BMS-536924 sensitzes human epithelial ovarian cancer cells to the PARP inhibitor, 3-aminobenzamide

Marie-Claude Beauchamp a,b, Ariane Knafo a,b, Amber Yasmeen a,b, Joan M. Carboni c, Marco M. Gottardis c, Michael N. Pollak b,d, Walter H. Gotlieb a,b,*

a Division of Gynecologic Oncology, Jewish General Hospital, McGill University, Montreal, Quebec, Canada
b Segal Cancer Center, Lady Davis Institute of Medical Research, McGill University, Montreal, Quebec, Canada
c Bristol-Myers-Squibb Research Institute, Princeton, NJ 08543, USA
d Department of Oncology, McGill University, Montreal, Quebec, Canada

A B S T R A C T

Objective. To evaluate the anti-neoplastic activity of BMS-536924, an IGF-1R inhibitor, in epithelial ovarian cancer and its capacity to potentiate the effect of a PARP inhibitor, 3-aminobenzamide.

Methods. OVCAR-3, OVCAR-4, SKOV-3 and TOV-81D cell lines were investigated in low-serum tissue culture conditions (1%FBS). Cytotoxicity assays were performed in quadruplicates using the Alamar colorimetric assay in the presence of BMS-536924 and/or 3-aminobenzamide. The levels of phospho-AKT, phospho-S6, PARP-1 and phospho-H2AX were evaluated by western blotting in the presence of BMS-536924.

Results. BMS-536924 induced a time and dose inhibitory effect on cell survival. This effect seemed to be mediated by a reduction of pAKT and pS6 in a dose-dependent manner. The drug also provoked cell death by apoptosis as suggested by the increase in PARP-1 cleavage. It also induces DNA damage as demonstrated by the increased phosphorylation of histone H2AX and the augmentation of the comet tail moment. Finally, BMS-536924 sensitized cells to the effect of the PARP inhibitor, 3-aminobenzamide.

Conclusion. Our study reinforces the concept that IGF-1R is a good therapeutic target in ovarian cancer. Moreover, it suggests that combination therapy using BMS-536924 with a PARP inhibitor might be an effective strategy to circumvent resistance to treatment in clinical settings.

Introduction

Ovarian cancer is the leading cause of death among all gynaecological cancers in western countries. It is estimated that this year in North America, 24150 women will be newly diagnosed with ovarian cancer and that 17220 women will die of the disease [1]. Although most patients with epithelial ovarian cancer (EOC) experience a reasonable initial clinical response to treatments, the majority of these patients will not be cured. Approximately 70% will experience a recurrence and ultimately die of the disease [2]. Presently, there are no available treatments capable of curing recurrent ovarian carcinomas due to their rapid evolution into chemoresistant disease. It has therefore become essential to introduce new therapeutic modalities that will salvage these patients.

The insulin-like growth factor 1 (IGF-1) and its receptor (IGF-1R) are important regulators of growth and proliferation under normal physiological conditions. It stimulates cell survival activity, cell proliferation, translation, and inhibits apoptosis through the activation of the PI3K/AKT pathway [3]. Studies have recently reported that under pathophysiological conditions, the IGF-1R is implicated in tumorigenesis and neoplasia progression in EOC [4]. It has been shown that its signalling facilitates malignant transformation, inhibits apoptosis, drives the growth and progression of established tumours, and promotes invasion and metastasis [5]. Because of its presence on all ovarian cancer cells [6] it represents a good therapeutic target. Moreover, we have previously reported the presence of an autocrine loop in the human epithelial ovarian cancer cell lines, the OVCAR-3 and OVCAR-4 [7]. In this previous study, we also showed that the NVP-AEW541, an IGF-1R kinase inhibitor, reduced cell growth and promoted apoptosis through the inhibition of AKT [7]. Bristol-Myers-Squibb developed BMS-536924, an insulin-like growth factor-1 receptor (IGF-1R) kinase inhibitor used in the present study [5]. This compound has been shown to have in vitro and in vivo anti-tumour effect on ovarian cancer cells [8].

Poly (ADP-ribose) polymerase (PARP-1) is an abundant nuclear protein that has been implicated in DNA repair and the maintenance of genomic integrity [9]. PARP-1 also plays a role in the regulation of cell death, acting as a molecular switch between apoptosis and necrosis [10]. Indeed, over-activation of PARP-1 diminishes NAD+ and ATP storages, which are necessary for the apoptotic process [11]. Therefore, inhibition of PARP-1 will preferably lead to cell death by apoptosis. In the context of the implication of PARP-1 in DNA repair,
the use of PARP inhibitors has been heavily investigated in cells lacking other DNA repair mechanisms like homologous recombination, such as BRCA1 or BRCA2 deficient cells [12,13]. However, it has been recently reported that BRCA2 deficient cells can acquire resistance to PARP inhibitors [14,15]. This acquired resistance appears to result from revertent mutations that lead to the expression of functional BRCA2 proteins.

Several interactions have recently been described between the IGF-1R cascade and PARP activity in vitro, including PARP-1 cleavage [7,8,16]. In view of the importance of the IGF-IR pathway in cell survival and inhibition of apoptosis, and the potential interaction between this pathway and the PARP pathway, we investigated whether the combination of BMS-536924 with the PARP-1 inhibitor, 3-aminobenzamide (3-AB) could enhance cell death in EOC cells.

Materials and methods

Cells lines and treatment

The ovarian cancer cell line OVCAR-3, SKOV-3 (American Tissue Culture Collection, Manassas, VA) and OVCAR-4 were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 10 μg/ml gentamicin. The cells were routinely passaged every 5 to 7 days. TOV-81D cells were kindly provided by Dr Anne-Marie Mes-Massons [17,18]. Cells were cultured in OSE medium containing 15% FBS, 10 ng/ml endothelial growth factor, 5 μg/ml bovine pituitary extract, 5 μg/ml insulin and 0.5 μg/ml hydrocortisone and passaged every week. The BRCA status of each cell line has been well defined in the literature [19,20] and only the TOV-81D were reported to carry a BRCA2 mutation, namely the 8765delAG [17,18]. All cells were maintained at 37 °C in 5% CO₂, 95% air atmosphere incubator. Assays were performed in medium containing 1% FBS. BMS-536924, an IGF-IR kinase inhibitor, was obtained from Bristol-Myers-Squibb and kept as a stock solution of 10 mM in DMSO. 3-aminobenzamide was obtained from Sigma (#A0788) and kept as a stock solution of 1 M in DMSO.

Western blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Briefly, clarified protein lysates (50 μg) were resolved electrophoretically on 10% denaturing SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking in 5% milk, membranes were probed with the following primary antibodies specific for: phospho-AKTser473, phospho-S6 ribosomal proteinser235/236, total AKT, PARP (New England Biolabs, Pickering, Ontario), phospho-histone H2AX (Millipore, Etobicoke, Ontario) and actin (Cederlane, Burlington, Ontario). Proteins were visualized with Horseradish peroxidise (HRP)-conjugated secondary antibodies (Amersham Biosciences, Baie d’Urfe, QC). Antigen-antibody complexes were detected using the ECL system (Amersham Biosciences).

Cytotoxicity assays

For the cytotoxicity assay, monolayers of 2000 cells were plated into 96-well flat-bottom cell culture plates (Corning Incorporated, NY, USA). Twenty-four hours after plating, when the cells had attached and reached ~40% confluency, cells were washed and the medium was replaced with medium containing 1% FBS for the indicated time periods. Controls included equal amount of DMSO. BMS-536924 was used at concentrations ranging from 1 μM to 20 μM for 24 to 72 h. For the combination of 3-aminobenzamide and BMS-536924, both drugs were added at the same time at the indicated doses for 72 h. All experiments were performed in quadruplicates and were reproduced and confirmed in three independent experiments. Cell viability was assessed by visual inspection of the plates and by using the AlamarBlue colorimetric assay as described in the Materials and methods section. Results are mean of two independent experiments.
After cells were treated, 6 μl of AlamarBlue was added into each well. When the color of the dye changed (approximately 4 h), plates were read in an ELISA plate reader at 2 different wavelengths, 562 nm and 620 nm to plot the graph. Percentage of reduced AlamarBlue was calculated using the following equation:

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\text{Reduced AlamarBlue} (\%) = A_{562} - (A_{620} \times R_0);
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where \(A_{562}\) and \(A_{620}\) are sample absorbencies minus the media blank; \(R_0 = A_{562}/A_{620}\) where \(A_{562}\) is the absorbance of oxidized form at 562 nm, and \(A_{620}\) is the absorbance of oxidized form at 620 nm.

**Determination of protein concentrations**

Total protein content was measured according to the Lowry method [21] using a colorimetric assay (Bio-Rad, Mississauga, Ontario).

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**Fig. 3.** Dose-dependent effect of BMS-536924 on PARP cleavage. Proteins extracted from OVCAR-3 and OVCAR-4 cells exposed to DMSO or increasing doses of BMS-536924 for 48 h in medium containing 1% FBS were subjected to western blotting for PARP expression, and actin. One representative blot out of three is shown. Expression of cleaved PARP between 0 and 5 μM BMS-536924 is shown in the bottom of the figure.

**Fig. 4.** BMS-536924 increases the cytotoxic effect of 3-aminobenzamide. (A) OVCAR-3, (B) OVCAR-4, (C) TOV-81D and (D) SKOV-3 cells were incubated for 72 h in 1% FBS with increasing doses of BMS-536924 and/or 3-aminobenzamide (3-AB) and assessed for viability using the AlamarBlue colorimetric assay as described in the Materials and methods section. Results are mean of four independent experiments.
**Comet assay**

To measure the DNA damage, the single cell gel electrophoresis assay from Trevigen was used (#4250-050-K) [22]. Briefly, 50 µl of cells at 1 × 10^5/ml was mixed with 500 µl of comet LMAgarose, and from this mixture, 50 µl was applied onto a comet slide well. Slides were allowed to solidify at 4 °C for 30 min. All slides were then placed in pre-chilled lysis solution for 45 min at 4 °C. After lysis, slides were treated with an alkali solution (300 mM NaOH and 1 mM EDTA) at room temperature for 1 h and electrophoresed in 1× TBE at 1 V/cm for 20 min. Slides were fixed in 70% ethanol for 5 min and stored at 4 °C until image analysis. Cellular DNA was stained with 1:10 000 SYBR Green in TE buffer. The slides were analyzed using the CometScore 15 software. The results were expressed as the mean comet tail moment of ~50 cells. The comet tail moment is defined as the product of the percentage of cellular DNA in the comet tail and the length of DNA tail migration. The higher the comet tail moment value, the greater the amount of cellular DNA strand breaks.

**Results**

**Effect of BMS-536924 on cell toxicity in OVCAR-3 and OVCAR-4**

First, we evaluated the effect of BMS-536924 on OVCAR-3 and OVCAR-4 cell lines. Cells were incubated with 1 to 10 µM BMS-536924 for 24 to 72 h and demonstrated a dose and time-dependent cytotoxicity (Fig. 1).

**Effect of BMS-536924 on phospho-AKT and phospho-S6 in OVCAR-3 and OVCAR-4**

We next verified the signalling pathways associated with BMS-536914 at the same concentrations that induced cytotoxicity in epithelial ovarian cancer cells. Cells were exposed to increasing doses of BMS-536924 for 30 min in medium containing 1% FBS. As shown in Fig. 2, BMS-536924 inhibited the phosphorylation of AKT and one of its downstream targets, ribosomal protein S6. In both cases, BMS-536924 caused a dose-dependent inhibition of phosphorylation, without affecting the total protein.

**BMS-536924 induces apoptosis in vitro**

IGF-1R signalling is known to inhibit apoptosis in tumour cells. When OVCAR-3 and OVCAR-4 cells were treated with BMS-536924, cell death was observed. We therefore investigated whether BMS-536924 could induce apoptosis in both cell lines. Cells were treated with increasing doses of the drug (1, 5, 10, 20 µM) in 1% FBS for 48 h. Cleaved PARP, an early indicator of apoptosis, was increased in a dose-dependent manner in response to BMS-536924 (Fig. 3).

**Additive effect of BMS-536924 and 3-AB on cell toxicity in ovarian cancer cells**

We next assessed the combination effect of the PARP inhibitor 3-AB and BMS-536924. Various cell lines were treated with increasing doses of 3-AB in the presence of increasing doses of BMS-536924 for 72 h in medium containing 1% FBS. As shown in Fig. 4, the combination of BMS-536924 and 3-AB showed increased cytotoxicity in the four cell lines tested.

**Induction of DNA damage by BMS-536924**

It has been reported that hyperphosphorylation of histone H2AX is a sensor of DNA damage in cells [23]. We next evaluated if BMS-536924 could enhance DNA damage in our cellular model, thereby sensitizing cells to PARP inhibition. OVCAR-3 and OVCAR-4 cells were treated with increasing concentrations of BMS-536924 (5, 10, 20 mM) in 1% FBS for 48 h. As demonstrated in Fig. 5A, phospho-H2AX was induced in a dose-dependent manner in the presence of the drug. To further confirm these results, the more sensitive comet assay was used. OVCAR-3 cells were treated with 1 or 10 µM BMS-536924 for 24 h and the comet assay was performed as described in the Materials and methods section. As shown in Fig. 5B, BMS-536924 increased dose-dependently DNA damage.

**Discussion**

Epithelial ovarian cancer is the leading cause of death among gynaecological cancers and close to 70% of patients with advanced-stage disease will experience recurrence [24]. This is caused by the development of resistance to present therapies, implying the need to develop new therapeutic modalities.

One promising target is the IGF-1 receptor [7,8]. In the present study, we confirmed our previous data using a novel IGF-1R kinase inhibitor, BMS-536924 that inhibited cell survival in a time and dose-dependent manner (Fig. 1), at concentrations that have previously been reported [8,25]. Our study suggests that the mechanisms involved in the inhibition of cell proliferation by BMS-536924 include, at least partly, a reduction of the phosphorylation of AKT as well as one of its downstream targets, ribosomal protein S6 (Fig. 2).

Our results suggest that BMS-536924 induced apoptosis as indicated by the elevation of PARP-1 cleavage (Fig. 3). There has been a growing interest in the use of PARP inhibitors in the treatment of cancer, especially breast and ovarian cancer, as shown by the increasing number of clinical trials with these molecules [26,27]. However, resistance to PARP inhibitors has become a potential challenge in their use [14]. In this manuscript, we show that the IGF-IR kinase inhibitor BMS-536924 increases the cytotoxicity...
induced by the PARP inhibitor, 3-aminobenzamide in human epithelial ovarian cancer cell lines (Fig. 4). As shown in Fig. 4, the PARP inhibitor alone reduced cell survival in the four cell lines tested regardless of their BRCA status since only the TOV-81D were reported to bear a BRCA2 mutation, the 8765delAG [17–20]. This is consistent with previously published data in human breast cancer cells [28], and suggests that the clinical relevance of PARP inhibition is not restricted to patients carrying a BRCA mutation [29]. The selective effect of PARP inhibition on BRCA deficient cell lines seems to be restricted to very potent PARP inhibitors [30], 3-aminobenzamide, used in this study, is not part of this family of PARP inhibitors, and shows a similar effect on ovarian cancer cell lines irrespective of BRCA mutation status. Most studies using PARP inhibitors described a reduced cell survival when treated in combination to DNA damaging agents, such as ionizing radiation or chemotherapeutic agents [31]. Although our findings do not unravel the various mechanisms involved, one possible explanation is that the induction of DNA damage by BMS-536924 sensitizes cells to PARP inhibition. This is supported by our results showing an increase of the phosphorylated histone H2AX, a known sensor of DNA damage [23], as well as the increase of the tail moment observed in the comet assay (see Fig. 5). Also, we showed that BMS-536924 provoked the cleavage of PARP-1 (see Fig. 3). It has been reported that this cleavage is caused by the caspase-3, -6, -7 complex during the execution phase of apoptosis and leads to inactivation of PARP-1. This allows the prevention of an over-activation of PARP-1 by DNA damage, preserving cellular energy (NAD+ and ATP) for a proper apoptotic process, which is consistent with our findings.

Experiments aimed at describing interactions between the IGF-1 and the DNA repair PARP-1 pathways are presently ongoing. We showed that the BMS-536924-induced cytotoxicity of ovarian cancer cells was associated with a dose-dependent inhibition of Akt phosphorylation. Intriguingly, as shown in Fig. 4, the TOV-81D cells seemed less sensitive to BMS-536924 alone compared to the other cell lines. All cell lines used in our study express detectable levels of IGF-1R (data not shown) which by itself cannot explain the discrepancy observed between the cell lines. In this context, it is interesting to note that only few studies in breast cancer reported increased IGF-1R expression in patients carrying BRCA mutations [32,33]. Moreover, BRCA1 was also recently reported to decrease activation of Akt [34] and to inhibit IGF-1R expression in vitro [35,36], suggesting some common aspects of the IGF-1R and the DNA repair pathways.

Previous studies have demonstrated that the inhibition of the IGF-1R pathway in addition to other cytotoxic treatments, such as cisplatin [7] or irradiation [37], increases cancer cell death. One recent publication revealed that combination of BMS-536924 to an inhibitor of the HER family of receptors might be an effective strategy for breast and ovarian cancer treatment [25]. In this report we show that the combination of IGF-1R kinase inhibition and PARP-1 inhibition increases ovarian cancer cell death in vitro. These data justify further investigation in the mechanisms underlying the increased cytotoxicity and its potential application in patients with cancer.

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References


