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Original article

The effect of Azo-dyes on glioblastoma cells *in vitro*

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ABSTRACT

Despite the multidisciplinary standard treatment of glioblastoma (GB) consisting of maximal surgical resection, followed by radiotherapy (RT) plus concomitant chemotherapy with temozolomide (TMZ), the majority of patients experience tumor progression and almost universal mortality. In recent years, efforts have been made to create new agents for GB treatment, of which azo-dyes proved to be potential candidates, showing antiproliferative effects by inducing apoptosis and by inhibiting different signaling pathways. In this study we evaluated the antiproliferative the effect of six azo-dyes and TMZ on a low passage human GB cell line using MTT assay. We found that all compounds proved antiproliferative properties on GB cells. At equimolar concentrations azo-dyes induced more cytotoxic effect than TMZ. We found that Methyl Orange required the lowest IC_{50} for 3 days of treatment (26.4684 μM), whilst for 7 days of treatment, two azo dyes proved to have the highest potency: Methyl Orange IC_{50} = 13.8808 μM and Sudan I IC_{50} = 12.4829 μM. The highest IC_{50} was determined for TMZ under both experimental situations. Conclusions: Our research represents a novelty, by offering unique valuable data regarding the azo-dye cytotoxic effects in high grade brain tumors. This study may focus the attention on azo-dye agents that may represent an insufficient exploited source of agents for cancer treatment.

1. Introduction

Glioblastoma (GB) is one of the most malignant forms of solid brain tumor, with more than 10,000 cases newly reported each year in USA only (Ostrom et al., 2018).

Conventional treatment strategies, such as chemotherapy, radiotherapy (RT) and surgery have been used for the treatment of various cancers. Until now, chemotherapy remains the most commonly used treatment worldwide (Amjad et al., 2022; Sevastre et al., 2021b), but with many limitations due to toxic side effects or multidrug resistance (Bukowski et al., 2020; Fernandes et al., 2017; Seliger et al., 2022).

There are several types of treatment for GB (Torres-Martinez et al., 2021), of which the standard of care includes surgical resection, followed by RT and concomitant administration of temozolomide (TMZ) (Fernandes et al., 2017). Regardless the addition of secondary treatment using other agents such as receptor tyrosin kinases (RTK) inhibitors and the multidisciplinary treatment, the majority of patients experience a tumor progression with median survival of not more than 15 months (Stupp et al., 2005). By adding of bevacizumab to standard treatment, progression-free survival (PFS) has been improved, but no increase in overall survival (OS) and higher adverse events rate have been observed (Chinot et al., 2014). Furthermore, significant advances have been made by using: targeted therapy (Alexandru et al., 2015; Artene et al., 2018; Sevastre et al., 2019; Sevastre et al. 2021a), cell therapy (Alexandru et al., 2015; Artene et al., 2018; Sevastre et al. 2021a), ablation therapy (Debela et al., 2021), nanoparticles (Sevastre et al., 2019), radionics, chemo- and sono-dynamic therapy (Debela et al., 2021).

Cancer cell biology plays an important role in elucidating the signaling and in identifying potential targets for cancer drugs.

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Deregulations of signaling pathways have been found in many types of cancer, such as disturbances in cell metabolism and cell cycle (Alexandru et al., 2020; Oprita et al., 2021), angiogenesis (Alexandru et al., 2020; Oprita et al., 2021;), apoptosis (Rodriguez et al., 2022; Sevastre et al.,2021b), or epigenetic modifications (Alexandru et al., 2020; Rodriguez et al., 2022; Sevastre et al., 2021b).

Also, microenvironmental signals may contribute to cancer development. The development of anticancer agents has changed from serendipitous discoveries in the past to cancer cells targeting therapies of today. The aim is to disrupt the cancer cell functions while sparing normal cells, therefore limiting the side effects. There is an urgent need of novel or even repurposed treatments. Considering this, in recent years, the natural sources chemical agents seem to be a very attractive alternatives to synthetic compounds that exhibit similar anti-cancer properties, and reduced side effects. Many research studies are focusing on already known natural compounds, for which novel properties are continuously discovered (Al Jourdi et al., 2019; Alexandru et al., 2019; Bhambhani et al., 2021; Mogosanu et al., 2016; Tataranu et al., 2017).

It was reported that natural compounds from vegetables and fruits might be effective against cancer cells both *in vitro* and *in vivo* (Persano et al., 2022; Zhai et al., 2021). Results indicate their activity on protein expression due to their influence on transcription factors. They are also involved in cellular response modifications, cellular signaling and epigenetic alterations. Azo-dye is a class of natural and synthetic components which have been intensively studied in the last years for their carcinogenesis and antiproliferative effect (Ravichandran et al., 2021; Udoikono et al., 2022; Zahara Fiza et al., 2022).

Several dye compounds have been proved to have antiproliferative effects on various types of cancer cells (Alexandru et al., 2011; Alexandru et al., 2016; Alexandru et al., 2019; Khedr et al., 2021; Saeed et al., 2020; Sevastre et al., 2021c), and their involvement in mutagenesis and carcinogenesis have been also reported (Chung et al., 2000). In addition, some azo dyes have been proved to inhibit intracellular signal transduction pathways *in vitro* (Powis et al., 1992; Ngo et al., 2022). These properties of azo compounds may represent a therapeutic window for different anticancer strategies.

In this study we performed the screening of six azo-dyes: Methyl Orange, Alizarin Yellow, Lithol Rubine, Butter Yellow Methyl Red and Sudan I, for their cytotoxic effect using a human GB cell line. TMZ was taken as a reference drug that is an alkylating agent used as standard first line treatment in GB treatment after maximal surgical resection and RT (Stupp et al., 2005).

2. Materials and methods

2.1. Reagents

Alizarin Yellow GG (206709–25 g, Aldrich), Butter Yellow (73225–25 mg, Fluka), Helianthin (Methyl Orange, 68250–25 g, Fluka), Methyl Red (Alizarin Red S, 250198–25 g, Sial), Lithol Rubine (90689–25 mg, RDX), Sudan I (C.I. 12055, 103624–25 g, Sial) and were purchased from Redox Life Tech. TMZ (T2577-25 mg) was purchased from Sigma-Aldrich (Germany). TRYPSIN-EDTA solution 10X (T4174-100 ml, Sigma), Fetal Bovine Serum (F7524-500 ml, Sigma), DULBECCO'S Modified EAGLE medium (DMEM)/nutrient mixture F-12 HAM, with L-glutamine and sodium bicarbonate, without HEPES, S (MEM, D8062-500 ml, Sigma) were purchased from Redox Life Tech. Cell Proliferation Kit I (MTT, RO11465007001, Roche Diagnostics) was purchased from Redox Life Tech.

2.2. Cell line establishment

The GB cell line GB1B was established in our laboratory according to standard procedures from brain tumors as residual biological material provided by Bagdasar-Arseni Emergency Hospital, Bucharest, Romania (Farr-Jones et al., 1999). At hospitalization, all patients agreed to donate the tissue for research proposes, by signing the consent forms. In brief, fresh tumor tissues were minced with a sterile blade in a Petri dish, then mixed with 0.25 mg/ml collagenase IV, 0.4 mg/ml DNase, and 0.5 mg/ml pronase. The samples were transferred in Hank's buffered saline solution and maintained in a shaking incubator at 37 °C for 30 min at 37 °C, followed by another 30 min at 4 °C. Further, a cell strainer was used to pass the cell mixture through, in order to obtain a single cell suspension. The cells were washed two times with phosphate buffer saline (PBS) and seeded in six well plates. In the end, cell suspension was transferred into tissue culture flasks and passaged for 2–3 times (Mogosanu et al., 2016). For experimental purposes, same passages cells were used.

2.3. Cell culture

The cell lines were cultured in DMEM containing 10 % fetal bovine serum (FBS), 2 mM glutamine and antibiotic (100 UI/ml penicillin and 100 UI/ml streptomycin).

The cells were grown in tissue-culture flasks kept in a humidified incubator at 37 °C in an atmosphere with 95 % air/5% CO_2. Cell cultures have been amplified 2–3 passages from the original biological material and passage 3 has been preserved. Experiments were initiated when the confluence reached 50 %.

For our experimental purposes, cells seeded in monolayers in 96-well culture plates (1–10X103 cells/well) in the same environmental conditions as in amplification phase, were treated with different concentrations (0,5, 1, 2, 4, 8, 16, 32, 64 and 128 μM) of Methyl Orange, Methyl Red, Butter Yellow, Lithol Rubine, Sudan I, Alizarin Yellow, and TMZ for 3 days and 7 days. Appropriate control groups with only diluents and blank controls consisting of each reagent used in the experiment (without any sample) were included.

2.4. Preparation of solutions

According to each azo-dye solubility, various solvents have been used to obtain stock solutions, which have been further diluted to achieve the final set of concentrations: 0,5μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM and 128 μM.

Therefore, Methyl Orange (Helianthin, 68250–25 g, Fluka) and Methyl Yellow (Alizarin Yellow, 206709–25 g, Aldrich) were separately dissolved in distilled water in order to obtain 10 mM stock solutions. Lithol Rubine (90689–25 mg, RDX) and Butter Yellow (73225–25 mg, Fluka) were separately dissolved in distilled water:ethanol (1:3, v/v) in order to obtain stock solutions of 2,5mM. Methyl Red (Alizarin Red S, 250198–25 g, Sial) and Sudan I (C.I. 12055, 103624–25 g, Sial) were separately dissolved in ethanol 96° in order to obtain stock solutions in concentration of 2,5mM.

Further, all stock solutions were stored at −20 °C, protected from light. For our experiments, we used various final concentrations (0,5, 1, 2, 4, 8, 16, 32, 64 and 128 μM) by diluting the stock solution with standard DMEM medium..TMZ (T2577-25 mg) was used in concentrations of 0,5μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM, 32 μM, 64 μM and 128 μM in aqueous solution. The set of concentrations was chosed based on usually tested values for IC_{50} determination (Chiu et al., 2016, Zargar et al., 2018).

2.5. MTT cellular proliferation assay

The antiproliferative effects of Methyl Orange, Alizarin Yellow, Lithol Rubine, Butter Yellow, Methyl Red and Sudan I were examined by using the MTT assay (Roche Diagnostics). The assay is based on the activity of metabolically active cells to cleave the MTT yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan crystals. Tests were conducted with 4,000 cells/well, plated in 200 µl media in 96-well plates, with four replicates. Cells proliferation was quantified 3 days and 7 days after treatment. To each well 10 µl MTT reagent was added, incubated for 4 h at 37 °C. Cells were then lysed by adding 100 µl solubilization buffer. The experiments were repeated 3 times in the same conditions. Each experiment had its own set of control. Optical density (OD) was measured using a spectrophotometer at 595 nm and relative cell viability was expressed as percentage of untreated control cultures.

2.6. IC_{50} calculation

The formula to estimate the inhibitory concentration that leads to death of 50 % of cells (IC_{50}) was calculated with the free online Quest Graph™ IC_{50} Calculator provided by https://www.aatbio.com (AAT Bioquest, 2022).

For promotion activity, the Hill coefficient of the equation is negative, and for inhibition effect, the coefficient is positive.

2.7. Statistical analysis

All data are presented as mean ± SD. Data were analyzed using an ANOVA two-tailed *t* test. Only values $p < 0.05$ were considered statistically significant.

3. Results

3.1. The effect of azo-dye compounds on cell viability

In this section, we investigated the effect of several azo-dye compounds to inhibit the cellular growth of GB1B cells *in vitro*. For this propose, six azo-dyes (Methyl Orange, Alizarin Yellow, Lithol Rubine, Butter Yellow, Methyl Red, Sudan I) were tested for the concentration-effect relation and corresponding IC_{50} values. The azo-dye concentrations ranged from 0.5 µM to 128 µM. The proliferation rates were evaluated by performing the MTT assay at 3 and 7 days after the treatment.

3.1.1. The effect of Alizarin Yellow on cell viability

Our results showed that Alizarin Yellow induced dose- and time-dependent cytotoxic effect on GB1B cells. The three out of nine concentrations used in this study, did not induce a significant decrease in cell viability neither at 3 nor at 7 days, as shown in Supplementary Fig. 1A and Supplementary Fig. 2A. The treatment with 2 µM Alizarin Yellow for 3 days resulted in 17 % cell death and the cytotoxicity reached a plateau level at the concentrations of 4, 8 and 16 µM, where approximately 30 % of the cells were killed, at 3 days (Supplementary Fig. 1A). The treatment with 32 µM Alizarin Yellow for 3 days induced 52 % cytotoxicity (Supplementary Fig. 1A). Higher dose of Alizarin Yellow (64 µM) induced 59 % cytotoxicity, while at highest used dose of Alizarin Yellow (128 µM), only 32 % of GB1B cells survived at 3 days (Supplementary Fig. 1A).

As seen in Supplementary Fig. 2A, prolonged exposure to treatment for 7 days produced a more prominent cytotoxicity in GB1B cells, compared to 3 days treatment. After 7 days of treatment with 2 µM Alizarin Yellow we determined 11 % cell death, while concentrations of 4, 8 and 16 µM Alizarin Yellow determined similar cytotoxicities, with approximately 35 % of killed cells (Supplementary Fig. 2A). The treatment with 32 µM Alizarin Yellow for 7 days induced 57 % cytotoxicity (Supplementary Fig. 2A). By increasing the dose to 64 µM, Alizarin Yellow induced 65 % cytotoxicity, while only 25 % of GB1B cells survived at 7 days after the treatment with the highest used dose of Alizarin Yellow (128 µM) (Supplementary Fig. 2A).

The IC_{50} value for Alizarin yellow was 32.5852 µM at3 days of treatment (Supplementary Fig. 1B) and 20.5722 µM at at 7 days of treatment (Supplementary Fig. 2B).

3.1.2. The effect of Butter Yellow on cell viability

Also, our results proved that Butter Yellow induced dose- and time-dependent cytotoxic effect on GB1B cells. The lowest concentrations used in this study, did not induce a significant decrease in cell viability neither at 3 nor at 7 days, as shown in Supplementary Fig. 3A and Supplementary Fig. 4A. By treating the GB1B cells with 2 µM Butter Yellow for 3 days, a 10 % cellular death resulted and by using the concentrations of 4, 8 and 16 µM, we observed similar levels of cytotoxicity, with approximately 30 % of killed cells, at 3 days (Supplementary Fig. 3A). The treatment with 32 µM Butter Yellow for 3 days induced 48 % cytotoxicity (Supplementary Fig. 3A), and 64 µM dose of Butter Yellow induced 55 % cytotoxicity, while the 3 days of treatment with the highest used dose (128 µM) of Butter Yellow led to only 33 % cell survival (Supplementary Fig. 3A).

Prolonged exposure to treatment for 7 days (Supplementary Fig. 4A) produced a more prominent cytotoxicity in GB1B cells, compared to 3 days treatment (Supplementary Fig. 3A). After 7 days of treatment with 4 µM Butter Yellow we determined 16 % cell death, and concentrations of 8 µM Butter Yellow determined approximately 19 % of killed cells. By increasing the dose to 16 µM Butter Yellow, cellular death increased to 37 % (Supplementary Fig. 4A). The treatment with 32 µM Butter Yellow for 7 days induced approximately 49 % cytotoxicity (Supplementary Fig. 4A). Higher dose (64 µM) of Butter Yellow induced 64 % cytotoxicity, while only 31 % of GB1B cells survived at 7 days after the treatment with the highest used dose (128 µM) of Butter Yellow (Supplementary Fig. 4A).

At 3 days of treatment, the IC_{50} for Butter yellow was determined 37.033 µM (Supplementary Fig. 3B), and the value was 26.0696 µM (Supplementary Fig. 4B) for 7 days of treatment.

3.1.3. The effect of Helianthin on cell viability

Furthermore, our results showed that also Helianthin (Methyl Orange) induced dose- and time-dependent cytotoxic effect on GB1B cells. We did not observe a significant decrease in cell viability neither at 3 nor at 7 days, for the lowest three out of nine concentrations used in this study, as shown in Supplementary Fig. 5A and Supplementary Fig. 6A. The treatment with 2 µM Methyl orange for 3 days resulted in 5 % cell death and the cytotoxicity reached a plateau level at the concentrations of 4, 8 and 16 µM, where approximately 33 % of the cells were killed, at 3 days (Supplementary Fig. 5A). The treatment with 32 µM Helianthin for 3 days induced 54 % cytotoxicity (Supplementary Fig. 5A). Higher dose (64 µM) of Methyl orange induced 59 % cytotoxicity, while at highest used dose (128 µM) of Helianthin, only 28 % of GB1B cells survived at 3 days (Supplementary Fig. 5A).

As seen in Supplementary Fig. 6A, prolonged exposure (7 days) produced a more prominent cytotoxicity in GB1B cells, compared to 3 days treatment. After 7 days of treatment with 2 µM Methyl orange, we determined 15 % cell death, while concentrations of 4, 8 and 16 µM Helianthin determined similar cytotoxicities, with approximately 40 % of killed cells (Supplementary Fig. 6A). The treatment with 32 µM Methyl orange for 7 days induced 62 % cyto-

toxicity (Supplementary Fig. 6A). By increasing the dose to 64 μM, Helianthin induced 73 % cytotoxicity, while only 18 % of GB1B cells survived at 7 days after the treatment with the highest used dose (128 μM) of Methyl orange (Supplementary Fig. 6A).

Methyl Orange (Helianthin) demonstrated the highest cytotoxicity compared with the other 5 tested azo-dyes and also with TMZ, with the lowest IC_{50} value of 26.4684 μM for 3 days of treatment (Supplementary Fig. 5B), and 13.8808 for 7 days of treatment (Supplementary Fig. 6B).

3.1.4. The effect of Lithol Rubine on cell viability

A dose- and time-dependent cytotoxic effect on GB1B cells induced also Lithol Rubine. The lowest concentrations used in this study, did not induce a significant decrease in cell viability neither at 3 nor at 7 days, as shown in Supplementary Fig. 7A and Supplementary Fig. 8A. By treating the GB1B cells with 2 μM Lithol Rubine for 3 days, 8 % cellular death resulted and by using the concentrations of 4, 8 and 16 μM, we observed a plateau level of cytotoxicity, with approximately 28 % of killed cells, at 3 days (Supplementary Fig. 7A). The treatment with 32 μM Lithol Rubine for 3 days induced 47 % cytotoxicity (Supplementary Fig. 7A), and 64 μM dose of Lithol Rubine induced 51 % cytotoxicity, while the 3 days of treatment with the highest used dose (128 μM) of Lithol Rubine led to only 35 % cell survival (Supplementary Fig. 7A).

Prolonged exposure to treatment for 7 days (Supplementary Fig. 8A) produced a more prominent cytotoxicity in GB1B cells, compared to 3 days treatment (Supplementary Fig. 7A). After 7 days of treatment with 4 μM Lithol Rubine we determined 26 % cell death, and concentrations of 8 μM Lithol Rubine determined approximately 38 % of killed cells. By increasing the dose to 16 μM Lithol Rubine, cellular death increased to 51 % (Supplementary Fig. 8A). The treatment with 32 μM Lithol Rubine for 7 days induced approximately 62 % cytotoxicity (Supplementary Fig. 8A). Higher dose (64 μM) of Lithol Rubine induced 70 % cytotoxicity, while only 26 % of GB1B cells survived at 7 days after the treatment with the highest used dose (128 μM) of Lithol Rubine (Supplementary Fig. 8A).

For both 3 and 7 days after the treatment, Lithol Rubine registered good IC_{50} 40.4851 μM (Supplementary Fig. 7B) and 15.084 μM, repectively (Supplementary Fig. 8B).

3.1.5. The effect of Methyl Red on cell viability

Furthermore, our results showed that also Methyl Red induced dose- and time-dependent cytotoxic effect on GB1B cells. For the lowest three out of nine concentrations used in this study, we did not observe a significant decrease in cell viability neither at 3 nor at 7 days, as shown in Supplementary Fig. 9A and Supplementary Fig. 10A. The treatment with 2 μM Methyl Red for 3 days resulted in 12 % cell death and the cytotoxicity reached a plateau level at the concentrations of 4, 8 and 16 μM, where approximately 30 % of the cells were killed, at 3 days (Supplementary Fig. 9A). The treatment with 32 μM Methyl Red for 3 days induced 50 % cytotoxicity (Supplementary Fig. 9A). Higher dose (64 μM) of Methyl Red induced 57 % cytotoxicity, while at highest used dose (128 μM) of Methyl Red, only 35 % of GB1B cells survived at 3 days (Supplementary Fig. 9A).

As seen in Supplementary Fig. 9A and 10A, prolonged exposure with Methyl Red for 7 days of treatment, produced a more prominent cytotoxicity in GB1B cells, compared to 3 days of treatment. After 7 days with 2 μM Methyl Red, we determined 11 % cell death, while concentrations of 4, 8 and 16 μM Methyl Red determined similar cytotoxicities, with approximately 35 % of killed cells (Supplementary Fig. 10A). The treatment with 32 μM Methyl Red for 7 days induced 57 % cytotoxicity (Supplementary Fig. 10A). By increasing the dose to 64 μM, Methyl Red induced 65 % cytotoxicity, while only 25 % of GB1B cells survived at 7 days after the treatment with the highest used dose (128 μM) of Methyl Red (Supplementary Fig. 10A).

Methyl Red had intermediary IC_{50} at 3 and 7 days after the treatment: 35.65 μM (Supplementary Fig. 9B), and 19.5441 μM (Supplementary Fig. 10B).

3.1.6. The effect of Sudan I on cell viability

Our results showed that Sudan I induced dose- and time-dependent cytotoxic effect on GB1B cells. Same as for the other tested compounds, the three out of nine concentrations used in this study, did not induce a significant decrease in cell viability neither at 3 nor at 7 days, as shown in Supplementary Fig. 11A and Supplementary Fig. 12A. 2 μM treatment with Sudan I for 3 days resulted in 8 % cell death and the cytotoxicity reached a plateau level at the concentrations of 4, 8 and 16 μM, where approximately 26 % of the cells were killed, at 3 days (Supplementary Fig. 11A). The treatment with 32 μM Sudan I for 3 days induced 43 % cytotoxicity (Supplementary Fig. 11A). Higher dose (64 μM) of Sudan I induced 48 % cytotoxicity, while at highest used dose (128 μM) of Sudan I, 44 % of GB1B cells survived at 3 days (Supplementary Fig. 11A).

As seen in Supplementary Fig. 12A, prolonged exposure to treatment for 7 days produced a more prominent cytotoxicity in GB1B cells, compared to 3 days treatment. After 7 days of treatment with 2 μM Sudan I we determined 20 % cell death, while concentrations of 4 and 8 Sudan I determined similar cytotoxicities, with approximately 35 % of killed cells (Supplementary Fig. 12A). After 7 days of treatment with 16 μM Sudan I, 57 % cytotoxicity was observed, and 32 μM induced 65 % cytotoxicity (Supplementary Fig. 12A). By increasing the dose to 64 μM, Sudan I induced 70 % cytotoxicity, while only 26 % of GB1B cells survived at 7 days after the treatment with the highest used dose of Sudan I (128 μM) (Supplementary Fig. 12A).

Sudan I had the highest IC_{50} value at 3 days after the treatment (60.8639 μM) compared to the other tested azo-dyes (Supplementary Fig. 11B), but the lowest IC_{50} value after 7 days of treatment (12.4829 μM) (Supplementary Fig. 12B).

For the tested azo-dyes, a slight increase in proliferation has been observed up to 1 μM concentration, at 7 days after the treatment. This increase in cell survival was not statistically significant. Instead, the decrease in cell survival observed for higher concentrations was statistically significant compared with untreated control ($p < 0.05$), for all tested compounds.

3.2. The effect of TMZ on GB cells

First developed in the early 1980 s and despite the "blockbuster" status as standard first line treatment for GB in 2010, TMZ remains the only approved agent in its class (second generation imidazotetrazinone). In fact, it is an orally administrated prodrug that changes *in vivo* to the active alkylating form, commonly used for the treatment of GB (Moody et al., 2014). TMZ is stable at acidic pH, but at neutral or slightly basic pH it suffers spontaneous nonenzymatic hydrolysis, starting further reactions that liberate a reactive methyl diazonium cation which methylates various residues on guanine and adenosine bases, causing DNA lesions and eventually apoptosis (Zhang et al., 2012).

As expected, TMZ proved to be cytotoxic in our experiment performed on GB cell line. In low concentrations (0.5 μM-2 μM) for 3 days treatment TMZ was not cytotoxic for GB cells, as seen in Supplementary Fig. 13A. Further, higher concentrations led to an increase in cellular death. For example, while a concentration of 4 μM TMZ induced only approximately 13 % cellular death, 128 μM TMZ reduced cell survival by 32.23 % when compared with untreated GB1B cancer cells, at 3 days after the treatment (Supplementary Fig. 13A). This decrease in cell survival was statistically

significant compared with untreated control ($p < 0.05$). When using a treatment of 7 days, TMZ induced a maximum cytotoxicity of 61.19 % (128 μM) compared with control (Supplementary Fig. 14A). The results proved that 7 days cytotoxicity was directly proportional to TMZ concentrations, for all the tested values.

The IC_{50} for TMZ was 558.9811 μM (Supplementary Fig. 13B) after 3 days of treatment, and 62.0346 μM (Supplementary Fig. 14B) after 7 days of treatment.

3.3. Comparison of cytotoxicity induced of azo-dyes and TMZ by equimolar doses on GB cells

Considering that TMZ is used as standard first line treatment for GB, we aimed to compare each azo-dye effect with the effect of TMZ, in the same experimental situations.

We obtained good cytotoxic effects for each azo-dye, compared to TMZ. The results stand as critical reasoning of further developing process of the tested azo-dyes as antineoplastic agents.

3.3.1. Comparison of cytotoxicity induced of Alizarin Yellow and TMZ by equimolar doses on GB cells

The comparison between the cytotoxic effect induced on GB1B by Alizarin Yellow and /TMZ is shown in Fig. 1. At 3 days after the treatment (Fig. 1A), the difference between the cytotoxic effect induced by the lowest used dose of 0.5 μM Alizarin Yellow and the equimolar concentration of TMZ was 25.65 %. When 1 μM was used, Alizarin Yellow proved a cytotoxic effect with 7.4 % higher than TMZ at equimolar dose. The difference between the cytotoxic effect induced by Alizarin Yellow and TMZ treatment at equimolar dose increased directly proportional with concentrations: 16.02 % for 2 μM, 16.09 % for 4 μM, 22.49 % for 8 μM, 23.77 % for 16 μM, 36.93 % for 32 μM, and 43.96 % for 64 μM. At the highest used concentration (128 μM), the difference between Alizarin Yellow and TMZ cytotoxicity values decreased at 35.55 %.

When prolonged exposure (7 days of treatment) was used, the difference in cell killing efficiency between azo-dye and TMZ cells was slightly higher, compared to short term exposure (Fig. 1B).

At low concentrations (0.5–2 μM), TMZ had higher cytotoxicity than Alizarin Yellow (16.45 % for 0.5 μM, 24.12 % for 1 μM and 13.2 % for 2 μM), but when higher equimolar concentrations were used, Alizarin yellow achieved better cytotoxicity. The treatment with 2 μM TMZ resulted in a 13.2 % lower cytotoxic effect that the same concentration of Alizarin Yellow and 4 μM TMZ treatment proved a cytotoxic effect with 5.26 % lower that when the same concentration of Alizarin Yellow was used. The difference was 3.38 % for 8 μM, 10.15 % for 16 μM, 11.53 % for 32 μM, and 10.86 % for 64 μM. At the highest used concentration (128 μM), the difference between Alizarin Yellow and TMZ cytotoxicities was 13.45 %.

3.3.2. Comparison of cytotoxicity induced of Butter Yellow and TMZ by equimolar doses on GB cells

We compared the cytotoxicity induced by Butter Yellow and TMZ on GB1B cell line, for both experimental situations: 3 and 7 days of treatment. The difference between the cytotoxic effect induced by the lowest used dose of 0.5 μM Butter Yellow and the equimolar concentration of TMZ was 20.74 % at 3 days after the treatment (Fig. 2A). When 1 μM was used, Butter Yellow proved a cytotoxic effect with 5.44 % higher than TMZ, for equimolar dose. Further, the difference increased directly proportional with concentrations: 9.28 % for 2 μM, 12.94 % for 4 μM, 19.16 % for 8 μM, 23.73 % for 16 μM, 33.13 % for 32 μM, and 40.25 % for 64 μM. At the highest tested concentration, Butter Yellow was with 35.55 % more cytotoxic than TMZ at equimolar concentration.

At 7 days of treatment the difference in cell killing efficiency between azo-dye and TMZ cells oscillated in both positive and negative sense. At low dose TMZ had higher values of cytotoxicity compared to Butter yellow. The difference was 31.64 % for 0.5 μM, 41.88 % for 1 μM, 27.51 % for 2 μM, 9.33 % for 4 μM and 11.86 % for 8 μM. Interestingly, at increased equimolar concentrations, Butter yellow was more cytotoxic. For example, at 16 μM TMZ proved a cytotoxic effect with 5.85 % lower that when the same concentration of Butter Yellow was used. The difference was 3.53 % for 32 μM, 9.99 % for 64 μM, and 7.42 % for 128 μM (Fig. 2B).

3.3.3. Comparison of cytotoxicity induced of Helianthin and TMZ by equimolar doses on GB cells

The analogy between the cytotoxic effect induced on GB1B by Helianthin and TMZ is presented in Fig. 3. The lowest tested dose of 0.5 μM Helianthin and the equimolar concentration of TMZ induced a difference in the cytotoxic effect of 17.27 % for the 3 days of treatment situation (Fig. 3A). 1 μM Helianthin proved a cytotoxic effect with 6.25 % higher than TMZ, at equimolar dose. Further, the difference augmented directly proportional with dose: 11.97 % for 2 μM, 15.58 % for 4 μM, 22.99 % for 8 μM, 26.8 % for 16 μM, 38.89 % for 32 μM, and 44.49 % for 64 μM. The difference between Helianthin and TMZ cytotoxicity values decreased slowly up to 39.29 % for the highest tested concentration (128 μM).

When prolonged exposure was used, the difference in cell killing efficiency between azo-dye and TMZ cells was not so evident, compared to short term exposure (Fig. 3B). At low concentrations (0.5–2 μM), TMZ killed more efficiently the GB1B cells than Helianthin with differences of 17.29 % for 0.5 μM, 24.07 % for

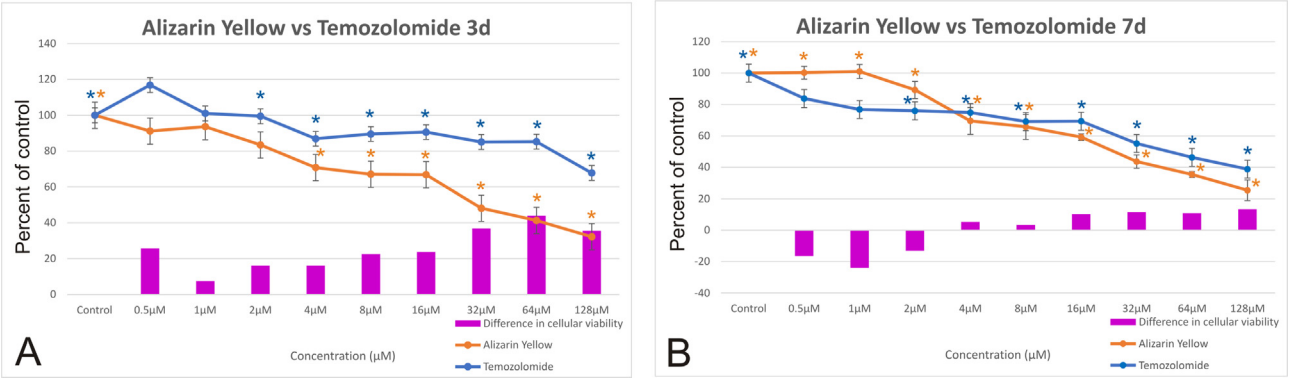

Fig. 1. Comparison between the cytotoxic effects on GB cells after 3 days (A) and 7 days (B) of treatment with Alizarin Yellow or TMZ. Notes: Results are expressed as percent of control. Positive bars represent increase in cytotoxicity of azo-dye compared to TMZ. Negative bars represent decrease in cytotoxicity of azo-dye compared to TMZ.* represents statistically significant values ($p < 0.05$).

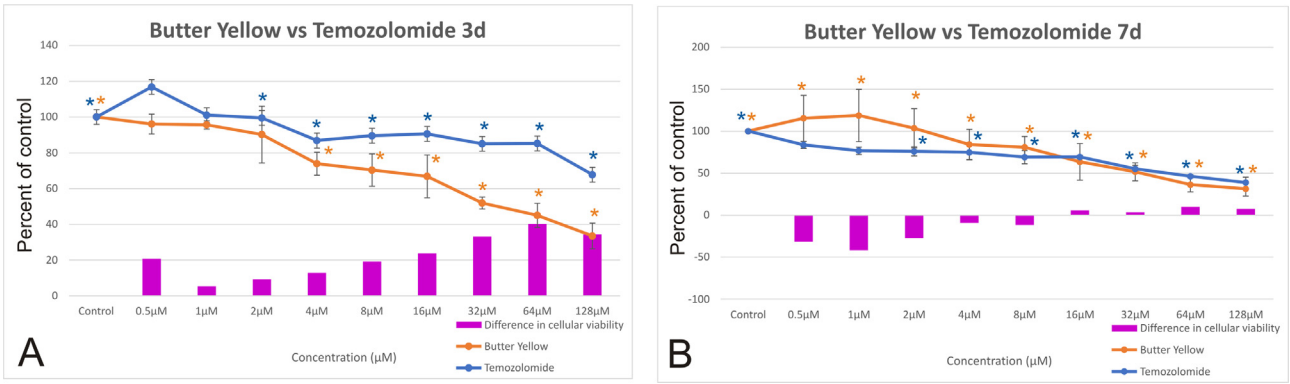

Fig. 2. Comparison between the cytotoxic effect of Butter Yellow (orange bars) and TMZ (blue bars) on GB cells after 3 days (A) and 7 days (B) of treatment with Butter Yellow or TMZ. Notes: Results are expressed as percent of control. Positive bars represent increase in cytotoxicity of azo-dye compared to TMZ. Negative bars represent decrease in cytotoxicity of azo-dye compared to TMZ..* represents statistically significant values ($p < 0.05$).

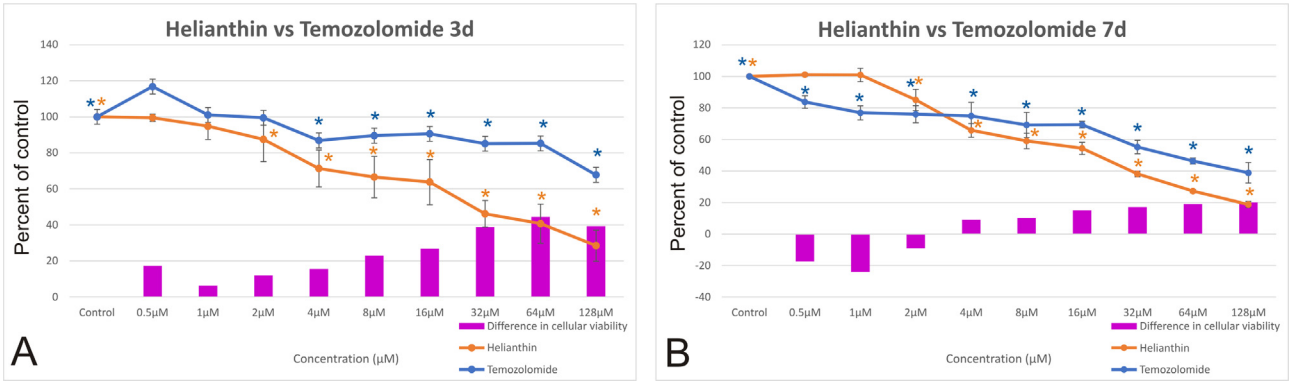

Fig. 3. Comparison between the cytotoxic effects on GB cells after 3 days (A) and 7 days (B) of treatment with Helianthin or TMZ. Notes: Results are expressed as percent of control. Positive bars represent increase in cytotoxicity of azo-dye compared to TMZ. Negative bars represent decrease in cytotoxicity of azo-dye compared to TMZ..* represents statistically significant values ($p < 0.05$).

1 μM and 9.04 % for 2 μM, but when higher equimolar concentrations were used, Helianthin achieved better cytotoxicity.

For example, at 4 μM TMZ proved a cytotoxic effect with 9.08 % lower that when the same concentration of Helianthin was used. The difference was 10.15 % for 8 μM, 15.03 % for 16 μM, 17.09 % for 32 μM, and 19.08 % for 64 μM. At the highest equimolar tested dose, Helianthin was with 20.05 % more cytotoxic than TMZ.

3.3.4. Comparison of cytotoxicity induced of Lithol Rubine and TMZ by equimolar doses on GB cells

We compared the cytotoxicity induced by Lithol Rubine and TMZ on GB1B cell line, for foth experimental situations: 3 and 7 days of treatment (Fig. 4). The lowest tested dose of 0.5 μM Lithol Rubine and the equimolar concentration of TMZ induced a difference in the cytotoxic effect of 15.97 % for the 3 days of treatment situation (Fig. 4A). At 1 μM, Lithol Rubine proved a cytotoxic effect with 5.11 % higher than TMZ, at equimolar dose. Further, the difference augmented directly proportional with dose: 7.73 % for 2 μM, 13.4 % for 4 μM, 18.02 % for 8 μM, 23.19 % for 16 μM, 31.52 % for 32 μM, and 36.56 % for 64 μM. For the highest tested concentration, Lithol Rubine was with 32.6 % more cytotoxicic than TMZ.

The second experimental situation was of 7 days of treatment with the corresponding agent (Fig. 4B). For this situation, concentrations of TMZ killed more efficiently the GB1B cells than Lithol Rubine with differences of 18.38 % for 0.5 μM, 25.81 % for 1 μM and 10.14 % for 2 μM. On the other hand, when higher equimolar concentrations were used, Lithol Rubine achieved better cytotoxicity. For example, at 8 μM TMZ proved a cytotoxic effect with 6.88 % lower that when the same concentration of Lithol Rubine was used. The difference was 20.77 % for 16 μM, 17.25 % for 32 μM, 16.47 % for 64 μM, and 13 % for 128 μM.

3.3.5. Comparison of cytotoxicity induced of Methyl Red and TMZ by equimolar doses on GB cells

Furthermore, the analogy between the cytotoxic effect induced on GB1B by Methyl Red and TMZ is shown in Fig. 5. The lowest tested dose of 0.5 μM Methyl Red was with 17.27 % more cytotoxic than the equimolar concentration of TMZ for the 3 days of treatment situation (Fig. 5A). 1 μM Methyl Red proved a cytotoxic effect with 3.90 % higher than TMZ, at equimolar dose. For doses in the range 4 μM – 64 μM, the difference increased directly proportional with dose: 9.01 % for 4 μM, 20.44 % for 8 μM, 22.51 % for 16 μM, 35.35 % for 32 μM, and 41.58 % for 64 μM. Further, the cytotoxic effect of Methyl Red was with 33.08 higher than and TMZ for equimolar dose.

For prolonged treatment (7 days), the difference in cell killing efficiency between azo-dye and TMZ cells ranged between −24.14 % and 13.49 % (Fig. 5B). At low concentrations (0.5–2 μM), TMZ killed more efficiently the GB1B cells than Methyl Red with differences of 16.42 % for 0.5 μM, 24.14 % for 1 μM and 12.40 % for 2 μM, but when higher equimolar concentrations were used, Methyl Red achieved better cytotoxicity. For example, 4 μM Methyl Red achieved a cytotoxic effect with 6.52 % higher that when the same concentration of TMZ was used. The difference was 4.44 % for 8 μM, 11.08 % for 16 μM, 12.08 % for 32 μM, and 11.32 % for 64 μM. After a treatment of 7 days with 128 μM dose, Methyl Red was with 13.49 % more cytotoxic than an equimolar dose of TMZ.

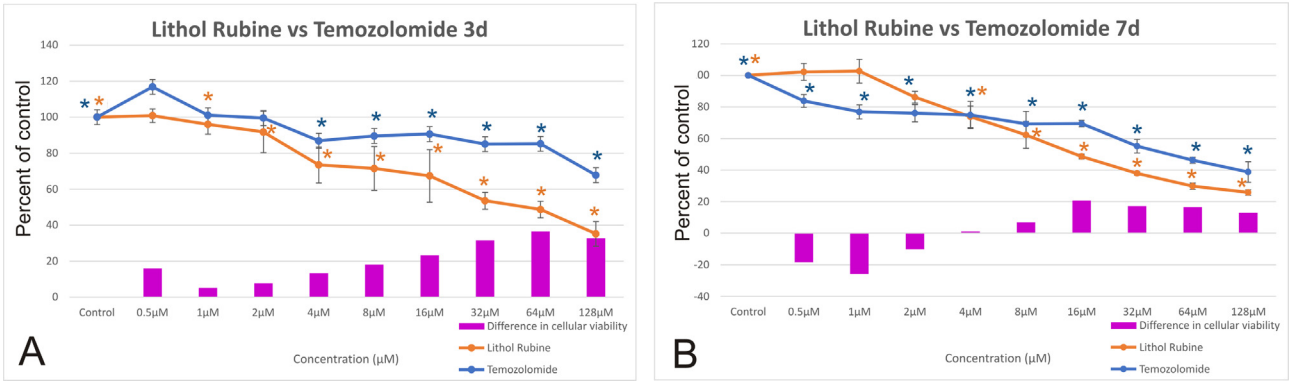

Fig. 4. Comparison between the cytotoxic effects on GB cells after 3 days (A) and 7 days (B) of treatment with Lithol Rubine or TMZ. Notes: Results are expressed as percent of control. Positive bars represent increase in cytotoxicity of azo-dye compared to TMZ. Negative bars represent decrease in cytotoxicity of azo-dye compared to TMZ..* represents statistically significant values ($p < 0.05$).

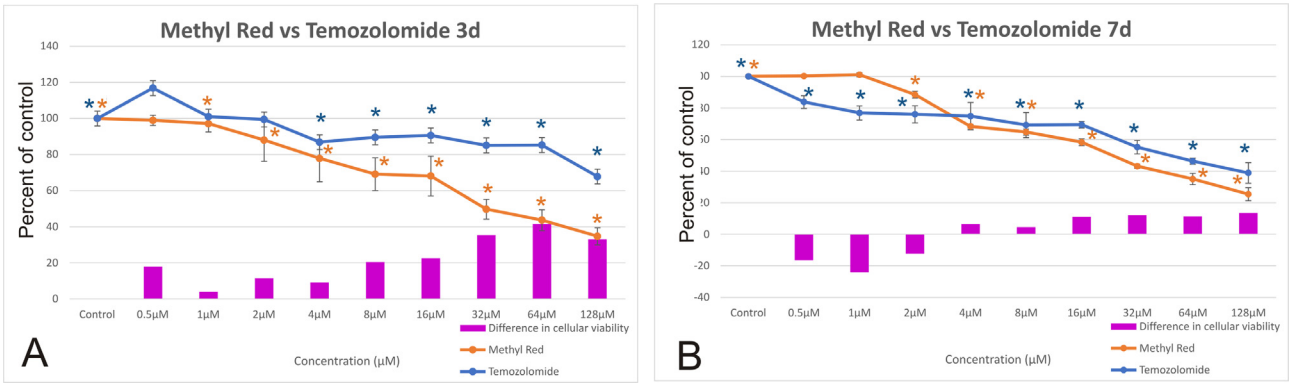

Fig. 5. Comparison between the cytotoxic effects on GB cells after 3 days (A) and 7 days (B) of treatment with Methyl Red or TMZ. Notes: Results are expressed as percent of control. Positive bars represent increase in cytotoxicity of azo-dye compared to TMZ. Negative bars represent decrease in cytotoxicity of azo-dye compared to TMZ..* represents statistically significant values ($p < 0.05$).

3.3.6. Comparison of cytotoxicity induced of Sudan I and TMZ by equimolar doses on GB cells

The efficiency with which equimolar concentrations of Sudan I and TMZ induced cytotoxicity on GB1B is presented in Fig. 6. The analysis was performed for azo-dye agent compared to TMZ, for both experimental situations. At 3 days after the treatment (Fig. 6A), 0.5 μM Sudan induced a cytotoxic effect with 17.76 % higher than the same dose of TMZ. When 1 μM was used, Sudan I proved a cytotoxic effect with 3.25 % higher than TMZ at equimolar dose. Further, the difference increased directly proportional with concentrations: 7.36 % for 2 μM, 11.98 % for 4 μM, 17.93 % for 8 μM, 17.53 % for 16 μM, 27.64 % for 32 μM, and 33.18 % for 64 μM. At the highest used concentration (128 μM), Sudan I was with 23.6 % more cytotoxic than the equimolar dose of TMZ.

The second experimental situation involved a 7 days treatment (Fig. 6B). At prolonged exposure and at low concentrations (0.5–2 μM), TMZ had higher cytotoxicity than Sudan I (14.25 % for 0.5 μM, 15.62 % for 1 μM and 4.62 % for 2 μM). Interestingly, at higher equimolar concentrations, Sudan I achieved better cytotoxicity. For example, at 4 μM TMZ proved a cytotoxic effect with 8.8 %

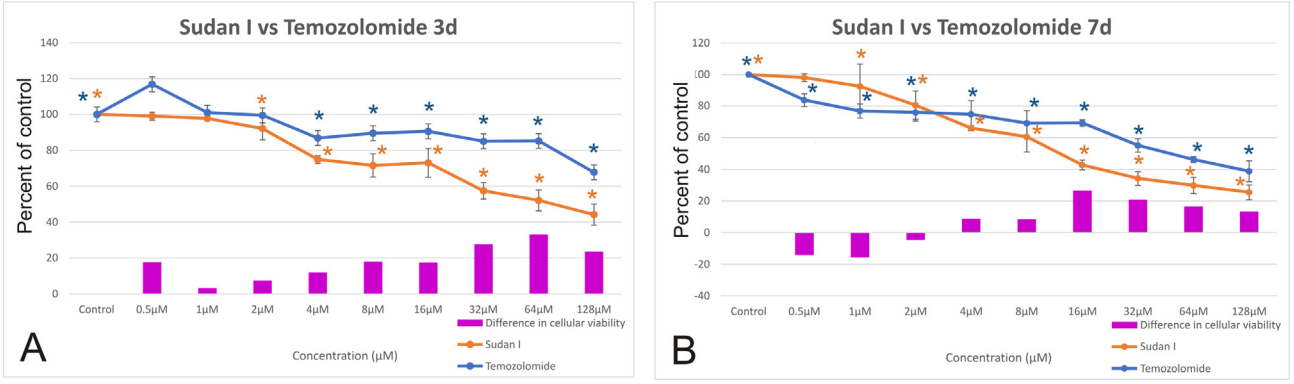

Fig. 6. Comparison between the cytotoxic effects on GB cells after 3 days (A) and 7 days (B) of treatment with Sudan I or TMZ. Notes: Results are expressed as percent of control. Positive bars represent increase in cytotoxicity of azo-dye compared to TMZ. Negative bars represent decrease in cytotoxicity of azo-dye compared to TMZ..* represents statistically significant values ($p < 0.05$).

lower that when the same concentration of Sudan I was used. The difference was 8.51 % for 8 μM, 26.6 % for 16 μM, 20.88 % for 32 μM, and 16.43 % for 64 μM. At the highest tested concentration (128 μM), the difference between Sudan I and TMZ cytotoxicity effect was 13.30 %.

4. Discussion

Although there are several approaches used in the treatment of GB and several drugs have been approved by the FDA, the outcomes remain poor (Rong et al., 2022; Poon et al., 2021).

In recent years, azo-dye compounds have gained more and more attention as anticancer drugs (Sevastre et al., 2021c). The carcinogenicity and mutagenicity of azo dyes have been intensively discussed over the years. Nevertheless, the correlation between the mutagenicity tests and carcinogenicity of azo dyes *in vivo* on animal experiments remains poor (Chung et al., 2000). Their use as anticancer drugs is supported by the fact that these compounds have a very weak cytotoxic effect on normal cells compared to malignant cells (Venugopal et al., 2019).

The antineoplastic activity of azo dyes has been very little studied, there are only a few dozen studies in the literature that show their *in vitro* cytotoxic effect on malignant cell lines and there is no study that analyzes their effect *in vivo*, on the animal model. In a study by Tadić, the antitumor activity of seven novel azo-dyes have been reported on prostate adenocarcinoma, lung carcinoma and chronic myelogenous leukemia cell lines (Tadić et al., 2021).

The antiproliferative effect of azo-dye compounds has also been demonstrated on other types of human cancer such as: cervical carcinoma, leukemia (Zahara Fiza et al., 2022), breast cancer (Kantar et al., 2015; Harisha et al., 2020), pancreatic cancer (Harisha et al., 2020; Maliyappa et al., 2022), hepatocellular carcinoma (Khedr et al., 2021).

Here, we found that six dyes compounds that have an azo-linkage (-N=N-) as a key functional group, induced antiproliferative effect of on human GB cells.

At this time, there are few studies in the literature that show the effect of azo dye in brain tumors, GB. Few doors have been opened for azo-compounds as potential chemotherapeutic drugs for the treatment of GB. There is a single work that presented the azo-compounds effect on GB is an *in silico* study. Using *in silico* molecular docking approach, the binding affinities of two azo compounds and the standard drug (TMZ) against 4 different GBM proteins: 6 s79, 6bft, 1Is5, and 1z2b have been evaluated and compared. Both compounds (E)-6-((4,6-dichloro-1,3,5-triazin-2-yl)amino)-3-((4-for-mylphenyl)diazenyl)-4-hydroxynaphthalen-2-yl hydrogen sulfite (compound D) and (E)-6-((4,6-dichloro-1,3,5-triazin-2-yl)amino)-4-hydroxy-3-(phenyldiazenyl)naphthalen-2-yl hydrogen sulfite showed good water solubility, good lipophilicity and relatively higher binding affinities with 6 s76, 6bft and 1Is5 proteins compared with the standard drug (Udoikono et al., 2022).

The molecular mechanisms underlying azo dyes cytotoxicity in cancer cells has been extremely little studied. Qi Liu et al recently proved that azobenzene-based prodrug possesses desirable targeting ability against tumor-hypoxia and can eliminate chemoresistance when compared with the conventional chemotherapy drugs as chloroethylnitrosoureas (Liu et al., 2021).

In our previous research, we analyzed the effect of Methyl Red, Methyl Yellow and Methyl Orange in concentrations from 0.01 μM to1 μM, on HGG cells viability (Alexandru et al., 2011). These concentrations induced cytotoxicity in high grade glioma (HGG) but did not reach the IC_{50} value. Furthermore, we demonstrated that Fe_3O_4 magnetic nanoparticles loaded with Helianthin induced cytotoxicity of human GB cells (Costachi et al., 2021). Our previous results have been very promising, therefore we continued our experiments by using a different HGG cell line and higher concentrations of several azo-dyes to be able to calculate the IC_{50} values. Currently, there are few experimental and theoretical articles published in the field of cancer, focusing on azo-dye cytotoxic effect in various types of cancer. It is worth mentioning that at the moment, the only experiments testing the cytotoxic effect of azo-dyes in HGG tumors have been performed only by our research group.

Azo-compounds have been reported to be involved in various biological reactions. For example, Alizarin yellow GG known as Mordant Yellow 1 or Metachrome Yellow, contains an azo-linked salicylate moiety with structural resemblance to sulfasalazine and can interact with the cell membrane antiporter protein (Nehser et al., 2019). In a study performed by Brown et al., the mutagenic and possibly carcinogenic potential of short-term treatment of Alizarin yellow was reported using *in vitro* tests. The carcinogenic potential was explained by the presence of mono group nitro-substituted in the molecule (Brown et al., 1978). In our experiment, after 3 days of treatment with Alizarin Yellow, the results showed the 67.8 % reduction of cell survival for the maximum tested concentration, compared with control cells (Supplementary Fig. 1A). Once the concentration of Alizarin Yellow used as treatment increased, the cytotoxicity also increased in a direct proportional manner. The inhibition of cellular viability was more evident at 7 days of treatment, when Alizarin Yellow induced a maximum cytotoxicity of 75 % compared with control (Supplementary Fig. 2A). There are no recent published articles reporting the cytotoxic effect of Alizarin yellow GG, so our results could represent its initial stage as novel drug development.

Butter yellow (Methyl yellow, 4-Dimethylamino-azobenzene) has been banned for use in foods in late 80′s (U.S. Department of Health and Human Services. Public Health Service CfDaH, Division of Standards, Development and Technology Transfer, 1988) for its carcinogenicity (Tokiwa et al., 1976). More recent results have been published suggesting that Butter yellow has cell proliferative potential in gall bladder cells following up-regulation of Proto oncogene Neu (ErbB2) and Cyclooxigenase-2 (COX-2) (Biswas et al., 2005; Mishra et al., 2012). Nevertheless, there is few evidence regarding Butter yellow's antiproliferative effect *in vitro*. In our previous research, we found that two azo-dyes Butter yellow and Methyl red (4-Dimethylaminoazobenzene-2′-carboxylic acid) did not inhibit the growth of human GB cells 18 and 38 (Alexandru et al., 2011). For this matter, we included both compounds in the azo-dye palette screening. Our results show that Butter Yellow treatment reduces GB1B cell survival by a maximum of 68.61 %, compared with control, at 7 days after the treatment (Supplementary Fig. 4A). In these experimental conditions, we found good IC_{50} values of Butter Yellow (37.033 μM and 26.0696 μM for 3 and 7 days of treatment).

Regarding Methyl Red, there were evidence ever since 1978 that short-term treatment had mutagenic and carcinogenic effect on *in vitro* tests (Nehser et al., 2019). In our current article, we report that Methyl Red treatment has cytotoxic effect on GB1B cell line. At 3 days after the treatment, the minimum used concentration induced only approximately 1.04 % cellular death, while 128 μM Methyl Red reduced cell survival by approximately 65.31 % when compared with untreated GB1B cancer cells (Supplementary Fig. 9A). This decrease in cell survival was statistically significant compared with untreated control ($p < 0.05$). Methyl Red induced a maximum cytotoxicity of 74.68 % compared with control cells for the maximum tested concentration of 128 μM, 7 days after the treatment (Supplementary Fig. 10A). In our experimental conditions, we determined reliable IC_{50} values for Methyl red (35.65 μM and 19.5441 μM for 3 and 7 days of treatment) (Supplementary Fig. 9B, Supplementary Fig. 10B). The results that we obtained could represent the beginning of a repurposing process involving these two azo-dyes, considering their significant cyto-

toxic effects on GB1B under both experimental conditions (3 and 7 days of treatment).

An azo-dye agent that gain a lot of interest form researchers in the last decades is Helianthin. In previous studies, the synthetic dye Methyl orange induced apoptotic cellular death in HGG cells, used both as water solution (Alexandru et al., 2011) and as magnetic nanoparticles (Costachi et al., 2021). Also, Helianthin manifested antiproliferative effect on human GB cells (lines 18 and 38), which involved two mechanisms: by inducing apoptosis and by inhibiting specific signaling pathway on GB cell lines by down-regulating RTK activity (Alexandru et al., 2011). In 18 GB cells, Helianthin treatment down-regulated ERK1/2 phosphorylation without affecting Phosphoinositide 3-kinases (PI3K) activity, and both PI3K and ERK1/2 have been reduced in GB 38 cell line. PI3-K and ERK1/2 are key proteins in the downstream signaling of most growth factor receptors, therefore targeting the common pathway of epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R) may be particularly useful due to these kinases. Furthermore, we demonstrated that Fe_3O_4 magnetic nanoparticles loaded with the same azo dye (Helianthin) induced dose and time dependent cytotoxicity in human GB cells (Costachi et al., 2021).

In the current experiment, Helianthin decreased the cellular viability of GB1B for all tested concentrations both for short and long-term treatment. While minimum used concentration (0.5 μM) induced only approximately 0.48 % cells death, 128 μM Helianthin reduced cell survival by approximately 71.52 % when compared with untreated control GB1B cancer cells 3 days after the treatment (Supplementary Fig. 5A). Helianthin demonstrated the highest cytotoxicity compared with the other 5 tested azo-dyes and also with TMZ, with the lowest IC_{50} value of 26.4684 μM for 3 days of treatment (Supplementary Fig. 5B), and 13.8808 for 7 days of treatment (Supplementary Fig. 6B), which proves that it can be a reliable potential candidate as anticancer agent for GB treatment.

Lithol Rubine also known as E180 or 3-Hydroxy-4-[(4-methyl-2-sulfophenyl)azo]-2-naphthoic acid calcium salt is a synthetic azo dye. The *in vitro* data available for genotoxicity suggested that Lithol Rubine BK is not mutagenic neither clastogenic. The azo reduction *in vivo*, leads to the formation of a carboxylated amino-naphthol and a sulphonated aromatic amine that is not obtained in the standard *in vitro* tests. Although aromatic amines have been associated with genotoxicity and carcinogenicity in *in vitro* assays, Lithol Rubine BK was negative in these tests (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2010). In our experiment, Lithol Rubine proved significant cytotoxic effect on GB1B line. Lithol Rubine and Methyl Red showed intermediary IC_{50} at 3 and 7 days after the treatment (Supplementary Fig. 7A-B, Supplementary Fig. 8A-B, Supplementary Fig. 9A-B, Supplementary Fig. 10A-B). At maximum tested concentration for 3 days of treatment, Lithol Rubine reduced cell survival by approximately 64.83 % and by 74.19 % for 7 days of treatment, when compared with control.

For almost all tested azo-dyes, a slight increase in proliferation has been observed up to 1 μM concentration, at 7 days after the treatment, but it was not statistically significant. Instead, the decrease in cell survival observed for higher concentrations was statistically significant compared with untreated control ($p < 0.05$), for all tested compounds.

Furthermore, International Agency for Research on Cancer classifies Sudan I as a Category 3 carcinogen agent (Annex I of the Directive 67/548/EC). However, little is known regarding the dose response of more biologically relevant low doses of Sudan-1. Studies report that its genotoxic activity was greatly increased in MCL-5 cells transfected to stably express CYP1A2, CYP2A6 and CYP3A4, enzymes that metabolically activate Sudan-1. This data suggested that the oxidation metabolites were both more clastogenic and more mutagenic than the parent agent. By testing the cytotoxicity of Sudan I on GB1B, we registered the highest IC_{50} value at 3 days after the treatment, but the lowest IC_{50} value after 7 days of treatment compared to the other tested azo-dyes (Supplementary Fig. 12B). As expected, the cytotoxicity of Sudan I was directly proportional to concentrations under both experimental situations (3 and 7 days of treatment).

Differently from the little data regarding azo-dyes cytotoxicity, TMZ (methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide) received great attention respecting to GB treatment. It is well known that TMZ induces cell cycle arrest in G2/M, eventually leading to apoptosis (Chien et al., 2021). At the moment, this alkylating agent has been widely used to treat GB, and many methods have been developed both to enhance its activity and to reduce the chemoresistance (Akbarnejad et al., 2017; Gerlach et al., 2022; Kang et al., 2022; Shao et al., 2019). Also, attention must be paid to concomitant administration of other medication, due to their influence on the TMZ efficacy (Drljača et al., 2022). Different strategies have been used to elucidate the TMZ influence on cancer cells. It has been reported that TMZ induced apoptosis in U87MG GB cell line at therapeutic dose, and decreased the expression levels of Vascular Endothelial Growth Factor (VEGF) and C-X-C chemokine receptor type 4 (CXCR4) (Mirabdaly et al., 2020). Recently, another GB cell line T98G who underwent two days treatment with TMZ showed significant deregulation of 7 genes: BCL2L1, BBC3, BIRC2, RIPK1, CASP3, DAPK1 and CARD6 involved in apoptosis (Vidomanova et al., 2022).

In our experiment, we determined significantly higher TMZ IC_{50} values (558.9811) compared to the tested azo-dyes for 3 days of treatment (Supplementary Fig. 13B), but in the range reported by researchers for different GB cell lines (94–1049 μM) (Ferretti et al., 2013). We now report that our results of *in vitro* cytotoxicity screening revealed that all the tested compounds proved promising activity against GB1B cell line. Antiproliferative evaluation of the members of the azo-dye series against GB cell line showed moderate to high potency to inhibit cellular viability. According to American National Standards Institute, agents with IC_{50} lower than 30 μM on experimental cancer cell lines may represent promising anticancer compounds for drug development (Hughes et al., 2011). In our experimental conditions of 7 days of treatment, all tested azo-dyes showed IC_{50} values < 30 μM against GB1B cell line, and proved to be more cytotoxic than TMZ at the same dose of either medication. Minimum inhibitory concentration was 0.5 μM for all tested agents and generally provoked −15 – 16 % cell death in GB1B cell line, whereas the highest concentrations (128 μM) drastically reduced cell viability, for example up to 18.74 % cell viability when Helianthin was used for 7 days of treatment. However, after 3 days of treatment, only Helianthin depicted an IC_{50} < 30 μM. The GB1B cell line answered in a dose dependent manner to the treatment with all studied agents when concentrations greater than 2 μM have been used. Compared with TMZ, all azo dyes required lower doses to achieve the same results, promoting them as possible therapeutic window for novel anticancer strategies. After 7 days of treatment, we registered an IC_{50} of 62.0346 μM for TMZ (Supplementary Fig. 14B), approximately-two folds higher that for the tested azo-dyes under the same experimental conditions. All tested azo-dyes proved to be more cytotoxic than TMZ at equimolar concentration, at 3 days of treatment, for the same experimental conditions (Figs. 1-6). For long term treatment, TMZ proved to be less cytotoxic than any other tested azo-dye, when used in concentrations higher than 8 μM (Figs. 1-6).

We obtained good cytotoxic effects for each azo-dye, compared to TMZ. These current results come to confirm the high potential of azo-dyes compounds as cytotoxic drugs in the battle against cancer.

The results stand as important motivation for further developing process of the tested azo-dyes as antineoplastic agents. There are few experimental and theoretical articles published in recent years focusing on azo-dye cytotoxic effect in various types of cancer. This study may focus the attention on azo-dye agents as unexploited source of drugs for cancer treatment.

5. Conclusions

The cytotoxic activity of azo-dye compounds on cancer was very little studied and their effect on brain tumors even less understood. Only few *in silico* studies demonstrated the potential of some azo dye compounds as chemotherapy drugs in the treatment of GB. At this time, there are few experimental and theoretical articles published in the literature, regarding the cytotoxic effect of azo-dyes in various types of cancer. The current study proved that azo-dye compounds induced antiproliferative effect against GB cells *in vitro* and compared with TMZ (taken as a reference drug for GB treatment), the compounds required lower doses to achieve the same results. That represents the novelty of the research. However, due to its novelty, the question remains open, whether the studied azo-dyes might be effective as antitumor agents *in vivo*.

This study may focus the attention on azo-dye agents that may represent an unexploited source of agents for cancer treatment. Given their cytotoxicity on GB cell line *in vitro*, their effect both *in vitro* and *in vivo* on other glioma subtypes and further other types of cancer would be of interest.

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CRediT authorship contribution statement

Ani-Simona Sevastre: Funding acquisition, Project administration, Writing – review & editing, Conceptualization, Investigation. **Carina Baloi:** Writing – original draft, Conceptualization, Investigation. **Oana Alexandru:** Visualization. **Ligia Gabriela Tataranu:** Methodology. **Oana Stefana Popescu:** Supervision, Writing – original draft, Investigation, Formal analysis. **Anica Dricu:** Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103599.

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