UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
PhD SCHOOL

PhD THESIS

INOVATIVE APPROACHES IN THE TREATMENT OF ASTROCITIC BRAIN TUMORS: MOLECULAR THERAPY AND CELLULAR APOPTOSIS

PhD COORDINATOR:

Prof. Univ. Dr. Doina Cârstea

PhD STUDENT:

Dr. Ada Maria Georgescu

CRAIOVA

2018

TABLE OF CONTENTS

Keywords: High-grade gliomas, Curcimine, Heliantine, Temozolomide, *Ligustrum Vulgare*

STATE OF KNOWLEDGE

Over 120 types of tumors of the nervous system have been reported in current medical literature. These tumors may arise in different areas of the brain, develop from different cell types and may have different therapeutic options. Astrocytic tumors derive from cells called astrocytes and are the most common type of brain cancer. Astrocytomas make up about 80% of brain tumors, and about 75% of them are glioblastomas (GBM), highly aggressive high-grade tumors.

Despite the high number of discoveries made in oncology, survival for patients with GBM has remained unchanged in recent years. Lack of early diagnosis due mainly to nonspecific signs and symptoms, high heterogeneity of tumor populations found within the same tumor, inability of the major therapeutic agents to cross the blood brain barrier (BBB), limiting the therapeutic options,has had a major contribution to the slow progress of developing new treatments for this type of tumors. An important step would be to discover the intrinsic mechanisms that give the GBM its particularly aggressive phenotype characterized by rapid progression, resistance to treatment and high heterogeneity. This rapid evolution's main promoter is somatic evolution, a process based on the progressive accumulation of mutations that give the cancer cell more and more distinct characteristics from those of a healthy cell. This development also involves tyrosine kinase receptors (RTKs) and their ligands, their particularly important role being linked to increased replication, invasion of surrounding tissues, promotion of angiogenesis, apoptosis inhibition and distant metastasis. Various therapeutic agents targeting these cellular structures have produced a major impact in the treatment of other cancers such as colon cancer, non-small cell lung cancer or melanoma. Unfortunately, the results could not be transposed to malignant gliomas where survival, disease free progression and quality of life were not improved by the introduction of these new therapies. An approach that is becoming more popular is the use of natural dyes as complementary treatment in various cancers.

Natural compounds extracted from plants are being used at an increasing rate in the treatment of cancer, particularly in the form of chemotherapeutic drugs. Many of the anticancer drugs used in recent decades are obtained directly from plants or are synthetic products derived from natural compounds. Agents such as vinca alkaloids (vincristine, vinblastine, vinorelbine and vindesine) are extracted from *Catharanthus roseus*, taxanes (paclitaxel) extracted from the Pacific yeast tree bark while topoisomerase I and II inhibitors (irinotecan, extracted from the Camptotheca acuminate Chinese ornamental tree and etoposide, extracted from wild mandarin Podophyllum peltatum) have been proven to be among the most effective anticancer drugs. Other agents, such as catechins (3-gal-epigallocatechin) extracted from green tea, the isothiocyanates found in cruciferous vegetables, isoflavones (pomiferina) have shown to have anti-tumor effects in both *in vitro* and *in vivo* experiments.

Curcumin (Difluolmethane) is a polyphenol derived from the rhizome of the *Curcuma Longa* plant, known as turmeric. Turmeric has been used since ancient times

(over 2000 years ago) in Ayurvedic Indian medicine to treat a wide range of disorders such as infections, burns, allergies, rheumatism, liver disorders and many more [1, 2].

PERSONAL CONTRIBUTIONS

OBJECTIVES OF THE STUDY

Objective no. 1 Effects of natural and synthetic dye compounds on GBM cell lines. Dye compounds are natural products (curcumin, quercetin) or synthetic (helianthin, methyl yellow, methyl red) which is a promising new category of antitumor drugs. In recent years, these substances have captured the attention of the scientific community because of their cytotoxic effect on malignant cells and their favorable *in vivo* toxic profile.

In study no. 1 we investigated the ability of curcumin and heliantin to inhibit the growth of malignant glioma cells in vitro. The cells were exposed to progressive doses of curcumin and helicin, respectively. The concentrations of the two coloring compounds studied ranged from 0.1 μM to 150 μM. Proliferation rates were assessed 3 days after treatment by performing the MTT assay.

Objective no. 2. Effect of curcumin treatment on primary GBM lines and correlations with growth rate and doubling time

Curcumin is a yellowish-orange natural dye that has been shown minimal toxicity in humans and is considered safe for human use; no toxicity associated with drug administration at doses up to 10 g / day has been reported. We also analyzed the effect of curcumin on three low passage GBM cell lines: GB3B, GB4B and GB5B. To examine the effect of the drug on cell viability, the cells were exposed at increasing doses of curcumin (0.1, 1, 2, 5, 10, 20, 50, 100 and 150 μM) for three days and the inhibitory effect was quantified by counting cells using a Burker haemocytometer.

Objective no. 3 The effect of targeted anti-EGFR targeted treatment on the viability of GBM cell lines and on caspase activation.

As a result of the frequent disruption of EGFR in HGG, this receptor represents a promising therapeutic target in these tumors. AG556 is a synthetic small molecule inhibitor of EGFR activity. In study no. 3, we investigated the ability of this substances to inhibit growth of HGG cells *in vitro*. For this purpose, MTT cell proliferation assays were performed on three HGG cell lines: 8, 18 and 38. Cells were exposed to progressive doses of AG556 (10 μM, 20 μM, 30 μM), and the cytotoxic effect of the inhibitor was evaluated after three days.

Objective no. 4 Effect of Ligustrum Vulgare (LV) Hydroalcoholic Extract (HALE) alone or together with Temozolomide on GBM cell lines.

In the study no. 4 we analyzed the effect of LV HALE on a number of four cell lines derived from brain tumors in vitro. Further, we analyzed the effect of the combination of LV HALE and TMZ chemotherapeutic agent in the four cell lines.

RESULTS AND DISCUSSIONS

Frequent genetic alterations encountered in gliomas result in aberrant activation of mitogenic signaling pathways that govern cellular proliferation, cell survival (apoptosis and necrosis), invasion and angiogenesis. In high-grade brain tumors, the most frequently activated proto-oncogene, by either amplification or mutation, is EGFR. Thus a series of EGFR receptor-specific inhibitors are under development or in clinical trials for the treatment of GBM. The most common mutant form of EGFR in GBM is the receptor variant III (EGFRvIII), which exhibits constitutive activation independent of ligand binding. In addition, the presence of EGFRvIII has been associated with increased human GBM tumorigenesis by reducing apoptosis and increasing proliferation.

Previous studies have shown that both curcumin and heliantin interact with EGFR, diminishing its activity. In our study, we analyzed the antiproliferative and apoptotic effect of curcumin and heliantin treatment on the low pass GBM cell line GB10B, *in vitro*. The major advantage of this approach is the high availability of these products, the particularly low toxic profile and the low costs involved in such treatment. Clinical protocols are often preceded by preclinical cytotoxicity studies using immortalized cell lines. However, immortalized cell lines are often incapable of reproducing the original features of the tumor. Also, high passage cell lines tend to accumulate a series of genetic mutations, changes in cell morphology, proliferation rate and expression of dysfunctional proteins [3, 4]. In this study we used a low-pass GBM line obtained in our laboratory from fresh tumor tissue.

The maximum antiproliferative effect of the drug was obtained using the 100 μM dose and determined a 77% reduction in cell viability. This curcumin concentration of 100 μM is considered safe since turmeric has been used in the Indian diet for thousands of years and has shown no toxicity when administered at doses up to 10 g per day.

Compared to curcumin, helianthin has so far been studied only on high-grade glioma cell lines *in vitro*. In fact, we have shown in previous studies that the substance induced apoptosis in several high-grade glioma cell cultures [5]. In this study we found out that 100 μM helianthin induced a drastic decrease in cell viability, killing 85% of GB10B cells. Thus, our data showed that helianthin had a greater cytotoxic effect on GB10B cells compared to the naturally occurring coloring compound curcumin. In line with our previous work, we noticed that 1 μM heliantin killed about 40% of our GB10B cells [5].

Caspases are a family proteases of that bring several key links in cell regulatory pathways that control programmed cell death also known as apoptosis. It is a well known fact that GMB cells present frequent disruptions of the mechanisms involved in regulating apoptosis [6].

In our paper we investigated the ability of curcumin and helianthin to induce apoptosis in GB10B cells. It is already known that curcumin is able to induce apoptosis in GBM by activating pro-apoptotic proteins and inhibiting anti-apoptotic signals. The drug is also involved in the activation of non-apoptotic autophagia, induction of differentiation cascade signaling, inhibition of matrix metalloproteinases (MMPs) and gene expression of the glucose 6-phosphate transporter (G6PT) and also activation of proteolytic pathways [7-9]. In this study we noticed that after treatment of GB10B cells with curcumin, caspase 3 became active 12 hours after treatment and remained active

until 48 hours after drug administration. Caspase 8 was active from 4 hours to 12 hours after curcumine administration while caspase 9 became active 4 hours after drug administration and remained activated until 48 hours after curcumin administration.

In our previous studies, we reported that heliantin treatment was associated with PARP degradation without affecting Bcl-2 protein expression in high-grade glioma cell lines [5]. In this study we noticed that helianthin activated caspase 3 in GB10B cells only 12 hours after drug administration. Caspase 8 became active 4 hours after drug administration and remained active until 48 hours after. Unlike caspase 3 and 8, early caspase 9 activation (4 hours after treatment) was detected, increasing activity successively until the end of treatment. These results suggest that treatment with curcumin and helicin can induce programmed cell death in GB10B cells by activating the pathways of both extrinsic and intrinsic apoptotic pathways. However, further studies are needed to better understand the apoptotic mechanisms produced by this substance class in GBM.

In order to evaluate the effect of treatment with curcumin and how it correlates with growth rate and doubling time (DT), we used three low passage primary cell lines isolated from GBM tumors (GB3B, GB4B and GB5B) in our study. The proliferation rate was approximately the same for all three cell lines. In fact, GB3B cell DT was found to be shorter than that of GB4B and 10 hours shorter than GB5B, but the difference between them was not statistically significant. All studied cell lines responded in a dose-dependent manner. The lowest curcumin concentration that determined the death of GBM cells was 1 μM for all cell lines, the cytotoxicity being 4%. The highest curcumin concentrations used in our study were 150 μM which drastically reduced cell viability to 24.5% in GB3B, 11.8% in the GB4B line and 11% in GB5B cells.

Preclinical studies have shown that it is possible to anticipate cellular response to drug therapy with curcumin correlating IC50 with DT measurements for each cell line [10]. Unfortunately, we did not notice a statistically significant correlation between curcumin IC50 values and DT ($P = 0.2$, $P = 0.552$) for the GBM cell lines used in this study. For example, the GB4B cell line which showed the highest IC50 value did not show different DTs when compared to GB3B and GB5B. Furthermore, the GB5B cell line with the longest DT was not the least sensitive to curcumin. The mechanism of action of curcumin on GB cells may probably explain this result. It is known that several targeted anti-neoplastic agents act by inactivating malignant cell survival structures (e.g., growth factor receptors, apoptosis activators, etc.) that can not be directly linked to cell growth rate or DT. In this context, curcumin-induced cell death in malignant glioma cells is due to the interference of the therapeutic agent with vital structures of tumor growth and progression.

In the second part of the study, we analyzed if AG556 is capable of activating caspases 3, 8 and 9 in cell lines 8, 18 and 38. For our experiments, we used 3 high-grade immortalized cell lines: 8, 18 and 38. HGG cells responded in a dose-dependent manner to EGFR inhibition using AG556. In cell line 18, the cytotoxic effect for 30 μM AG556 was 36%, whereas in line 38, the decrease in cell survival was about 26.5%. The most potent cytotoxic effect of the 30 μM AG556 dose was obtained in cell line 8, at around 38.3%.

While treatment with AG556 activated caspase 3 in all 3 cell lines, 3 hours after treatment and remained active for 24 hours, the treatment failed to activate caspase 8 in

cell line 38. However, in cell lines 18 and 8, administration of AG556 caused activation of caspase 8. This protease was activated 3 hours after treatment and remained active for up to 24 hours.

Several studies have highlighted the role of herbal extracts in the treatment of cancer [11]. In our study, we found that LV HALE exhibited inhibitory properties on primary cerebral tumor cells in vitro. It is known that ligustrum species contain different active molecules, such as flavonoids, secoyridoids, mono- and triterpenoids, coumarins, polyphenolic carboxylic acids and derivatives, lignans and proteins [12].

For our experiment, we used four low passage cell cultures derived from primary cerebral tumors: three cell lines derived from HGG-GBM (GB1B, GB2B and GB8B) and a cell line derived from a LGG tumor (Grade II astrocytoma) (AC1B).

The results of our study have shown that LV HALE induced cell death in human glial cells in a time and dose-dependent manner. LGG AC1B cells were more susceptible to treatment with LV HALE compared to GBM-derived GB1B, GB2B or GB8B.

In our study, we found that treatment with EHAL LV induced caspase 3 activation in all GBM cell lines. However, we noticed that the treatment effect was more pronounced in the GB1B and AC1B cell lines in which caspase 3 was activated at 48h and 72h after LV HALE administration, while in the GB2B and GB8B cell lines, caspase 3 was activated only 48 hours after treatment. In normal human HUC-1 stem cells, caspase 3 was activated only 72 hours after drug administration.

Several studies reported that concomitant use of various compounds had a synergistic effect on tumor cytotoxicity and prolonged survival of brain cancer patients. Gliadel, a biodegradable polymer containing the bis-chloroethylnitrosourea (BCNU) alkylating agent, in combination with radiation, chemotherapy and surgery, produced results in the treatment of high-grade malignant glioma [13]. Preclinical studies have suggested that medicinal herbs in combination with TMZ have been useful for the treatment of cancer, including brain tumors [14].

Our results showed that LV HALE had a clear impact on the viability of brain tumor cells, and additionally, the combination of LV HALE and TMZ resulted in an enhanced cytotoxic effect as expected. Even though combined treatment was stronger than single agent treatment, more data is needed to support the effectiveness of the combination of drugs.

CONCLUSIONS

Study no. 1 Effects of natural and synthetic dyes on GBM cell lines.

This study demonstrates that treatment with curcumin and helicin induces cell death in GBM cells in vitro, and helianthin exhibits an antiproliferative capacity superior to curcumin. We also found that treatment with curcumin and helianthin caused activation of caspase 3, 8 and 9 in GB10B cells. At this point, more studies are needed to conclude if curcumin and helianthin could be effective chemotherapeutic agents *in vivo*.

Study no. 2. Effect of curcumin treatment on primary GBM lines and correlations with growth rate and doubling time.

In our study, we found out that curcumin kills GBM cells in vitro in a dose-dependent manner. Unfortunately, we did not notice a statistically significant correlation between the IC50 and curcumin DT (P = 0.2, P = 0.552) for the GBM cells used in this study.

Study no. 3. The effect of targeted anti-EGFR treatments on the viability of GBM cell lines and on caspase activation.

In our paper we found out that treatment with AG556 produced similar cytotoxic effects in the high grade 18 and 8 malignant glioma cell lines, while in cell line 38, drug efficacy was more limited. The drug was able to activate caspases 3, 8 and 9 in cell lines 18 and 8. In cell line 38, administration of AG556 only activated caspase 3 and 9, while caspase 8 remained inactive. All of these experiments have once again demonstrated that EGFR remains a promising target in HGG therapy, and small molecule inhibitors such as AG556 can be used as personalized therapies.

Study no. 4 Effect of Hydroalcoholic Extract of Ligustrum Vulgaration alone or together with Temozolomide on GBM cell lines

Our results showed that LV HALE had a clear impact on the viability of brain tumor cells and, moreover, the combination of LV HALE and TMZ resulted in an enhanced cytotoxic effect, as expected. Even though combination treatment was stronger than single agent treatment, more data are needed to support the efficacy of the drug combination.

REFERENCES

1. Aggarwal BB, Sundaram C, Malani N, Ichikawa H. Curcumin: the Indian solid gold. Adv Exp Med Biol 2007; 595: 1-75.
2. Araujo MC, Antunes LM, Takahashi CS. Protective effect of thiourea, a hydroxyl-radical scavenger, on curcumin-induced chromosomal aberrations in an in vitro mammalian cell system. Teratog Carcinog Mutagen 2001; 21: 175-180.
3. Sambuy Y, De Angelis I, Ranaldi G et al. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 2005; 21: 1-26.
4. Chang-Liu CM, Woloschak GE. Effect of passage number on cellular response to DNA-damaging agents: cell survival and gene expression. Cancer Lett 1997; 113: 77-86.
5. Alexandru O, Dragutescu L, Tataranu L et al. Helianthin induces antiproliferative effect on human glioblastoma cells in vitro. J Neurooncol 2011; 102: 9-18.
6. Eisele G, Weller M. Targeting apoptosis pathways in glioblastoma. Cancer Lett 2013; 332: 335-345.
7. Nagai S, Kurimoto M, Washiyama K et al. Inhibition of cellular proliferation and induction of apoptosis by curcumin in human malignant astrocytoma cell lines. J Neurooncol 2005; 74: 105-111.

8. Kang SK, Cha SH, Jeon HG. Curcumin-induced histone hypoacetylation enhances caspase-3-dependent glioma cell death and neurogenesis of neural progenitor cells. Stem Cells Dev 2006; 15: 165-174.
9. Romero-Hernandez MA, Eguia-Aguilar P, Perezpena-DiazConti M et al. Toxic effects induced by curcumin in human astrocytoma cell lines. Toxicol Mech Methods 2013; 23: 650-659.
10. Fallahi-Sichani M, Honarnejad S, Heiser LM et al. Metrics other than potency reveal systematic variation in responses to cancer drugs. Nat Chem Biol 2013; 9: 708-714.
11. Ullah MF, Khan MW. Food as medicine: potential therapeutic tendencies of plant derived polyphenolic compounds. Asian Pac J Cancer Prev 2008; 9: 187-195.
12. Gao BB, She GM, She DM. Chemical constituents and biological activities of plants from the genus Ligustrum. Chem Biodivers 2013; 10: 96-128.
13. Limentani SA, Asher A, Heafner M et al. A phase I trial of surgery, Gliadel wafer implantation, and immediate postoperative carboplatin in combination with radiation therapy for primary anaplastic astrocytoma or glioblastoma multiforme. J Neurooncol 2005; 72: 241-244.
14. Mittal A, Tabasum S, Singh RP. Berberine in combination with doxorubicin suppresses growth of murine melanoma B16F10 cells in culture and xenograft. Phytomedicine 2014; 21: 340-347.