The Influence of Glutamate Receptors on Proliferation and Metabolic Cell Activity of Neuroendocrine Tumors

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Abstract. *Neuroendocrine tumors are relatively insensitive to radiation therapy, as well as chemotherapy. Thus, new approaches for alternative therapies are needed. We found that glutamate receptor antagonists are capable of suppressing tumor growth and cell activity of different peripheral malignancies. In the present article we review scientific literature in this field of science. Subtype-specific, non-competitive, metabotropic glutamate receptor-1 antagonists differently suppressed the growth and metabolic cell activity of one human medullary thyroid carcinoma cell line, as well as of four different human midgut neuroendocrine tumor cell lines. Furthermore, PCR analyses revealed that this subtype of glutamate receptor is expressed in these cell lines. These first results indicate that specific metabotropic glutamate receptor antagonists suppress the proliferation and cell activity of neuroendocrine tumor cells, which makes them possible targets in cancer therapy.*

Neuroendocrine tumors (NETs) are a broad group of neoplasms whose clinical behavior varies from an indolent, benign course to an aggressive, rapidly progressive, and deadly disease depending on the primary tumor site, size, grade of differentiation and proliferation (1-5). Medullary thyroid carcinoma (MTC) is a rare malignancy derived from parafollicular C-cells of the thyroid (1, 3-4). It can occur in sporadic and hereditary forms and is relatively insensitive to radiation therapy, as well as chemotherapy (multi-drug resistance) (3, 6). Thus, new therapeutic approaches are needed. Promising novel therapy concepts comprise of the inhibition of the Rearranged during transfection (*RET*) tyrosine kinase domain, as well as inhibitors of Vascular endothelial growth factor receptors (VEGFR) (7-9). Recently, our group showed that particular plant extracts [*e.g.* of *Stemona tuberosa* Lour, *Cautleya gracilis* (Smith) and *Uncaria tomentosa* (Willd.)] are effective against MTC *in vitro* (10-13). NETs of the gastrointestinal tract arise from enterochromaffin (EC) cells and secrete bioactive substances which, in the case of metastasis, are responsible for the characteristic clinical symptoms of the carcinoid syndrome, consisting of flushing, diarrhea, heart valvular lesions and abdominal cramping (14-16). Similar to MTCs, chemotherapy and radiotherapy are usually ineffective and no curative treatment exists except for radical surgery. For this reason, the treatment of carcinoid tumors (NETs) also needs a multidisciplinary approach. In this context, somatostatin analogs, such as octreotide and pasireotide, are commonly used in therapy (17-19). In this respect, our group showed that novel plant extracts from *Trailliaedoxa gracilis* (W.W. Smith & Forrest) exert a strong anti-proliferative effect in the small intestine SI–NET cell line KRJ-I and in SCID-mice transplanted with KRJ-I (20).

Concerning the effect of neurotransmitters on (patho)physiological functions in the periphery, it has been unequivocally proven that neurotransmitters and their receptors are not only expressed in the central – and peripheral nervous systems, but also in non-neuronal peripheral tissues, in order to enable local auto – as well as paracrine communication from cell to cell (21). This also applies to the major excitatory neurotransmitter glutamate and its receptors, and it is generally accepted that glutamate, apart from mediating synaptic transmission, can also regulate a broad area of other biological responses (22). Peripheral glutamate receptors (GluRs) are involved in nociception, the modulation of stomach motility, skeletal biology, immune functions, and, last but not least, tumor biology (22). However, little is known about the impact of glutamate signaling in NETs. Only one report described that low doses of ketamine, an ion-channel associated GluR

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antagonist, were effective in treating neuropathic pain in a patient with metastasized thyroid carcinoma (23). Chang *et al*. (24) investigated the expression of a special type of G-protein coupled, metabotropic, GluR (mGluR), in particular the mGluR4 subtype, in malignant tissues from various organs by immunohistochemistry. They found that mGluR4 was expressed in one case of 12 tested patients with papillary thyroid carcinoma and one case of one tested person with medullary thyroid carcinoma (24). Furthermore, Rzeski *et al*. (25) and Stepulak *et al*. (26) described the expression and role of different types of GluRs in human follicular thyroid carcinoma (FTC238) cells. Kidd *et al*. (27) showed that normal EC cells, but not tumor cells, express the mGluR4 subunit. In contrast, the ion-channel associated *GluR2* gene is highly expressed in gastrointestinal NET cells, whereas it was not detected in EC cells (28). In the last years, our group has shown that the subtype-specific, non-competitive, mGluR1 antagonist, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) significantly inhibits the proliferation and metabolic cell activity of the MTC cell line MTC-SK (29, 30). Litschig *et al*. (31) were the first to describe this negative allosteric modulator and its specific binding site within the transmembrane domain VII of the human mGluR1. In the course of our recent investigations, we used two more subtype-specific mGluR1 antagonists and extended our studies to midgut NETs. So far, our initial results emphasize the therapeutic value of subtype-specific, non-competitive mGluR reactive drugs in the treatment of drug-insensitive NETs (see below).

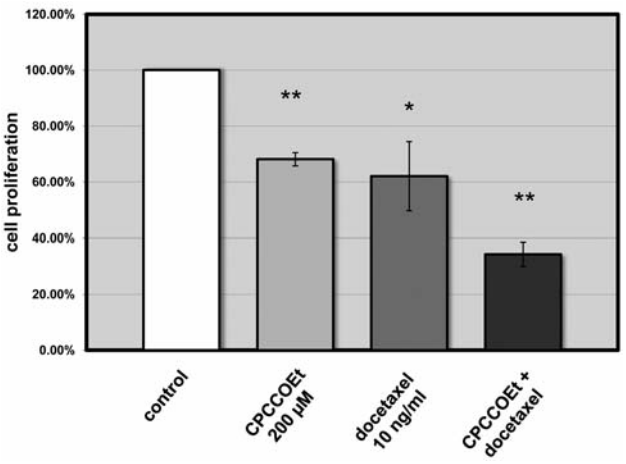

Figure 1. *Effect of a three-day treament with CPCCOEt and docetaxel. CPCCOEt (200 µM) and docetaxel (10 ng/ml) alone inhibited cell proliferation of HBMC cells. Treatment with both, CPCCOEt and docetaxel, resulted in a clearly more pronounced effect in comparison with either treatment alone (*p<0.05; **p≤0.01; Student's t-test). The bar diagrams illustrate overall effects on cell proliferation. Error bars represent SEM. Experiments were performed in triplicates.*

Glutamate and Tumor Growth Outside the CNS

The glutamate transmission system is the most complicated neurotransmitter system in the human body. Glutamate stimulates more than 20 receptors, which are divided into ionotropic, ligand-gated ion-channel-associated, receptors (iGluRs) and metabotropic, G-protein-coupled receptors (mGluRs). iGluRs are permeable to Na^+, K^+, as well as Ca^{2+} and, based on agonist specificities, traditionally subdivided into three subgroups, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors. mGluRs are also traditionally classified into three groups according to their pharmacology and preferred second messenger. Group I receptors (mGluR1, mGluR5) coupled to phospholipase C and increase phosphoinositide hydrolysis. Group II (mGluR2, mGluR3) and III (mGluR4, mGluR6, mGluR7, mGluR8) receptors are negatively linked to adenylate cyclase and commonly decrease cyclic adenosine monophosphate (cAMP) levels (32-37). Glutamate receptors are not only expressed in the central nervous system (CNS), but also in peripheral non-excitable cells (e.g. taste buds, dental regions, intestine, heart, lung, spleen, thymus, pancreas, adrenal gland, kidney, skin, bone, hepatocytes, megakaryocytes, platelets and lymphocytes) (22, 30, 38-40). Of particular interest is the discovery that GluR-reactive reagents can differentially modulate tumor cell proliferation and migration, and induce morphological alterations in tumor cells (25, 26, 41-45). In 2001, Rzeski *et al*. found that depending on components found in the external milieu (serum-containing medium *versus* serum-deprived medium), peripheral glutamatergic signaling differentially modifies the proliferation of tumor cells (25). This is in line with our findings, which describe the influence of iGluR antagonists on the growth of human promonocytic lymphoma (U937) cells in a serum- and glutamate-containing medium compared to serum- and glutamate-free conditions (46). An additional, clinically-relevant finding of Rzeski *et al*. was the synergistic effect of iGluR antagonists and common cytostatic agents (cyclophosphamide, thiotepa, vinblastin, cisplatin) used in cancer therapy (25). In our study with human Bowes melanoma (HBMC) cells we also observed that the combined administration of the specific mGluR-1 antagonist CPCCOEt and an established chemotherapeutic drug (docetaxel) resulted in a stronger cytostatic effect than either treatment alone (47) (Figure 1). Furthermore, we observed differential morphological changes after administration of GluR reactive drugs. For example, U937 cells exposed to the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) revealed enlarged mitochondria, suggesting changes in mitochondrial membrane depolarization (46), and HBMC cells appeared to be more spindle-shaped after treatment with

Table I. *The subtype-specific, non-competitive metabotropic glutamate receptor-1 antagonists cyclothiazide and YM298198 decreased cell proliferation of MTC-SK cells.*

MTC-SK cells

	200 μM	100 μM	50 μM	10 μM	Control
Cyclothiazide	61.81%	75.10%	91.26%	99.22%	100%
YM298198	68.47%	85.26%	91.69%	102.41%	100%

Table II. *The subtype-specific, non-competitive metabotropic glutamate receptor-1 antagonists cyclothiazide and YM298198 (both at 200 μM) decreased the cell proliferation of KRJ-I, P-STS, L-STS and H-STS cells.*

Midgut NETs

	200 μM Cyclothiazide	200 μM YM298198	Control
KRJ-I	57.80%	78.81%	100%
P-STS	80.49%	72.66%	100%
L-STS	58.49%	65.49%	100%
H-STS	61.03%	57.98%	100%

the mGluR1 antagonist CPCCOEt, which may indicate a loss of adherence (47). In recent years, an increasing number of data indicated the expression and underscored the significance of different GluR subunits in peripheral tumors such as lung carcinoma, colon carcinoma, pancreatic cancer, hepatocellular carcinoma, breast carcinoma, oral squamous cell carcinoma, tongue carcinoma, T-cell leukemia cells, human MG-63 osteoblast-like osteosarcoma cells, prostate cancer, thyroid carcinoma and melanomas, including benign tumors, such as uterine leiomyomata (24-26, 29-30, 42-43, 45-64). In this context, mGluRs, in particular positive and negative allosteric modulators, which offer the potential for improved selectivity and increased chemical tractability, have attracted increasing attention within the pharmaceutical industry as novel therapeutic agents for an increasing number of diseases (37, 65-68). The non-competitive mGluR1 antagonist (3aS,6aS)-6a-naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on (BAY 36-7620) inhibited cell growth of human melanoma cells, promoted apoptosis and decreased the levels of extracellular glutamate (45). BAY 36-7620 also suppressed melanoma migration, invasion and colony formation *in vitro* (69). Furthermore, it inhibited cell growth and increased apoptosis of breast cancer cell lines (63). We found that the non-competitive mGluR1 antagonist CPCCOEt inhibited cell proliferation as well as metabolic cell activity of two different human melanoma cells lines (47). However, we did not observe signs of apoptotic cell death following treatment with this mGluR1 antagonist (47). In summary, these data underscore the scientific relevance of specific allosteric modulators of GluRs and encourage the extension of the studies to other (for example therapy-resistant) tumors.

Glutamate and Neuroendocrine Tumors

As mentioned above, little is known about the significance of glutamate signaling in NETs. In our recent studies, we used two more subtype-specific, non-competitive mGluR1 antagonists, cyclothiazide and 6-amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide (YM298198). Cyclothiazide, initially described as a blocker of AMPA receptor desensitization (70, 71), was recently shown to inhibit mGluR1 by specifically interacting with the allosteric binding site of CPCCOEt (72). YM298198 is also a selective, but water-soluble, non-competitive mGluR1 antagonist, whose binding site is close to the CPCCOEt allosteric site, as well (73). We tested a human MTC cell line (MTC-SK) and four midgut NET cell lines (ileum) (KRJ-I, P-STS, L-STS, H-STS), which were established and characterized in our laboratory by Pfragner *et al*. (74-76). All cell lines were incubated for three days with different concentrations of the specific GluR antagonists and counted using the CASY-1® Cell Counter & Analyser (Roche Diagnostics GmbH/Innovatis, Vienna, Austria). This technology combines an established particle measurement counting and the resistance measuring principle, with pulse area analysis for cell particle size distribution with full computer support. To measure the metabolic cell activity, we performed the Cell Proliferation Assay WST-1 (Roche Diagnostics GmbH Vienna, Austria). This assay is a test for cell viability and proliferation based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenase. Quantification of the formazan dye was performed by O.D. measurements at 450/650 nm by an ELISA reader (Molecular Devices Corporation, Sunnyvale, California). We showed that both mGluR1 antagonists, cyclothiazide and YM298198 (both at 10-200 μM), inhibited the proliferation of MTC-SK cells in a dose-dependent manner, whereby the dosage of 200 μM proved to be the most effective (Table I). We then tested whether the same mGluR1 antagonists (10-200 μM) also affected the growth of the four human midgut NET cell lines (KRJ-I, P-STS, L-STS, H-STS). Similarly to MTC cells, both substances dose-dependently inhibited the growth of midgut NETs. Again the best results were obtained when cells were treated with a concentration of 200 μM (Table II). In accordance with the results obtained in the cell proliferation assays, both cyclothiazide and YM298198 (10-200 μM) dose-dependently inhibited the metabolic cell activity of all neuroendocrine cell lines, and

Table III. *The subtype-specific, non-competitive metabotropic glutamate receptor-1 antagonists cyclothiazide and YM298198 (both at 200 μM) decreased metabolic cell activity of MTC-SK, KRJ-I, P-STS, L-STS and H-STS cells.*

Suppression of metabolic cell activity

	200 μM Cyclothiazide	200 μM YM298198	Control
MTC-SK	69.55%	34.19%	100%
KRJ-I	50.73%	65.03%	100%
P-STS	79.03%	56.03%	100%
L-STS	41.86%	48.00%	100%
H-STS	39.99%	10.40%	100%

once again the best results were obtained when cells were treated with a concentration of 200 μM (Table III). Normal human fibroblasts as controls were less or completely unaffected by the same treatment. Furthermore, we carried out PCR analyses to investigate mGluR expression in the NET cell lines. The results indicated that the mGluR1 subtype is expressed in all cell lines.

Conclusion

In summary, data mentioned in the current report provide clear evidence that glutamate signaling plays a critical role not only in the CNS, but also in non-synaptic signaling in the periphery. Our findings suggest that, in particular, mGluR allosteric modulators may be promising new targets for the therapy of drug-insensitive tumors. However, additional studies are required to investigate the detailed mechanisms underlying these effects. For example, to examine whether the mGluR1 antagonists induce apoptosis or necrosis and/or influence the cell cycle. In addition, it will be necessary to determine the signaling cascades triggered by mGluR1 antagonists. Further studies are currently ongoing to elucidate synergistic actions between GluR antagonists and common cytostatic agents, similarly to studies with melanoma cells. Most importantly, however, it should be remembered that there are close interactions between the brain, peripheral nerves, neurotransmitters and tumor cells. These intricate interactions provide the basis, not only for further scientific investigations, but also for a better understanding of psychooncology, *i.e.* ultimately, mind-body medicine.

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