Gadobutrol Exerts a Cytogenotoxic Effect in SH-SY5Y Neuroblastoma Cells

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Abstract

Objective: Gadobutrol is a macrocyclic gadolinium-based contrast agent (GBCA) used in magnetic resonance imaging (MRI). Although it is also used in the imaging of malignant tumors, its effect on SH-SY5Y neuroblastoma cells remains unclear. The aim of this study was to investigate the effects of gadobutol on cytotoxicity and genotoxicity in SH-SY5Y neuroblastoma cells.

Methods: After incubation of neuroblastoma cells with gadobutrol (0.1 mM, 1 mM, 10 mM, and 100 mM), cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. After determining the IC_{50} value of gadobutrol, the genotoxicity effect of gadobutrol (1.75 mM, 3.50 mM, and 7 mM) on neuroblastoma cells was examined by Comet assay.

Results: Gadobutrol (0.1 mM, 1 mM, 10 mM, and 100 mM) statistically significantly decreased cell viability in SH-SY5Y neuroblastoma cells ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$, respectively). Gadobutrol (1.75 mM, 3.50 mM, and 7 mM) caused an increase in genotoxicity in neuroblastoma cells. However, gadobutrol (3.50 mM) and gadobutrol (7 mM) statistically significantly increased genotoxicity in neuroblastoma cells ($p < 0.05$ and $p < 0.01$, respectively).

Conclusion: The results of this in vitro study show that gadobutrol used as a contrast agent in MRI increases both cytotoxicity and genotoxicity in SH-SY5Y neuroblastoma cells. The cytogenotoxic effect of gadobutrol in SH-SY5Y neuroblastoma cells may point to it as a promising new strategy for the treatment of neuroblastoma.

Keywords: neuroblastoma; gadobutrol; cytotoxicity; genotoxicity; SH-SY5Y cell.

INTRODUCTION

Neuroblastoma is one of the most common extracranial solid tumors in children, accounting for approximately 8-10% of childhood cancers. Neuroblastoma represents a significant proportion of cancer-related deaths in childhood [1-3]. Despite advances in multimodal treatment including surgery, radiotherapy, chemotherapy and stem cell transplantation, unfortunately, outcomes in children with relapsed or refractory neuroblastoma remain unsatisfactory, with mortality rates well above 50% [4]. It is therefore vital to identify novel and high-potential therapeutics for the treatment of neuroblastoma.

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Gadobutrol is a well-known non-ionic macrocyclic gadolinium-based contrast agent (GBCA) used in magnetic resonance imaging (MRI) [5]. It is also used in the prognosis and diagnosis of tumors such as neuroblastoma. Clinical evidence suggests that gadobutol is a particularly effective GBCA for diagnostic MRI in pediatric patients [6]. Recent studies indicate that gadolinium may have therapeutic potential in addition to its diagnostic use [7]. Gadolinium may have satisfactory therapeutic potential in cancer treatment. In radiation therapy, gadolinium is assumed to sensitize tumor cells and kill cancer cells [7]. Furthermore, the combination of therapeutic and diagnostic tools in cancer research, referred to as theranostics, is exciting. Recently, the demand for new theranostic agents has been increasing. Among the most prominent and promising cancer theranostics is gadolinium [8]. However, more information is needed on the effects of the diagnostic gadolinium chelate gadobutrol on neuroblastoma, a childhood cancer.

Although studies have focused on the safety of gadobutrol, it has been reported that gadobutrol may affect cell viability and cell proliferation in different cancer cell lines [9]. Recent studies suggest that gadobutrol may have proliferation suppressive effects in cancer cells beyond alternative therapeutic options [10]. Furthermore, the fact that gadobutrol causes an increase in hypersensitivity in healthy or pathological conditions [11] and increases neurotoxicity in peripheral neurons [12] may suggest that it may also act on neuroblastoma cells. The most widely used cell line in neuroblastoma research is the human SH-SY5Y neuroblastoma cell line [13]. These cell lines play an important role in elucidating the pathophysiology of neuroblastoma and evaluating new therapeutic options [14]. There are grounds to investigate the cytogenotoxic effect of gadobutrol in the SHSY-5Y cell line.

The cytotoxic and genotoxic effects of gadobutrol in cell lines may be mediated by different molecular mechanisms. These mechanisms may include processes such as inflammation, DNA damage, and oxidative stress [10, 15]. It has been reported that gadobutrol administration may inhibit DNA synthesis in cells and cause an increase in apoptosis-related gene expression [16]. In addition, it is emphasized that the increase in reactive oxygen species (ROS), that is, the increase in oxidative stress, is directly related to genotoxicity [17, 18]. Gadobutrol may have anticancer potential through mechanisms such as oxidative stress, DNA damage and inflammation in SHSY-5Y neuroblastoma cells. The demonstration of the effects of gadobutrol in SHSY-5Y cells may contribute to the development of new therapeutics in the treatment of neuroblastoma and the improvement of existing treatment methods. It is also thought to be a

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new step for targeted applications of GBCAs such as gadobutrol in general. Further research is required to identify alternatives to existing treatment modalities, especially in high-risk tumors like neuroblastoma.[19].

Given the limitations of assessments of cytogenotoxicity in neuroblastoma patients in the clinic, the effect of gadobutrol in SH-SY5Y cells can be investigated and may support the provision of new opportunities for use in the clinic. It may be possible to reduce mortality rates of patients with new treatment options in addition to traditional therapies [20]. Therefore, investigating the cytogenotoxic effects of gadobutrol in SH-SY5Y neuroblastoma cells may provide additional important contributions to existing knowledge and enlighten new research.

The aim of the present study was to evaluate the effects of the contrast media gadobutrol on the cell viability and genotoxicity of SH-SY5Y neuroblastoma cell line.

MATERIALS AND METHODS

Cell lines and cell culture

Human neuroblastoma (SH-SY5Y) cells were purchased from the American Type Culture Collection (ATCC, Shanghai, China). Cells were fixed in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin with glutamine. Cells were plated at 37°C in a moisturized medium with 5% CO_2.

Incubation with Gadobutrol

To evaluate the potential cytotoxic effects of gadobutrol, neuroblastoma cells were incubated at concentrations of 0.1, 1, 10, and 100 mM. These concentration values were preferred because they were within safe limits in previous cellular studies in the literature in which gadobutrol was administered [21, 22]. The $LogIC_{50}$ value was determined from analysis-XY analysis-Nonlinear regression (curve fit) in GraphPad Prism version 9.0.0. The $logIC_{50}$ value was 0.8452 and the corresponding IC_{50} dose was 7.001 mM. To examine the genotoxic effect, gadobutrol was applied to neuroblastoma cells at concentrations quarter (1.75 mM), half (3.50 mM), and itself (7 mM) the IC_{50} value. Gadobutrol was diluted with sterile water and sterile water was applied to solvent/negative control groups.

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Evaluation of cytotoxic effect by MTT assay

Trypan blue at a concentration of 0.4% was utilized to evaluate cell viability, with experiments initiated once cell viability reached 90%. SH-SY5Y neuroblastoma cells were counted using a hemocytometer, after which they were plated at a density of 5×10^4 cells per well in 96-well plates in preparation for 3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide (MTT) assays [23]. The plates were organized into groups: control, solvent, and varying concentrations of Gadobutrol (0.1 mM, 1 mM, 10 mM, and 100 mM). Neuroblastoma cells in the gadobutrol groups were exposed to these different concentrations for a duration of 24 hours. The percentage of cell viability was measured to assess the cytotoxic effects of gadobutrol.

Evaluation of genotoxic effect by Comet test

The experimental groups used for genotoxicity evaluation were control, negative control (sterile water), positive control (H_2O_2), Gadobutrol (1.75 mM), Gadobutrol (3.50 mM) and Gadobutrol (7 mM) groups. The Comet assay protocol was adapted significantly from the method described by Singh et al [24]. Cells from the cell culture were added to each Eppendorf tube at a concentration of 5×10^5 cells per tube. Precise doses of the test substance (in 100 µl) were subsequently introduced into the tubes containing the cells. The samples were incubated at 37 °C for 1 hour before being centrifuged at 3000 rpm and +4 °C for 5 minutes. The supernatant was then discarded, and cell viability was assessed, ensuring that it remained above 90%. Following this, 100 µl of phosphate-buffered saline (PBS) was added to the remaining cell pellet.

Next, 75 µl of low melting agar (LMA) was quickly mixed with the 100 µl of cells and spread onto microscope slides, which were then covered with coverslips. The slides were stored in a closed box in the refrigerator for 20-25 minutes. After this incubation, the coverslips were removed, and the slides were placed in a dish containing a lysis solution. They were stored in the refrigerator for a minimum of 1 hour and up to a maximum of 16 hours, with the procedure continued the following day.

After lysis, the slides were placed in buffer within an electrophoresis tank and allowed to sit for 20-25 minutes before undergoing electrophoresis for 20 minutes at 25 V and 300 mA (while maintaining the tank temperature at +4 °C). Following electrophoresis, the slides were immersed in a dish containing a neutralization buffer and kept at +4 °C for 5 minutes, a process that was repeated twice. After completing these steps, 50 µl of SyBR-safe dye, prepared in

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buffer, was added to each slide, which was then covered with a coverslip. Finally, microscopic examination was conducted.

For each group, a total of 100 cell images were analyzed and documented. Cells were evaluated based on the severity of nuclear damage, with scores assigned in increasing order of damage. Grade 0 = No damage, Grade 1 = Slightly damaged, Grade 2 = Moderately damaged, Grade 3 = Highly damaged, Grade 4 = Extremely damaged.

Number of grade 0 cells (G0) × 0
Grade 1 cell number (G1) × 1
Grade 2 cell number (G2) × 2
Grade 3 cell number (G3) × 3
Grade 4 cell number (G4) × 4
N = Total number of cells examined (100)
Total Damage Score (Genetic Damage Index = GDI)
Genetic Damage Index (GDI)=(0×G0 + 1×G1 + 2×G2 + 3×G3 + 4×G4)/N

The previously proposed calculation method was used to determine the GDI. The sum of the mean number of damaged cells (Grade 2, Grade 3, and Grade 4) was evaluated as the damaged cell index (DCI).

Statistical Analysis

Statistical analysis was conducted with GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California, USA). The normal data distribution was verified with the Shapiro-Wilk normalization test. Cell viability and genotoxicity values were compared with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Results are reported as mean ± SD. Differences were considered statistically significant when $p < 0.05$. Significance levels are indicated in the figures as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and **** = $p < 0.0001$.

RESULTS

Cytotoxic effect of gadobutrol in SH-SY5Y neuroblastoma cells

When the solvent group as compared to the control group, no statistically significant change in cell viability of neuroblastoma cells was observed (Figure 1A; $p > 0.05$). In the comparison of

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Gadobutrol (0.1 mM) and the control group, it was determined that Gadobutrol (0.1 mM) caused a statistically significant decrease in cell viability in SH-SY5Y neuroblastoma cells (Figure 1A; $p < 0.0001$). When the Gadobutrol (1 mM) group was compared with the control group, it was determined that the cytotoxic effect of Gadobutrol (1 mM) was statistically significant in SH-SY5Y neuroblastoma cells (Figure 1A; $p < 0.0001$). When the gadobutrol (10 mM) group was compared with the control group, the viability level of the cells decreased statistically significantly (Figure 1A; $p < 0.0001$). The gadobutrol (100 mM) group also showed a statistically significant increase in the cytotoxic effect compared to the control group (Figure 1A; $p < 0.0001$). The cytotoxic effect of gadobutrol was found to be dose-dependent. The cytotoxic effect of gadobutrol was calculated as $LogIC_{50} = 0.8452$ with a corresponding IC_{50} value of 7.001 mM and is shown in the dose-response curve in Figure 1B.

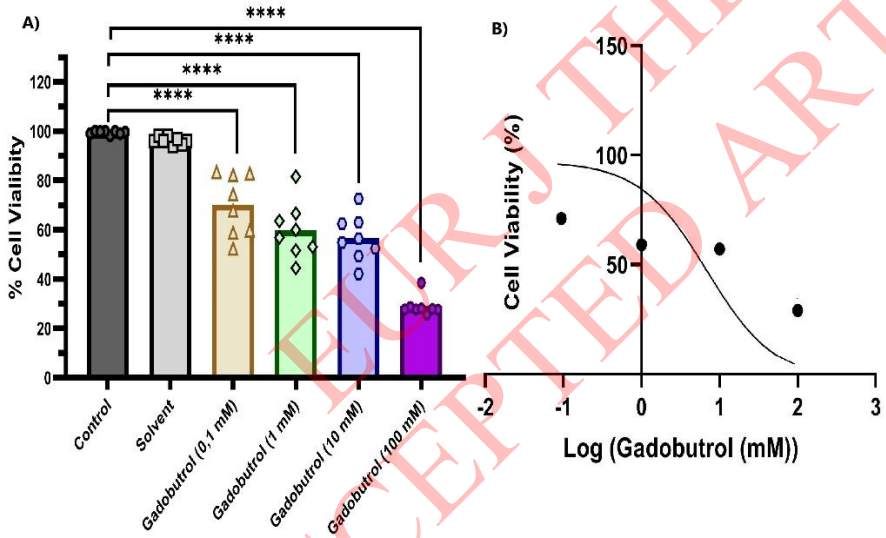

Figure 1. A) Effect of gadobutrol (0,1,1, 10 and 100 mM) on cell viability in SH-SY5Y neuroblastoma cells. ****P < 0.0001 vs control (one-way ANOVA, post-test Tukey). **B)** $LogIC_{50}$ dose-response curve for different Gadobutrol concentrations (0,1,1, 10 and 100 mM).

Genotoxic effect of gadobutrol in SH-SY5Y neuroblastoma cells

The data showing DNA damage in the Comet assay test are GDI and DCI values. When the positive control group was compared with the control group, a statistically significant increase

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in GDI was detected (Figure 2; $p < 0.0001$).Gadobutrol (1.75 mM) group showed an increase in GDI value compared to the control group. However, it was not statistically significant (Figure 2; $p > 0.05$). Gadobutrol (3.50 mM) group showed a statistically significant increase in GDI value compared to the control group (Figure 2; $p < 0.05$). A statistically significant increase in the GDI value was also observed in the gadobutrol (7 mM) group as opposed to the control group (Figure 2; $p < 0.01$).

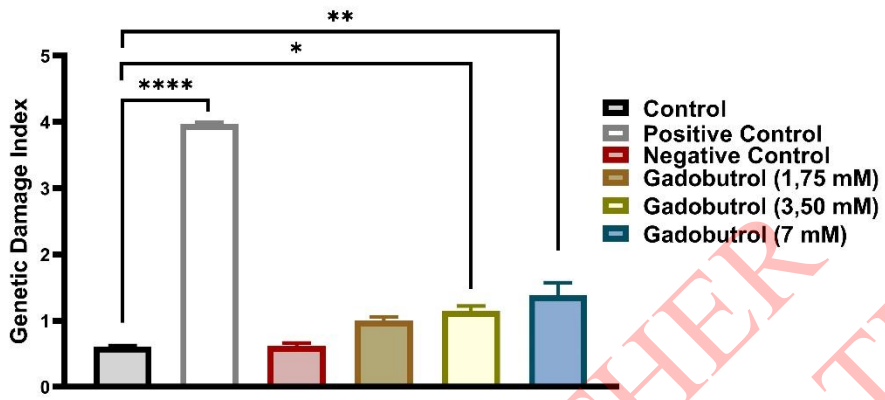

Figure 2. Mean ± SD values of Genetic Damage Index of gadobutrol (1.75 mM, 3.50 mM and 7 mM) in SH-SY5Y neuroblastoma cells *P < 0.05, **P < 0.01 or ****P < 0.0001 (one-way ANOVA, post-test Tukey) versus control.

The positive control group showed a statistically significant increase in DCI value when compared with the control group (Figure 3; $p < 0.0001$). Gadobutrol (1.75 mM) group showed an increase in DCI value compared to the control group. However, it was not statistically significant (Figure 3; $p > 0.05$). Gadobutrol (3,50 mM) group showed a statistically significant increase in DCI value when compared with the control group (Figure 3; $p < 0.05$). When gadobutrol (3.50 mM) group was compared with the control group, there was a statistically significant increase in DCI value (Figure 3; $p < 0.01$). The genotoxic effect of gadobutrol in SH-SY5Y neuroblastoma cells was dose-dependent.

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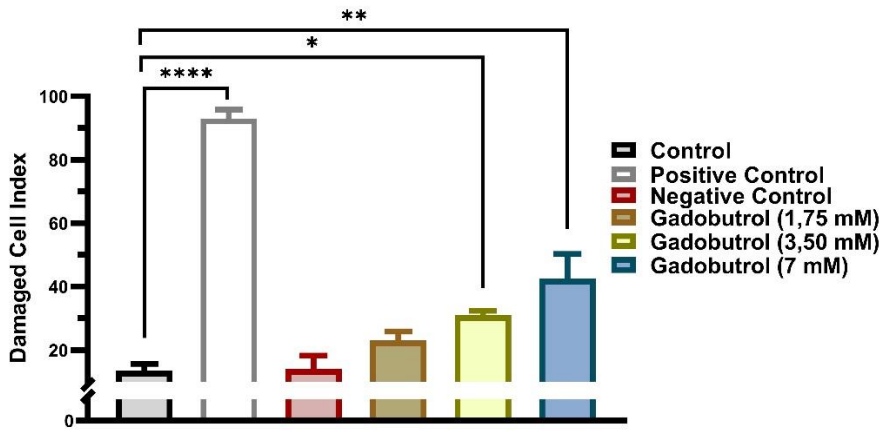

Figure 3. Mean ± SD values of Damaged Cell Index of gadobutrol (1.75 mM, 3.50 mM and 7 mM) in SH-SY5Y neuroblastoma cells. *P < 0.05, **P < 0.01 or ****P < 0.0001 (one-way ANOVA, post-test Tukey) vs control.

DISCUSSION

GBCAs are used as MRI contrast agents in cancer types [25, 26]. However, uncertainties remain about their therapeutic potential in cancer, not only for diagnostic purposes [8]. Results from existing studies suggest that some types of GBCAs may have therapeutic effects in cancer [8, 27], but the possible anti-cancer effect of gadobutrol in neuroblastoma remains a scientific gap. Moreover, theranostic agents are now gaining prominence in cancer treatment and these agents exhibit ideal distribution in diagnosis and treatment. Especially gadolinium-chelated theranostics may have therapeutic potential by accumulating specifically in tumors [28]. Our current findings shed light on the anti-cancer effect of gadobutrol in neuroblastoma. Although the study by Erdogan et al. focused more on the safety of GBCAs in neurons, it was reported that GBCAs reduced cell viability in neuroblastoma cells [9]. In this study, it was revealed that linear GBCAs showed more cytotoxic effect than macrocyclic GBCAs containing gadobutrol. This may be explained by the fact that linear GBCAs accumulate more Gd^{+3} in neurons compared to macrocyclic GBCAs [29, 30]. In addition, when compared with our study, the treatments at 0.1 and 1 mM concentrations are similar, but the decreasing effect on cell viability is higher in our study. The difference in these results in the same direction but in the amount can be speculated to the difference in the tests applied and the difference in the number of cells in the well. Our treatments at 10 and 100 mM concentrations showed a dose-dependent higher neurotoxic effect. However, in conclusion, although the study of Erdoğan et al. did not aim to

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investigate the anticancer effect of gadobutrol, their results were consistent with our finding of cytotoxic effect of gadobutrol in SH-SY5Y neuroblastoma cells. The result of this study is consistent with the finding of the cytotoxic effect of gadobutrol in SH-SY5Y neuroblastoma cells in our research.

In neuroblastoma treatment, cytotoxicity plays a pivotal role in the destruction of tumor cells [31]. The treatment process is based on various combinations of drugs that usually take about 5 months after complete removal of the tumor or biopsy [32]. These drugs target tumor cells and exert cytotoxic effects. It is also important to explore new therapeutic strategies to improve the efficacy of existing treatment modalities [33]. These methods, which are usually applied to pediatric patients, are vital for the management of such tumors in children. The inadequacy of these vital methods shows the necessity of investigating different therapeutics [34]. In this direction, gadobutrol contrast agent, which can also be used in imaging in neuroblastoma patients, has recently been emphasized to cause Gd^{+3} accumulation in neurons [35]. The effect of this accumulation in neurons in different diseases has not been sufficiently elucidated. A growing number of studies have shown that gadobutrol uptake and accumulation increase cytotoxicity in cells [36]. Although studies have generally focused on the safety of gadobutrol, it is thought that it may exert therapeutic effects on cancer cells. Gadobutrol-dendrimer caused increased expression of pro-apoptotic Bax and decreased expression of anti-apoptotic Bcl-2 in MCF-7 breast cancer cell line. It was also found to increase the expression of the metastasis suppressor gene KAI1. The apoptosis-promoting effects of gadobutrol-dendrimer via Bax/Bcl-2 and metastasis suppressing effects via KAI1 indicate its anticancer effect in breast cancer [10]. In this study, gadobutrol showed a cytotoxic effect in SHSY-5Y neuroblastoma cells, which may indicate that this agent may have therapeutic potential in neuroblastoma in addition to imaging.

Chemotherapy drugs used in the treatment of neuroblastoma (e.g. alkylating agents such as cyclophosphamide, ifosfamide, and antimetobolytics such as methotrexate, 5-fluorouracil) cause disruptions in the genetic structure of cells through DNA damage [37, 38]. By directly damaging DNA in cancer cells, these genotoxic agents can cause cell cycle arrest or apoptosis [39]. These effects are particularly pronounced in rapidly dividing cells. Neuroblastoma cells are sensitive to these genotoxic agents, underlining the importance of genotoxicity in their treatment [40]. Gadobutrol has shown genotoxic effects in many different cell types [36, 41, 42]. Our findings that gadobutrol induced genotoxicity in SHSY-5Y neuroblastoma cells

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provide evidence for this hypothesis. Gd^{+3} accumulation-induced pathological mechanisms may underlie the therapeutic potential of gadobutrol by causing genotoxicity in neuroblastoma cells. Previous studies have demonstrated that gadobutrol causes oxidative stress, increased inflammation and apoptosis [43] These mechanisms that play a role in the development of genotoxicity may suggest that gadobutrol causes genotoxicity in neuroblastoma cells through these mechanisms. In addition, disruption of the pathways that control cell cycle to repair damaged DNA or triggering the production of proinflammatory cytokines such as interleukin-13, interleukin-4 and interleukin-6 by Gd^{+3} [44] may have caused DNA damage.

Limitations

This study was conducted as an in vitro cellular evaluation and involved short-term exposure to gadobutrol, which significantly limits the applicability of the findings to real clinical settings. Furthermore, while in vitro studies are not considered as valuable as clinical trials, they occupy a position in the evidence hierarchy; however, their level of evidence is low since they are placed at the lower tiers of this hierarchy. Another significant limitation is that the mechanisms underlying the cytotoxic and genotoxic effects of gadobutrol have not been explored. This study could have benefited from support through in vivo and/or in silico research. Additionally, since gadobutrol is administered in its pharmaceutical form, its effects cannot be solely attributed to its chemical components. Moreover, the fact that gadobutrol is used in the same formulation as it is administered clinically may enhance the clinical validity of the results.

CONCLUSION

The results of this experimental in vitro study show that gadobutrol induces cytotoxicity in SHSY-5Y neuroblastoma cells. Furthermore, it was revealed for the first time that gadobutrol enhances genotoxicity in SHSY-5Y neuroblastoma cells. With these results, gadobutrol may be a promising candidate for neuroblastoma but further clinical studies are needed.

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