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Apoptosis is responsible for the cytotoxic effects of anti-GD2 ganglioside antibodies and aurora A kinase inhibitors on human neuroblastoma cells*

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In recent years, immunotherapy has been identified as an effective treatment method for high-risk neuroblastoma. A previous study demonstrated that an anti-GD2 ganglioside (GD2) mouse 14G2a monoclonal antibody (mAb) combined with a small molecule, i.e., an aurora A kinase inhibitor (MK-5108), significantly increased cytotoxicity against human neuroblastoma cells, as compared to monotherapy. This study aimed to demonstrate the mechanism of neuroblastoma cell death *in vitro* following the addition of an anti-GD2 human-mouse chimeric ch14.18/CHO mAb (presently used in clinics) and two aurora A inhibitors (MK-5108 and MK-8745). The effects of the aforementioned agents on neuroblastoma cells were determined by measuring the level of ATP, the level of apoptotic and necroptotic markers, and the activity of caspase 3/7. The results revealed that the ch14.18/CHO mAb decreased cellular ATP levels in the IMR-32 and CHP-134 neuroblastoma cell lines, similarly to the 14G2a mAb. Regarding ch14.18/CHO mAb treated IMR-32 cells, the observed cytotoxic effect was concomitant with induced caspase 3 cleavage, which indicated the induction of apoptosis in IMR-32 cells, but not in CHP-134 cells. Furthermore, the MK-5108 inhibitor induced apoptosis, as indicated by the increased cleavage of caspase 3 and increased activity of caspase 3/7. However, the presence of necroptosis was ruled out in MK-5108-treated IMR-32 and CHP-134 cells. In summary, the effects of the combination of ch14.18/CHO mAb and aurora A kinase inhibitors (MK-5108 and MK-8745) were shown to enhance apoptosis in IMR-32 cells compared to when used individually.

Keywords: neuroblastoma, ganglioside GD2, ch14.18/CHO, 14G2a, aurora A kinase, apoptosis.

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Abbreviations: GD2, GD2 ganglioside; mAb, monoclonal antibody; ER, endoplasmic reticulum; RIP, receptor-interacting serine/threonine-protein kinase; MLKL, mixed lineage kinase domain-like protein; MAPKs, mitogen-activated protein kinases

INTRODUCTION

Neuroblastoma is the most common type of solid extracranial tumor in children and is responsible for 7% of all pediatric neoplasms in patients under the age of 15. It accounts for 15% of all pediatric tumor-related deaths worldwide (Kholodenko *et al*., 2018). The greatest challenge for pediatric oncologists is the unfavorable outlook on treatment options for high-risk neuroblastoma, which is detected in ~60% of patients (Johnsen *et al*., 2019). The treatment methods currently in use include high-dose chemotherapy, autologous stem cell rescue, surgery, and radiotherapy. All exhibit low efficacy due to the presence of high cell heterogeneity in these tumor cells (Kholodenko *et al*., 2018; Garaventa *et al*., 2021; Seitz *et al*., 2021).

To date, the most effective treatment for patients with high-risk neuroblastoma is achieved through immunotherapy, which is based on GD2 ganglioside-specific antibodies combined with multimodal treatment (Kholodenko *et al*., 2018; Furman, 2021; Morandi *et al*., 2021). The GD2 ganglioside is a well-established molecular target in neuroblastoma due to its high expression in neuroblastic cells (Terzic *et al*., 2018). The GD2 ganglioside-targeting ch14.18/CHO monoclonal antibody (mAb, dinutuximab β) is produced in Chinese hamster ovarian cells and is an IgG1 human/mouse chimeric switch variant of the murine 14G2a mAb (Zeng *et al*., 2005). The ch14.18/CHO mAb was indicated to improve 5-year event-free and overall survival of patients, thus serving an important role in treatment regimens applied in the International Society of Pediatric Oncology Europe Neuroblastoma Group High-Risk Neuroblastoma 1 trial HR-NBL1/SIOPEN (Ladenstein *et al*., 2020; Barone *et al*., 2021). The functional properties of the ch14.18/CHO mAb had previously been identified in complement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC) against GD2 positive neuroectodermal tumor cell lines *in vitro* (Zeng *et al*., 2005).

To improve GD2-targeted therapy, it may be important to utilize the direct cytotoxic activity of GD2-specific antibodies. Antibodies are capable of inhibiting proliferation in GD2-expressing tumor cell lines through a number of different mechanisms including apoptosis, necrosis, autophagy, and oncosis-like cell death (Kowalczyk *et al*., 2009; Horwacik & Rokita, 2015; Durbas *et al*., 2018). Other observed effects of antibodies on GD2-expressing tumor cell lines involve morphological changes, cell aggregation, and detachment (Kowalczyk *et al*., 2009; Horwacik & Rokita, 2015). The molecular mechanisms behind these effects have not yet been fully determined and require further investigation.

To enhance the efficacy of high-risk neuroblastoma therapy, GD2-specific antibodies could be used with carefully selected chemotherapeutic drugs and/or small molecular inhibitors to induce a synergistic cytotoxic effect. The high heterogeneity of a neuroblastoma means that a personalized approach is required to select the optimal combination of therapies for each patient (Kholodenko *et al*., 2018). The identification of a potential novel target that could be used in neuroblastoma, i.e., aurora A kinase, has gained increasing attention (Hansen *et al*., 2017; Sankhe *et al*., 2021; Mou *et al*., 2021; Mesquita *et al*., 2021). Aurora A kinase plays a role in several stages during mitosis, including centrosome duplication and mitotic spindle formation (Hochegger *et al*., 2013). The gene encoding aurora A kinase (*AURKA*) is amplified in human neuroblastomas and has been shown to be associated with high levels of MYCN proto-oncogene, bHLH transcription factor (*MYCN*) and lower survival rates in patients with neuroblastoma (Brockmann *et al*., 2013). MYCN is protected from proteasomal degradation during mitosis by the aurora A protein, which forms a complex with MYCN in neuroblastoma cells (Otto *et al*., 2009). Small molecule inhibitors have been used to target aurora A kinase in an attempt to indirectly target the MYCN protein (Pastor & Mousa, 2019; Yi *et al*., 2021; Boi *et al*., 2021). The consequences of aurora A kinase inhibition include abnormal spindle pole formation, cell-cycle arrest between phases G2-M, polyploidy, and apoptosis (Boss *et al*., 2009).

A previous study demonstrated that anti-GD2 14G2a mouse mAb and a specific aurora A kinase inhibitor, MK-5108, significantly enhanced cytotoxicity against neuroblastoma cells *in vitro* compared to monotherapy (Horwacik *et al*., 2013). The aforementioned study reported that in combination 14G2a mAb and MK-5108 increased the nuclear level of P53 at 2 h and 24 h compared to treatments with 14G2a mAb alone, and decreased MYCN cytoplasmic levels at 2 h compared to control cells (Horwacik *et al*., 2013). Previously, 14G2a mAb and the MK-5108 inhibitor were indicated to induce autophagy in IMR-32 cells (Durbas *et al*., 2018). To our knowledge, this present study is the first study to demonstrate the potential of anti-GD2 ch14.18/CHO chimeric mAb, in order to induce cytotoxic activity against human neuroblastoma cells IMR-32 and CHP-134 *in vitro*. This study also aimed to gather more data on the cytotoxic effect exerted independently of ADCC and CDC, as well as to expand our knowledge on the roles GD2 plays in cell signaling (as gangliosides are, for example, important constituents of lipid rafts).

14G2a mAb has previously been observed to induce cell death partially by apoptosis (Kowalczyk *et al*., 2009). Following the observation of murine 14G2a mAb-induced apoptosis in IMR-32 cells, the current study also examined whether chimeric ch14.18/CHO mAb exhibited similar effects in terms of inducing cytotoxicity in selected neuroblastoma cell lines. Therefore, the current study examined how similar the cellular response was to the 14G2a mAb and the ch14.18/CHO mAb, which is currently used in clinics. The current study also aimed to investigate the effects of the ch14.18/CHO mAb in terms of determining processes that could be associated with the observed cytotoxic effects, such as apoptosis or necroptosis.

Furthermore, the mechanisms of aurora A kinase inhibitors, MK-5108 and MK-8745, in exerting a cytotoxic effect on IMR-32 and CHP-134 neuroblastoma cells were investigated. A total of two neuroblastoma cell lines were selected for the current study, IMR-32 and CHP-134, as these cell lines were indicated to be the most sensitive to 14G2a mAb among the GD2-positive and *MYCN*-amplified human neuroblastoma cell lines tested (Horwacik *et al*., 2013). Furthermore, IMR-32 and CHP-134 were previously identified as the most susceptible to the MK-5108 inhibitor compared to other cell lines (Horwacik *et al*., 2013). The ch14.18/CHO mAb and aurora A kinase inhibitors are two different groups of drugs that represent monoclonal antibodies and small molecule inhibitors, respectively (Pastor & Mousa, 2019). Several different approaches have been tested by combining immunotherapy with the pharmacological inhibition of molecular targets, chemotherapy or cytokines, to identify more efficient therapeutic treatments of neuroblastoma (Kowalczyk *et al*., 2009; Horta *et al*., 2016; Federico *et al*., 2017; Aravindan *et al*., 2020). Consequently, the present study aimed to determine the possible underlying mechanism of action of anti-GD2 ch14.18/CHO mAb and aurora A kinase inhibitors, as well as the effects of their combination on neuroblastoma cells in search of the most potent drug combinations.

MATERIALS AND METHODS

Cell culture

IMR-32 (CCL-127; American Type Culture Collection) and CHP-134 (ACC 653; German Collection of Microorganisms and Cell Cultures GmbH) human neuroblastoma cell lines were cultured as previously described by Durbas and others (Durbas *et al.,* 2018).

Treatment with drugs

IMR-32 and CHP-134 cells were treated with 14G2a mAb (obtained as previously described: Horwacik *et al*., 2013) at a concentration of 40 μg/ml, or ch14.18/CHO mAb which consists of variable regions derived from murine 14G2a mAb (Qarziba; cat. no. 1045413A; Salus International; EUSA Pharma) at a concentration of 40 μg/ml, MK-5108 (cat. no. S2770; Selleck Chemicals) at a concentration of 0.1 μM for both cell lines, or MK-8745 (cat. no. S7065; Selleck Chemicals) at a concentration of 0.3 μM for IMR-32 cells and 0.1 μM for CHP-134 cells. Furthermore, control cells (treated with PBS for 14G2a and ch14.18/CHO mAb or with DMSO for MK-5108 and MK-8745 inhibitors) were incubated with an equivalent volume of drug solvent. Necrostatin-1 (NEC-1; 20 μM for IMR-32; 10 μM for CHP-134; cat. no. 480065; Sigma-Aldrich; Merck KGaA) and GSK'872 (GSK) inhibitor (3 μM for IMR-32; 10 μM for CHP-134; cat. no. S8465; Selleck Chemicals) were also used as single agents or in combination with the MK-5108 inhibitor. Following appropriate treatment, cells were seeded in plates and cultured at 37°C.

Cell viability tests

Cells were treated for 24, 48 or 72 h. 96-well plates containing 2×10^4 IMR-32 cells/well and 5×10^3 CHP-134 cells/well were also prepared. Cellular ATP content was measured after a period of time using the ATPlite Luminescence ATP Detection Assay system (cat. no. 6016947; PerkinElmer, Inc.) and an Infinite M200 Reader (Tecan Group, Ltd.), according to the manufacturer's protocol.

Measurements of caspase 3/7 activity

After treatment, IMR-32 cells (1×10^4 per well) and CHP-134 cells (2.5×10^3 per well) were cultured in 96-well plates for 72 h in 50 µl complete media. On day 3, caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay (cat. no. G8090; Promega Corporation) according to the manufacturer's protocol. Generated luminescent signals were analyzed in triplicate using the Infinite M200 Reader and divided by the relative levels of ATP of the respective groups of cells (to normalize signals with the number of viable cells).

Protein isolation and immunoblotting

For protein analysis, IMR-32 cells (1×10^6 cells in one well of a six-well plate) and CHP-134 cells (0.25×10^6 cells in one well of a six-well plate) were prepared as previously described (Durbas *et al.*, 2018). The following rabbit anti-human antibodies were purchased from Cell Signaling Technology, Inc.: α-tubulin (cat. no. 2125), caspase 3 (cat. no. 9665), cleaved caspase 3 (cat. no. 9664), PARP (cat. no. 9542), cleaved PARP (cat. no. 5625), endoplasmic reticulum chaperone BIP (BIP; cat. no. 3177), protein-disulfide isomerase (PDI; cat. no. 3501), P38α (cat. no. 9218), ERK1/2 (cat. no. 4695), apoptosis signal-regulating kinase 1 (ASK-1; cat. no. 8662). All the aforementioned antibodies were diluted to 1:1,000. Secondary goat anti-rabbit IgG, HRP-linked antibodies (cat. no. 7074) were purchased from Cell Signaling Technology, Inc. and diluted to 1:2000.

Statistical analysis

All experiments were repeated at least three times. Data were presented as means ± SEM. To test for statistically significant differences for experiments with >two independent groups (Fig. 1C and D; Fig. 2; Fig. 3E, F, G and H, Fig. 4C, D, G and H), one-way ANOVA was performed using R i386 4.0.0 Patched software, followed by Tukey's post hoc test for pairwise comparisons between specific groups. For analysis of data presented in Fig. 3A, B, C, and D and 4A, B, E, and F, a two-way ANOVA was used with Tukey's post hoc test for pairwise comparisons between specific groups. One sample *t*-tests were also performed, allowing for the comparison of the means of treated cells *vs.* control cells, which were set as 1.

RESULTS

Analysis of ATP levels and apoptotic markers in IMR-32 and CHP-134 cells treated with ch14.18/CHO

14G2a mAb (40 µg/ml) has previously been indicated to induce cell death within IMR-32 cells in a partially caspase-dependent manner (Kowalczyk *et al.*, 2009). Based on the concentration curves obtained for ch14.18/CHO mAb, a concentration of 40 µg/ml was selected for IMR-32 and CHP-134 cells (Fig. 1A and B). The appropriate isotype control antibody (Rituximab) was also used (not shown). The current study compared the effects of 14G2a mAb on neuroblastoma cell ATP levels, with the effects observed after the addition of the ch14.18/CHO mAb (both, 40 µg/ml). The results indicated that the effect of the 14G2a mAb was stronger (relative ATP level decreased to 0.47) compared to the effect induced by the ch14.18/CHO mAb in IMR-32 cells (relative ATP level reduced to 0.65; Fig. 1C). The changes in relative ATP levels for IMR-32 cells between means were significantly different [ANOVA; $F(1,4)=40.423$; $P=0.0031$]. However, the cytotoxic effects of both antibodies (14G2a and ch14.18/CHO mAbs) on CHP-134 cells were comparable and reduced to 0.75 and 0.73, respectively; Fig. 1D). No significant changes were identified in CHP-134 cells [ANOVA; $F(1,4)=0.0491$; $P=0.8355$]. The results also demonstrated that ch14.18/CHO mAb improved caspase 3 cleavage in IMR-32 cells, while caspase 3 expression did not change (Fig. 1E, left panel). The expression of PARP, being one of the substrates of caspase 3, was also detected in IMR-32 cells. PARP expression (both 116 and 89 kDa forms) was not altered in IMR-32 cells treated with ch14.18/CHO mAb compared to control cells (Fig. 1E, right panel). However, the expression of cleaved PARP (89 kDa) in IMR-32 treated with ch14.18/CHO mAb was markedly induced, following the increase in the expression of cleaved caspase 3. These results allowed us to confirm the activity of caspase 3 towards its substrate, PARP, in IMR-32 cells. No changes in caspase 3 expression and cleavage were observed in the CHP-134 cell line (Fig. 1F). These effects were not surprising, as 14G2a mAb was previously indicated not to be an effective inducer of apoptosis in CHP-134 cells (Horwacik *et al.*, 2013). PARP and cleaved-PARP expression was not changed in CHP-134 cells treated with ch14.18/CHO mAb (not shown). This is not surprising because the expression of caspase 3 and cleaved caspase 3 in CHP-134 cells also remained unchanged. Furthermore, changes in the expression of necroptosis-related proteins were determined in IMR-32 and CHP-134 cells following the addition of ch14.18/CHO mAb. No changes in RIP1 and RIP3 protein expression were identified in IMR-32 cells and CHP-134 cells (not shown). Therefore, necroptosis is probably not involved in ch14.18/CHO mAb-induced cell death.

Analysis of apoptotic and necroptotic markers in IMR-32 and CHP-134 cells treated with MK-5108 and MK-8745 inhibitors

To distinguish between apoptosis, necroptosis, or a mixed type of cell death in MK-5108 aurora A inhibitor treated neuroblastoma cells, the expression of necroptosis and apoptosis-associated proteins was investigated. The concentration of MK-5108 (0.1 µM) which causes a similar inhibitory effect in both cell lines (up to ~0.7 of the control, evaluated by measuring ATP levels) was selected, compared to the control cells (Fig. S1A and B at https://ojs.ptbioch.edu.pl/index.php/abp). Changes in the expression of markers associated with apoptosis in MK-5108 treated and control IMR-32 and CHP-134 cells were determined. The results indicated an up-regulation of the cleaved caspase 3 expression signal in IMR-32 cells (Fig. 2A) and CHP-134 cells (Fig. 2B). No changes in the regulation of the other proteins associated with apoptosis: caspase 9, cleaved caspase 9, BCL-2, BCL-XL, BAX, BAD, and proteins associated with necroptosis: RIP1 and RIP3 kinases were observed in IMR-32 and CHP-134 cells (not shown). These results suggest an enhancement of apoptosis in IMR-32 and CHP-134 cell cultures treated with MK-5108.

MK-8745, another aurora A kinase inhibitor, was tested in IMR-32 and CHP-134 cells. The two cell lines show different sensitivities to MK-8745, and CHP-134 cells are more sensitive. Therefore, the concentration of MK-8745 that decreases ATP levels to the same extent in both cell lines (to ~0.7 of the control for 72 h) was used, compared to control cells, which were set

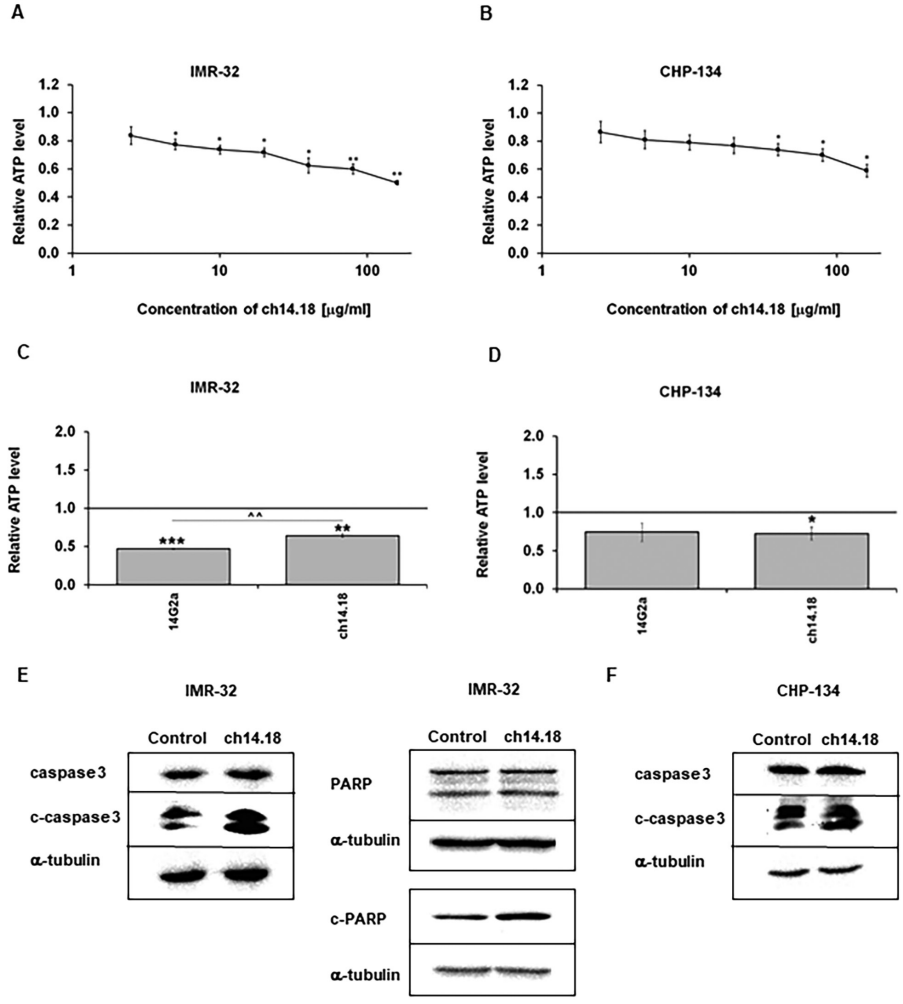

Figure 1. Comparison of the cytotoxic effects of 14G2a mAb and ch14.18/CHO mAb on relative ATP levels and on apoptotic markers in IMR-32 and CHP-134 cells.
(**A**) IMR-32 cells were treated with ch14.18/CHO mAb (2.5–160 μg/ml) for 72 h and (**B**) CHP-134 cells were treated with ch14.18/CHO mAb (2.5–160 μg/ml) for 72 h. Relative ATP levels were calculated versus control cells (PBS-treated cells), which were set as 1. Relative levels of cellular ATP were measured at 72 h in (**C**) IMR-32 and (**D**) CHP-134 cells treated with 14G2a mAb, ch14.18/CHO mAb (both, 40 μg/ml) or control cells. The means of three independent experiments (±S.E.M.) were calculated vs. control cells (PBS-treated cells, which were set as 1, black baseline). A one-way ANOVA was performed for the sections C and D of the figure, followed by Tukey's post hoc test; ^^P<0.01. t-tests were performed to compare the differences between the means of the agent versus the control; *P<0.05; **P<0.01; ***P<0.001. Representative immunoblots from three independent experiments were presented showing chemiluminescent signals for caspase 3, c-caspase 3 (left panel), PARP, c-PARP (right panel) and α-tubulin in (**E**) IMR-32 cells treated with ch14.18/CHO mAb, and chemiluminescent signals for caspase 3, c-caspase 3 and α-tubulin in (**F**) CHP-134 cells treated with ch14.18/CHO mAb. Control, PBS-treated cells; mAb, monoclonal antibody; 14G2a, 14G2a mAb treated cells; ch14.18, ch14.18/CHO mAb treated cells; c, cleaved; PARP, poly ADP ribose polymerase.

at 1. For IMR-32 cells, a concentration of 0.3 μM decreases the ATP level to 0.71 and for CHP-134 cells, a concentration of 0.1 μM decreases the ATP level to 0.74 (Fig. S1C and D, respectively at https://ojs.ptbioch.edu.pl/index.php/abp). MK-8745, an inhibitor, was shown to induce a level of cleaved caspase 3 in IMR-32 (Fig. 2C) and CHP-134 cells (Fig. 2D). The expression of RIP1 and RIP3 kinases remained unchanged at 72 h after the treatment with MK-8745 of IMR-32 and CHP-134 cells (data not shown). These results show that the MK-8745 inhibitor induces apoptosis in IMR-32 and CHP-134 cells.

Effects of ch14.18/CHO mAb and aurora A kinase inhibitors on IMR-32 and CHP-134 cells

The combination of ch14.18/CHO mAb (40 μg/ml) and the MK-5108 inhibitor or the the MK-8745 inhibitor used in 0.03 μM and 0.1 μM was shown to decrease relative ATP levels at 72 h in IMR-32 and CHP-134 cells, as compared to aurora A kinase inhibitors alone (Tukey's post hoc test, P<0.0001; Fig. 3A, B, C and D). The combination of ch14.18/CHO mAb and the MK-5108 inhibitor at 72 h induced caspase 3/7 activity 3.5-fold as compared to MK-5108 used alone and 1.7 times compared to ch14.18/CHO in IMR-32 cells (Fig. 3E). Significant changes between agents were identified [two-way ANOVA; F(2,6)=622.72; P=1.102e-07]. The combination of ch14.18/CHO mAb and the MK-8745 inhibitor at 72 h induced the activity of caspase 3/7 4.5 times compared to MK-8745 and 1.7 times compared to ch14.18/CHO used individually in IMR-32 cells (Fig. 3G). Significant changes among agents were identified [two-way ANOVA; F(2,6)=34.607; P=0.0005076]. This study showed that both aurora A inhibitors were capable of inducing the activity of caspase 3/7 in CHP-

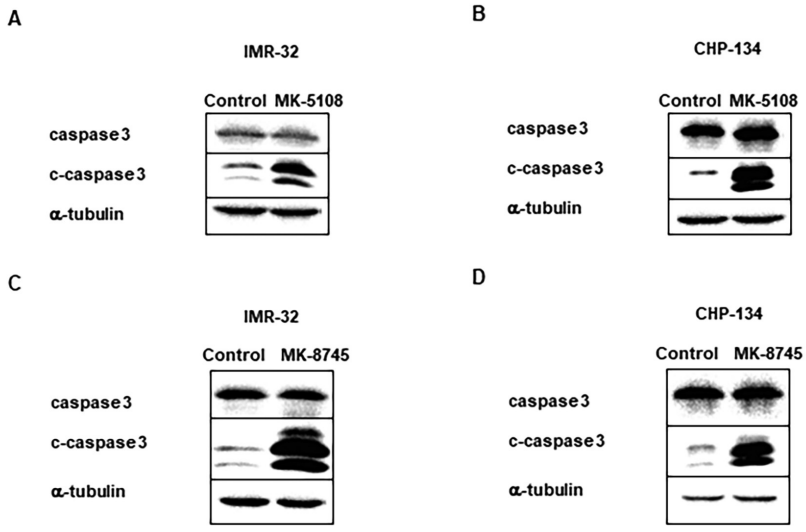

Figure 2. Analysis of caspase 3 cleavage in MK-5108-treated and MK-8745-treated IMR-32 and CHP-134 cells.
Representative immunoblots from three independent experiments were presented showing chemiluminescent signals for caspase 3, c-caspase 3 and α-tubulin in (**A**) IMR-32 and (**B**) CHP-134 cells treated with MK-5108 (0.1 μM) and (**C**) IMR-32 and (**D**) CHP-134 cells treated with MK-8745 (0.3 μM for IMR-32 cells; 0.1 μM for CHP-134 cells). Control, DMSO-treated cells; MK-5108, MK-5108-treated cells; MK-8745, MK-8745-treated cells; c, cleaved.

134 cells, while the ch14.18/CHO mAb is capable of inducing apoptosis in IMR-32 cells, but not in CHP-134 (Fig. 3F and H).

Inhibition of RIP1 and RIP3 kinases decreases cellular ATP and increases caspase 3 and 7 activity in IMR-32 and CHP-134 cells

The molecules associated with apoptosis and necroptosis that are regulated in cells treated with MK-5108 should not be considered as the only indicators of apoptosis and necroptosis. Therefore, the aforementioned results were supported by measuring cellular ATP levels and caspase 3 and 7 activities after pharmacological inhibition of RIP1 and RIP3 kinases by necrostatin-1 and GSK'872, respectively. To evaluate the effects of the combined treatment of MK-5108 and necrostatin-1/GSK'872, two different concentrations of necrostain-1 (i.e., 20 μM for IMR-32 cells; 10 μM for CHP-134 cells; Figs. S1E and F, respectively) and two different concentrations of GSK'872 (i.e., 3 μM for IMR-32; 10 μM for CHP-134; Figs. S1G and H, respectively) were used. This was done to ensure that relative ATP levels are not altered after the respective treatment with NEC-1/GSK'872 in both cell lines, compared to control cells. Furthermore, NEC-1/GSK'872 concentrations were selected to have an inhibitory effect on the expression and phosphorylation levels of the RIP1/RIP3 kinases (not shown). NEC-1 and MK-5108 inhibitors were shown to markedly reduce cell viability compared to control cells, which was assessed by measuring cellular ATP levels in both neuroblastoma cell lines (Fig. 4A and B). A two-way ANOVA revealed that for IMR-32 and CHP-134 cells, changes in relative ATP levels over time were statistically significant [$F(2,27)=3.3659$; $P=0.04953$ for IMR-32 cells; $F(2,18)=8.4743$; $P=0.00255$ for CHP-134 cells]. Statistically significant changes in agents were also observed in IMR-32 and CHP-134 cells [$F(2,27)=4.04$; $P=0.02918$, $F(2,18)=22.6936$; $P=1.201e-05$], respectively. Furthermore, a significant inhibition of ATP levels was also observed in IMR-32 and CHP-134 cells with combined treatment with MK-5108 and GSK'872 for 24, 48 and 72 h, compared to control cells (Fig. 4E and F). Statistically significant changes in time were observed [two-way ANOVA; $F(2,18)=18.576$; $P=4.202e-05$ for IMR-32 cells; $F(2,27)=9.2943$; $P=0.0008489$ for CHP-134 cells] and significant changes between agents were also identified [two-way ANOVA; $F(2,18)=43.758$; $P=1.224e-07$ for IMR-32 cells; $F(2,27)=15.9359$; $P=2.689e-05$ for CHP-134 cells]. The results also revealed the 2-fold increase in caspase 3 and 7 activities in IMR-32 cells after the combined treatment of MK-5108 and NEC-1 compared to cells treated with NEC-1 (Fig. 4C). A 6.4-fold increase was observed after MK-5108 and NEC-1 combination treatment in CHP-134 cells compared to NEC-1 (Fig. 4D). Relative caspase 3/7 levels were significantly different for a number of different agents, determined using ANOVA [$F(2,9)=27.183$; $P=0.0001534$ for IMR-32 cells; $F(2,9)=8.96$; $P=0.007224$ for CHP-134 cells]. The application of combined treatment with MK-5108 and GSK'872 was also indicated to induce a 2.5-fold increase in caspase 3 and 7 activities in the IMR-32 cell line, compared to cells treated with GSK'872 (Fig. 4G). The 7.3-fold increase after MK-5108 and GSK'872 combination treatment was observed in CHP-134 cells compared to GSK'872 (Fig. 4H). Relative caspase 3/7 levels differed between agents as assessed using ANOVA [$F(2,12)=14.412$; $P=0.00064511$ for IMR-32 cells; $F(2,9)=13.084$; $P=0.00217$ for CHP-134 cells].

Effects of MK-5108 inhibitor treatment on ER stress and MAPKs

The levels of some ER-associated stress proteins, calnexin, IREα and PERK, remained unchanged in the IMR-32 and CHP-134 neuroblastoma cell lines when treated with the MK-5108 inhibitor (not shown). However, there was disparate regulation of BIP and PDI proteins, whose levels decreased in IMR-32, but increased in CHP-134 cells (Fig. S2A at https://ojs.ptbioch.edu.pl/index.php/abp).

To investigate whether the MAPK signaling pathway was associated with the MK-5108 mode of action, immunoblotting assays were performed to detect MAPK-

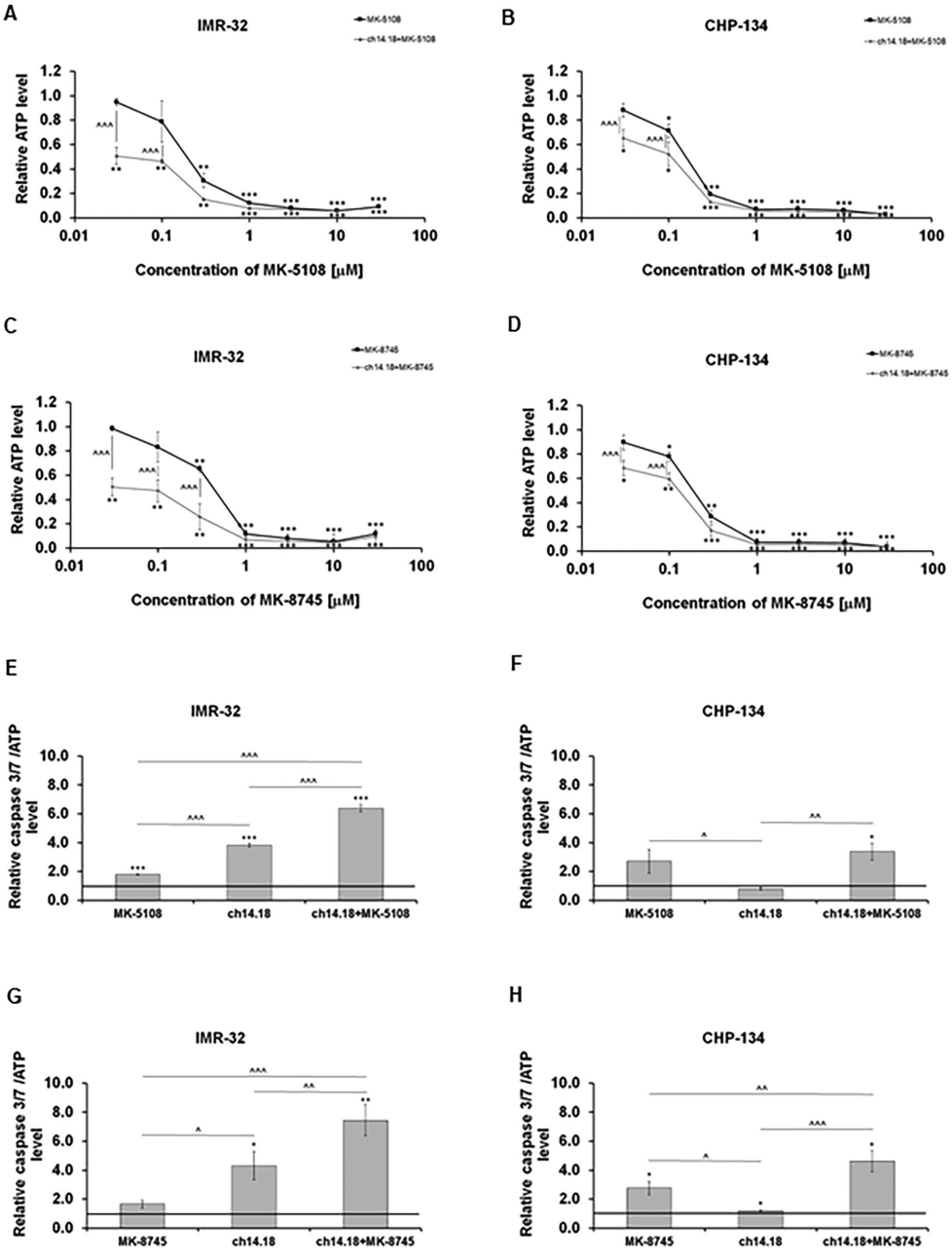

Figure 3. Ch14.18/CHO mAb and aurora A kinase inhibitors combined treatment of IMR-32 and CHP-134 cells.
Ch14.18/CHO mAbs were used at a concentration of 40 μg/ml and MK-5108/MK-8745 inhibitors were used in a concentration range of 0.03-30 μM. IMR-32 cells were treated with ch14.18/CHO mAb and MK-5108 inhibitor (**A**) or ch14.18/CHO mAb and MK-8745 inhibitor (**C**) or both agents used alone. CHP-134 cells were treated with ch14.18/CHO mAb and the MK-5108 inhibitor (**B**) or ch14.18/CHO mAb and the MK-8745 inhibitor (**D**) or both agents used alone. Cytotoxic effects were determined at 72 h by measuring the ATP content and compared with respective controls (set as 1) treated with PBS (for ch14.18 mAb) and/or DMSO (for MK-5108/MK-8745). Data are presented as means (± S.E.M.) from three experiments. Caspase 3/7 activities were determined at 72 h. (**E**) IMR-32 cells treated with ch14.18 mAb (40 μg/ml) or MK-5108 (0.1 μM) or combination of both agents. (**F**) CHP-134 cells treated with ch14.18 mAb (40 μg/ml) or MK-5108 (0.1 μM) or combination of both agents. (**G**) IMR-32 cells treated with ch14.18/CHO mAb (40 μg/ml) or MK-8745 (0.1 μM) or combination of both agents. (**H**) CHP-134 cells treated with ch14.18 mAb (40 μg/ml) or MK-8745 (0.1 μM) or combination of both agents. A one-way ANOVA was performed for the E, F, G and H sections of the figure, followed by Tukey's post hoc tests for pairwise comparisons; ^P<0.05; ^^P<0.01; ^^^P<0.001. *t*-tests were performed to compare the differences between the means of the agent *versus* the control; $^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001. ch14.18, cells treated with ch14.18/CHO mAb.

associated proteins. As presented in Fig. S2B, MK-5108 decreased the expression of P38α in IMR-32 cells. However, incubation of IMR-32 cells with MK-5108 did not affect the expression of total ERK1/2 kinase. Furthermore, the expression of ASK-1 upstream of MAPK decreased (Fig. S2B at https://ojs.ptbioch.edu.pl/index.php/abp). These observations indicated that the MK-5108 inhibitor exerts its effect in IMR-32 cells through the inhibition of the expression of ASK-1 and P38.

DISCUSSION

Most neuroblastomas express high levels of disialoganglioside GD2 (Keyel & Reynolds, 2019). Several

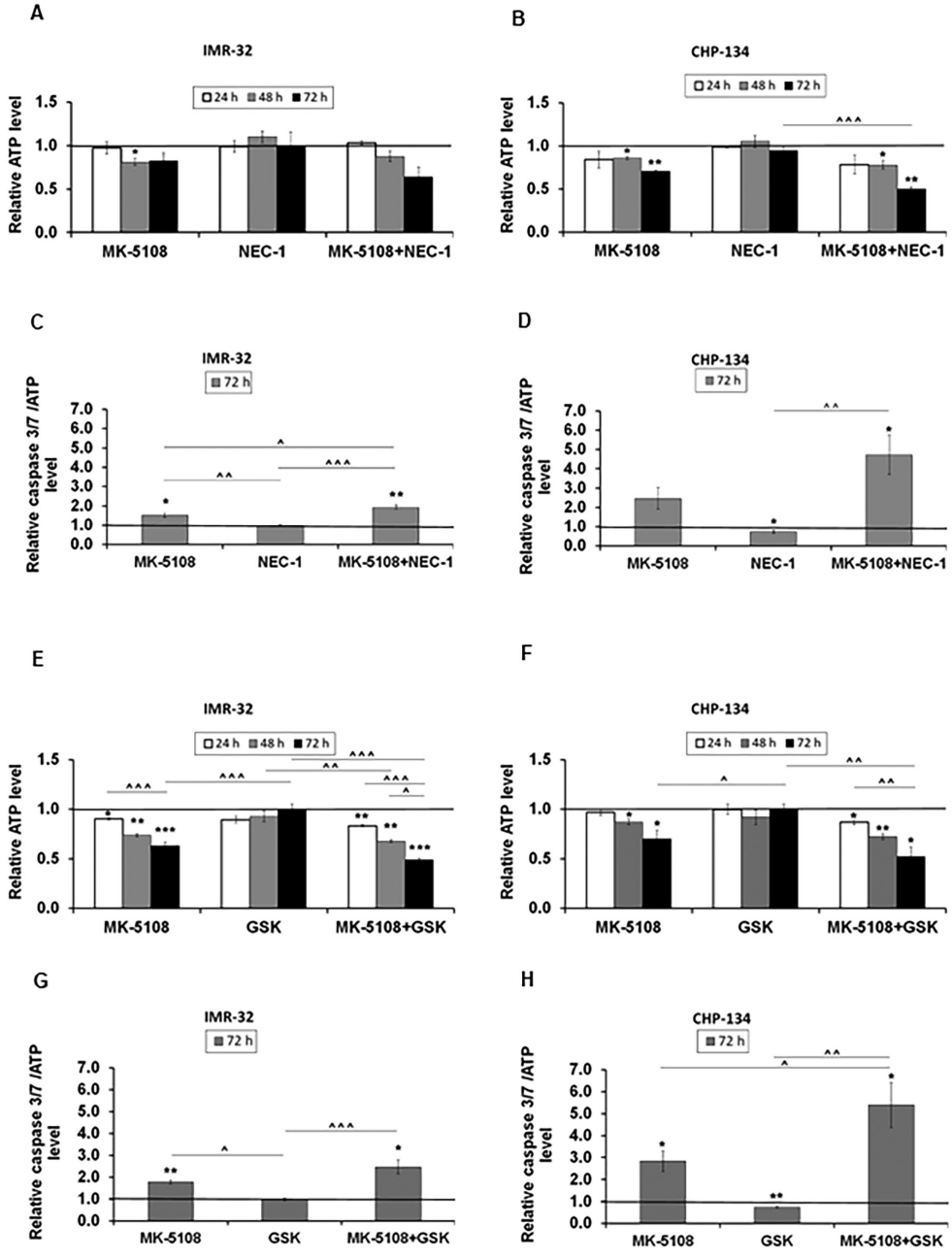

Figure 4. Analysis of apoptosis and necroptosis in IMR-32 and CHP-134 cells treated with MK-5108 and NEC-1/GSK'872 combined. Relative cellular ATP levels were measured at 24, 48 and 72 h in (**A**) IMR-32 and (**B**) CHP-134 cells treated with MK-5108 (0.1 μM) and NEC-1 (20 μM for IMR-32; 10 μM for CHP-134), cells treated with a single agent and control cells. Relative caspase 3/7 levels were measured at 72 h in (**C**) IMR-32 cells and (**D**) CHP-134 cells treated with MK-5108 (0.1 μM) and NEC-1 (20 μM for IMR-32; 10 μM for CHP-134), cells treated with a single agent and control cells, and divided by ATP levels of the respective groups of cells (to normalize signals to the number of viable cells). The means of three independent experiments (± S.E.M.) were calculated *vs*. control cells (cells treated with PBS and/or DMSO), which were set as 1 (black baseline). Relative cellular ATP levels were measured at 24, 48 and 72 h in (**E**) IMR-32 and (**F**) CHP-134 cells treated with MK-5108 (0.1 μM) and GSK'872 (3 μM for IMR-32; 10 μM for CHP-134), cells treated with a single agent and control cells. Relative caspase 3/7 levels were measured at 72 h in (**G**) IMR-32 cells and (**H**) CHP-134 cells treated with MK-5108 (0.1 μM) and GSK'872 (3 μM for IMR-32; 10 μM for CHP-134), cells treated with a single agent and control cells, and divided by ATP levels. A two-way ANOVA was performed for the A, B, E, and F sections of the figure and a one-way ANOVA was performed for the C, D, G, and H sections of the figure, followed by Tukey's post hoc tests. For clarity, only the *P* values for Tukey's post hoc test for pairwise comparisons were presented for the same time and different agents, and the same agent and different times were presented; ^P<0.05; ^^P<0.01; ^^^P<0.001. *t*-tests were performed to compare the differences between the means of the agent *vs*. control; *P<0.05; **P<0.01; ***P<0.001. Control, DMSO-treated cells; MK-5108, MK-5108-treated cells; NEC-1, necrostatin-1-treated cells; GSK, GSK'872-treated cells.

different antibodies have been developed to target the GD2 ganglioside in neuroblastoma cells (Kholodenko *et al*., 2018). Recent studies have elucidated the important role of ch14.18/CHO-based immunotherapy in the treatment of high-risk neuroblastoma, which is primarily based on Ab-dependent cellular cytotoxicity specific to GD2 (ADCC) (Siebert *et al*., 2017). As presented by Siebert and others (Siebert *et al*., 2017), the effects induced by ch14.18/CHO mAb increased the inhibitory immune checkpoint PD-1/PD-L1, while the combination of ch14.18/CHO mAb with a blockade of PD-1 results in synergistic treatment effects in mice. Strategies to fur-

ther enhance the efficacy of this treatment are important and are currently being explored in prospective clinical trials using ch14.18/CHO mAb and/or IL-2 treatment in patients with neuroblastoma (Ladenstein *et al*., 2018). Although the results of neuroblastoma therapy with ch14.18/CHO mAb are scrutinized in clinical practice, *in vitro* findings are underrepresented and require increased attention (Ladenstein *et al*., 2020).

The novelty of the study relies on showing the potential of a combination of ch14.18/CHO mAb and aurora A kinase-specific inhibitors, which are two distinct groups of drugs representing monoclonal antibodies and small molecule inhibitors, respectively. This approach based on the combination of immunotherapy with ch14.18/CHO mAb, used in clinics, with the pharmacological inhibition of a promising molecular target, i.e., aurora A kinase, was tested *in vitro* and shown to be an effective therapeutic strategy by means of potentiating apoptosis in neuroblastoma cells. This study compared the cytotoxic effects of two variants of anti-GD2 ganglioside-binding antibodies, the ch14.18/CHO mAb and the 14G2a mAb, on two neuroblastoma cell lines. Measurement of cellular ATP levels revealed that the effect exhibited by 14G2a mAb treatment was stronger, compared to ch14.18/CHO mAb treatment in IMR-32 cells (the identical concentration of both drugs was used). CHP-134 cells were identified to be similarly susceptible to treatment with either the 14G2a mAb or the ch14.18/CHO mAb. Treatment with the ch14.18/CHO mAb was associated with an increased level of cleaved caspase 3 in IMR-32 cells. These aforementioned effects were not observed in CHP-134 cells treated with the antibody. Similar observations have previously been made by us regarding the lack of apoptosis induction in CHP-134 cells after 14G2a mAb treatment (Horwacik *et al*., 2013).

AURKA is highly expressed in multiple types of human tumors, including neuroblastoma (D'Assoro *et al*., 2016). Previously, it has been shown that when combined with the GD2 ganglioside-specific 14G2a mAb, the specific small-molecule inhibitor of aurora A kinase, MK-5108, significantly enhances the cytotoxic effect against IMR-32 and CHP-134 cells (Horwacik *et al*., 2013). However, the molecular mechanism of inhibition of aurora A kinase through MK-5108 in neuroblastoma cells requires further clarification. Therefore, this study investigated the cytotoxic potential of MK-5108, which is a highly selective aurora A inhibitor, in the regulation of apoptosis in neuroblastoma cells. The results revealed that treatment with MK-5108 induced the expression of cleaved caspase 3 at 72 h in IMR-32 and CHP-134 cells. Additionally, caspase 3 and 7 activities increased in IMR-32 and CHP-134 cells treated with MK-5108, indicating induction of apoptosis in these neuroblastoma cultures. The results also indicated that MK-8745, another potent and selective aurora A kinase inhibitor tested, induced a level of cleaved caspase 3 at 72 h in both neuroblastoma cell lines. As presented by Nair and others (Nair *et al*., 2012), MK-8745 has been shown to induce apoptotic cell death in a P53-dependent manner when tested *in vitro* in pancreatic, sarcoma, and melanoma cells. Cells expressing wild-type P53 exhibited delayed mitosis followed by cytokinesis, resulting in 2N cells, along with apoptosis. However, cells with or without mutant P53 underwent a prolonged arrest in mitosis followed by endoreduplication and polyploidy (Nair *et al*., 2012).

In this study, a combination of ch14.18/CHO mAb and aurora A kinase inhibitors (MK-5108 and MK-8745) on IMR-32 and CHP-134 cells was also investigated. The most potent effect of this combination on the decrease in ATP levels, compared to both agents used individually, was observed at concentrations of 0.03 μM and 0.1 μM of inhibitors and ch14.18/CHO mAb (40 μg/ml) in both cell lines. Ch14.18/CHO mAb was confirmed to induce apoptosis in IMR-32 cells, but not in CHP-134, in a manner similar to the effect of 14G2a mAb reported previously. Most importantly, MK-5108 and MK-8745 aurora A inhibitors when used with ch14.18/CHO mAb further potentiate apoptosis in IMR-32 cells. It is postulated that the mechanisms of two drugs, i.e., ch14.18/CHO mAb and aurora A kinase inhibitors (MK-5108 or MK-8745) used in combination therapy, complement each other in the promotion of apoptosis by activating caspase 3 and caspase 7 activity in IMR-32 neuroblastoma cells. However, such apoptosis potentiation in CHP-134 cells is exclusively based on the effect of aurora A kinase inhibitors in inducing apoptosis (*via* involvement of caspase 3 and 7). Therefore, the observed mechanisms could be more cell line-specific and/or agent-dependent.

Necroptosis is an alternative mode of regulated cell death that combines features of apoptosis and necrosis (Dhuriya & Sharma, 2018). Necroptosis requires proteins such as RIP3 and its MLKL substrate, which play an important role in the pathway (Dhuriya & Sharma, 2018). Although RIP3 and MLKL are necessary for programmed cell death, necroptosis is confined to certain tissue types that express RIP3/MLKL (Dhuriya & Sharma, 2018). To investigate the role of necroptosis during MK-5108 treatment, the current study assessed the expression of key necroptosis-associated proteins. The expression levels of the RIP1 and RIP3 kinases were unchanged. To verify the involvement of necroptosis in MK-5108-stimulated cell death, specific inhibitors of RIP1 and RIP3 kinases, necrostatin-1 and GSK'872, were used, respectively. No recovery was recorded during the observed cytotoxic effect in MK-5108 and necroptosis inhibitor-co-treated cells, compared to control cells and MK-5108 used alone (as evaluated by measuring ATP levels), suggesting a low contribution of necroptosis to the observed cytotoxic effect. Remarkably, inhibition of RIP1 and RIP3 kinases combined with the MK-5108 inhibitor decreased ATP levels, advancing the observed cytotoxic effect, compared to control cells. Furthermore, the combination of MK-5108 inhibitor and necrosatin-1/GSK'872 was shown to significantly increase caspase 3 and 7, activities compared to control cells, indicating the improved efficacy of the proposed combined treatment. Similar results were also obtained for another aurora A kinase inhibitor used in this study, MK-8745, which caused an increase in the expression of cleaved caspase 3 in both cell lines. These aforementioned studies proved that the pharmacological inhibition of aurora A kinase by the two small molecule inhibitors examined, MK-5108 and MK-8745, causes cell death by apoptosis, but not necroptosis. Additionally, the results indicated that inhibitors of RIP kinases may potentiate apoptosis induced by aurora A kinase inhibitors. Most aurora A kinase inhibitors have been reported to induce apoptosis and the results of the current study are complimented by these reports (Boss *et al*., 2009; Nair *et al*., 2012; Shang *et al*., 2017; Das *et al*., 2021). However, Martens and others (2018) demonstrated that the pan-aurora kinase inhibitor, Tozasertib (VX-680; MK-0457), is a potent inhibitor of necroptosis. In contrast, Xie and others (Xie *et al*., 2017) identified the aurora kinase inhibitor, CCT137690, as an agent that induces necroptosis in pancreatic ductal adenocarcinoma cells *via* RIP1, RIP3, and MLKL. The

aforementioned study also reported that aurora A kinase interacts directly with RIP1 and RIP3 (but not MLKL) to reduce necrosome formation and activation.

Anomalies in protein synthesis are common during cancer treatment (Jaud *et al*., 2020). Several different stressors cause the accumulation of unfolded proteins in the ER, which is involved in protein synthesis, preservation of cellular homeostasis, and adaptation to unfavorable environments (Jaud *et al*., 2020). The accumulation of unfolded proteins in the ER causes a stress-induced unfolded protein response (UPR), which can result in the promotion of cell survival or the induction of apoptosis when stress is prolonged (Jaud *et al*., 2020). The mechanisms by which ER stress leads to cell death remain unclear. Therefore, defining which specific proteins serve a role in a particular drug response requires further study. The current study identified that the expression of ER stress transducers, the BIP protein, and the PDI protein decreased in IMR-32 cells treated with the MK-5108 inhibitor. However, CHP-134 cells exhibited increased expression of BIP and PDI, suggesting that activation of ER stress was initiated. As previously reported by Tanaka and others (Tanaka *et al*., 2000) the overexpression of the gene encoding PDI resulted in protection against the loss of cell viability induced by hypoxia in SK-N-MC neuroblastoma cells. Shen and others (Shen *et al*., 2021) also reported that combined inhibition of aurora A and HSF1 (Heat shock factor 1) promotes apoptosis in hepatocellular carcinoma by activating ER stress.

The ASK-1 kinase has been identified during the induction of apoptosis through signaling by the TNF-family of receptors (Matsukawa *et al*., 2004). During ER stress, ASK-1 is recruited to oligomerized IRE1 complexes containing TNF receptor associated factor 2, activating this kinase and causing downstream activation of JNK (c-Jun N-terminal kinase) and P38 MAPK (Matsukawa *et al*., 2004). ASK-1 has previously been reported to play a key role in ER stress-induced apoptosis (Hattori *et al*., 2009; Obsilova *et al*., 2021). Apoptosis signal-regulating kinase 1-/- neurons subjected to inducers of ER stress exhibit a requirement for this kinase for JNK activation and cell death (Nishitoh *et al*., 2002). The current study demonstrated that MK-5108 treatment reduced ASK-1 and P38 levels in IMR-32 cells. Similar observations have also been made for AGS and NCI-N78 gastric cancer cell lines treated with the small molecule aurora A kinase inhibitor, Alisertib, which exhibited an inhibitory effect on the activation of P38 MAPK at Thr180/Tyr182 (Yuan *et al*., 2015). Shang and others (Shang *et al*., 2017) revealed that Alisertib exerts antiproliferative, proapoptotic, and pro-autophagic effects on A375 and SK-Mel-5 melanoma cells by inhibiting P38 MAPK signaling.

CONCLUSION

As a consequence of the treatments with ch14.18/CHO and MK-5108, several different biological processes are significantly altered within the cells. Apoptosis is responsible for the cytotoxic effects of anti-GD2 ch14.18/CHO mAb in IMR-32 cells. Furthermore, the mechanism of MK-5108 and MK-8745 aurora A kinase inhibitors involves apoptosis, but not necroptosis, in both human neuroblastoma cell lines. This is accompanied by inhibition of the expression of ASK-1 and P38α kinases in IMR-32 cells treated with MK-5108 inhibitor. Finally, the combination of ch14.18/CHO mAb and aurora A kinase inhibitors enhanced the cytotoxic effect by way of apoptosis, compared to IMR-32 cells treated with a single agent. More studies are required to further elucidate biological processes and their interrelation within cells, and this may lead to the development of an effective neuroblastoma combination therapy. Revisiting the existing immunotherapeutic options and identifying suitable co-targets for the treatment of high-risk neuroblastoma requires further study.

Declarations

Author Contributions. MD, IH, and HR contributed to the study conception and design. Data collection and analysis were performed by MD and some complementary experiments were performed by AW, while analysis and interpretation of the results were performed by MD, HR and IH. The manuscript was written by MD. HR, IH, AW and IN read, interpreted and revised the manuscript critically for important intellectual content. All authors approved the final manuscript.

Availability of Data and Materials. The data generated during this study are included in the published article and the Supplemental data. Immunoblotting membranes derived from full gels are presented in Figs. S3-8 of the Supplemental data.

Conflict of Interest. The authors declare that they have no competing interests.

Acknowledgements. Declared none.

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