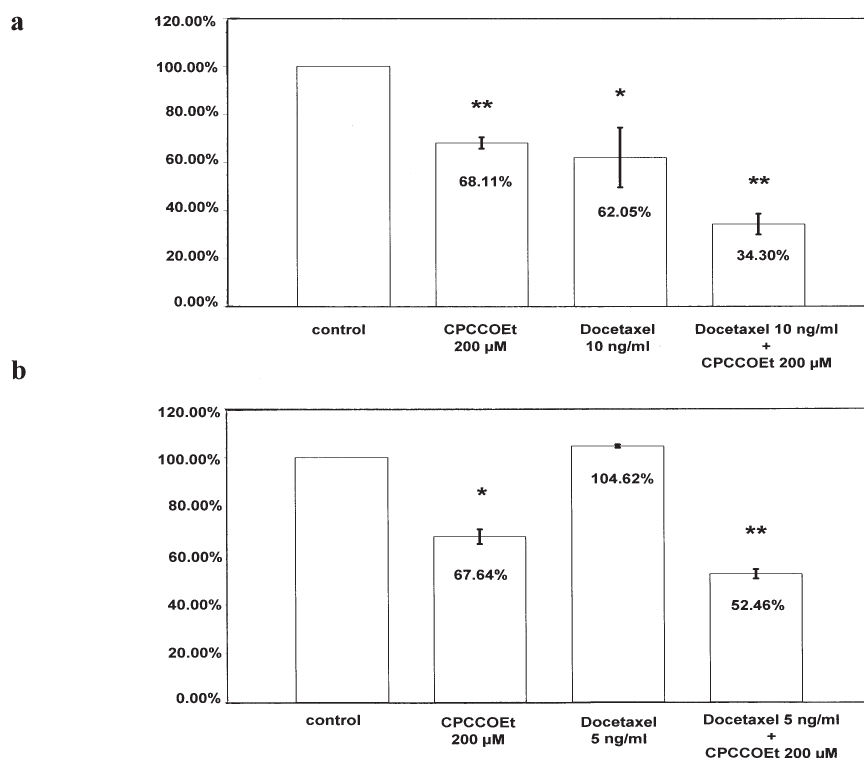
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Figure 5. Effect of a 3-day treatment with CPCCOEt, docetaxel alone, or CPCCOEt in combination with docetaxel on HBMC cells. (a) CPCCOEt (200 μM) as well as 10 ng/ml of docetaxel alone inhibited cell proliferation. Treatment with both CPCCOEt and docetaxel resulted in a clearly more pronounced effect in comparison with either treatment alone (*p<0.05; **p≤0.01). (b) Administration of 5 ng/ml of docetaxel did not affect cell proliferation. In combination with 200 μM of CPCCOEt a strong synergistic cytostatic effect was observed as compared to treatment with CPCCOEt alone (*p<0.05; **p≤0.01). The bar diagrams illustrate overall effects on cell proliferation. Error bars represent SEM. Experiments were performed in triplicate.

both CPCCOEt and docetaxel (Fig. 5a). Administration of 5 ng/ml of docetaxel did not affect cell proliferation. However, in combination with 200 μM of CPCCOEt a strong synergistic cytostatic effect was observed as compared to treatment with CPCCOEt alone (Fig. 5b). Similar effects were observed with WST-1 activity (not shown).

Discussion

It has been established that ionotropic glutamate receptor antagonists limit tumor growth in peripheral tissues and enhance the tumoricidal effects of cytostatic drugs (9). In line with this, our group observed that the competitive AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)/kainate receptor antagonist CNQX (6-cyano-7-nitroquin-oxaline-2,3-dione) significantly decreased growth and altered morphology of the human histiocytic lymphoma cell line U937 (10). Furthermore, the mGluR1 subtype-specific competitive antagonist LY367385 inhibited the agonist-induced IP$_3$ accumulation and ERK 1/2 activation in melanoma cells (18). In this study we showed that the selective non-competitive mGluR1 antagonist CPCCOEt significantly, dose-dependently, and reversibly inhibited the growth of two human melanoma cell lines. Presently, we do not know the mechanisms and signal cascades underlying this effect. TUNEL analysis has provided no results up to now, indicating that apoptosis-associated DNA strand breaks do not occur. This is in line with observations by others, who reported that human melanoma cell lines per se are very resistant to

apoptosis (23). Furthermore, the effect appeared to be independent of external calcium, since culturing the cells in a special calcium-magnesium-free medium resulted in the same inhibition of tumor growth (not shown). However, as mentioned above, ERK1/2 activation may play a role (18) that needs to be investigated in future experiments.

Scanning electron microscopy revealed a more spindle morphology of melanoma cells after treatment with CPCCOEt. At present, the significance of this result is still obscure. In 1986, Xiang and Kimura (24) reported that B16 melanoma cells assumed spindle morphology after a shift from Eagle's Hanks' medium with 10% newborn calf serum to Dulbecco's modified Eagle's medium with 10% fetal bovine serum. These morphological changes were characteristic of established criteria of melanoma differentiation, and alterations in the differentiation state had profound effects on metastatic potential. Overexpression of the mGluR1 subunit was recently described in metastatic melanoma (12). Studies are underway to examine whether metabotropic glutamate receptor antagonists, in particular CPCCOEt, interfere with *in vitro* migration and *in vivo* metastasis of melanoma cells.

Finally, we observed that the combined administration of CPCCOEt and an established chemotherapeutic drug (docetaxel) resulted in a stronger cytostatic effect than either treatment alone. This was in line with the data of Rzeski, who reported that NMDA and AMPA antagonists enhanced the tumoricidal effects of cytostatic drugs (cyclophosphamide, cisplatin, thiotepa, and vinblastin) *in vitro* (9). In general, selective allosteric modulators of mGluRs have a promising

therapeutic potential. Firstly, they act either as positive or negative modulators of the respective receptor without affecting the affinity or efficacy of the orthosteric ligand (25). Secondly, these compounds may improve the selectivity for individual mGluRs as well as enhance the scope for chemical tractability (26). In this direction, a new, promising, selective non-competitive mGluR1 antagonist, YM-298198, is now available, which is water soluble, whose binding site is close to the CPCCOEt allosteric site and which was shown to be highly active *in vivo* even with oral administration to exert an analgesic effect in streptozotocin-induced hyperalgesic mice (27). Recently, the group of Namkoong (28) suggested that the interference at specific points within GluR1 signal transduction should inhibit melanoma cell proliferation. The results of our study support this idea and may provide new concepts for cancer research and therapy.

Acknowledgements

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