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# Potentialization of N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK) cytotoxic activity by 2-(1-adamantylamino)-6-methylpyridine (AdAMP) in human ovarian cancer cells

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## ABSTRACT

**Objectives:** TNF is one of the key cytokines involved in cancer development. TNF signaling can result in both stimulating and inhibitory signals that can result in opposite biological effects in cancerogenesis. 2-(1-adamantylamino)-6-methylpyridine (AdAMP) enhances TNF secretion whereas N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a NF-κB inhibitor potentially stimulating proapoptotic TNF signals. The aim of the study was to assess the effect of TPCK in combination with AdAMP on human ovarian cells.

**Material and methods:** CAOV-1 human ovarian cell line was incubated with TPCK and AdAMP for 24 hours. The cytotoxic effect was evaluated in a crystal violet assay. A monoclonal antibody against TNF, Infliximab, was added to examine the possible mechanism of interactions.

**Results:** Depending on concentration, AdAMP potentialized cytotoxic activity of TPCK or had a synergistic effect with TPCK. Infliximab did not reverse cytotoxicity of AdAMP and TPCK and in some cytotoxic and non-cytotoxic concentrations even enhanced their cytotoxicity.

**Conclusions:** AdAMP and TPCK cytotoxicity seems to be dependent on TNF signaling, however, the exact mechanism of interactions remains unclear.

**Key words:** AdAMP, TPCK, TNF, NF-kB inhibitors, proteasome, ovarian cancer, cell line

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## INTRODUCTION

Ovarian cancer remains the main cause of mortality among malignancies of female reproductive tract in developed countries [1]. Unsatisfactory survival rates are associated with late diagnosis and in consequence with inadequate treatment [2]. Contemporary management of advanced ovarian cancers usually includes cytoreductive surgery combined with adjuvant platinium-taxane based chemotherapy. Although the response rate in the first line treatment is relatively high, relapses account for about 70% of cases [3]. Treatment of recurrent ovarian cancer that is based mainly on various chemotherapy regimen poses a problem of drug resistance, drug toxicity and decreased quality of life what in turn is the limitation of further treatment options and decreases overall survival [4]. Thus, intervention with chemopreventive agents or new adjuvant therapy may offer a desirable option for the improvement in ovarian cancer management [5].

TNF is a multipotent cytokine that plays an important role in cancer growth regulation. The main source of TNF

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are macrophages and monocytes but this cytokine can also be secreted by other cells including human ovarian cancer cells [6]. A biological effect of TNF is a result of stimulating and inhibitory signals that can give opposite results in different conditions. TNF-R1 is involved in most pathways, whereas signaling through TNF-R2 is less understood [7].

The pleiotropic actions of TNF range from proliferative responses such as cell growth and differentiation, through inflammatory effects and the mediation of immune responses, to destructive cellular outcomes such as apoptotic and necrotic cell death mechanisms [8]. Procaspase-8 activation is the essential pathway of TNF induced apoptosis, whereas there are two arms of proliferative signals transducing that ultimately result in the activation of two major transcription factors: NF-κB and c-Jun. The existence of extensive cross talk between apoptosis, NF-κB and c-Jun signaling pathways causes that in the absence of NF-κB activity cellular susceptibility to TNF-induced apoptosis increases, whereas enforced activation of NF-κB protects against apoptosis [9].

Several adamantylamino-pyridine and -pyrimidine derivatives have TNF production-enhancing properties. 2-(1-adamantylamino)-6-methylpyridine (AdAMP) is the most potent of these adamantane derivatives, on some biological functions and TNF production by normal and neoplastic cells [10, 11]. In the previous study we demonstrated a strong TNF production-enhancing activity of AdAMP in human ovarian cancer cell line CAOV1 [12].

Adamantane derivatives were used for treatment of type A Influenza and the Parkinson's disease [13, 14]. Antiproliferative activity of adamantane derivatives was found against murine leukemia cells (L1210), human T-lymphocyte cells (CEM), and cervix carcinoma cells (HeLa) [15].

NF-kB inhibitors constitute a heterogenic group of agents that can inhibit a proliferative pathway of TNF signaling. The well-known NF-kB inhibitors are some antioxidants, IkBa phosphorylation and/or degradation inhibitors, and proteasome inhibitors, i.e. N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK), pyrrolidine dithiocarbamate or MG-132 [16–18]. Some other agents like non-steroidal anti-inflammatory drugs, glucocorticoids, and cytokines (Il-10) have the activity of NF-kB inhibitors [18–20]. A reversible 26S proteasome inhibitor, bortezomib, that has been recently approved by the Federal Drug Administration (FDA) and the European Regulatory Agency (EMEA) for the treatment of multiple myeloma at least partly acts through the inhibition of NF-κB activity [21].

## OBJECTIVES

In this study we tried to investigate the effect of N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in combination with 2-(1-adamantylamino)-6-methylpyridine (AdAMP) on human ovarian cell line CAOV-1 growth and to explain the possible mechanism of their action.

## MATERIAL AND METHODS

### Human ovarian cancer cells

The CAOV-1 line was a human ovarian cell line established in Jagiellonian University in Krakow.

The cell line was cultured in Dulbecco'sMEM with 4.5 g/L glucose, sodium pyruvate and Glutamax-1 (high glucose DMEM) supplemented with antibiotic-antimycotic, 50 μM 2-mercaptoethanol and 10% fetal calf serum (FCS) (all from Gibco BRL, Life Technologies, Paisley, UK). Cells were maintained at 37°C in a humidified atmosphere containing 5% $CO_2$.

### Reagents

2-(1-adamantylamino)-6-methylpyridine (AdAMP) was synthesized at the Institute of Chemistry, Agriculture University of Warsaw, as described previously [22].

N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was purchased in Sigma (St. Louis, USA) . Both drugs were prepared as 100 mM stock solutions in DMSO and were diluted to the required concentration in culture medium.

Infliximab (Remicade®), a monoclonal antibody against TNF, was obtained from Janssen Biotech, Inc. (Belgium). The stock solution (10 mg/mL) was prepared in distilled water.

### Crystal violet assay

The cytotoxic effect of TPCK and/or AdAMP on ovarian cancer cells was tested in a standard crystal violet assay. The dye in this assay, crystal violet, stains DNA of adherent cells in monolayer. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture.

Cells were incubated in 96-well plates ($2 \times 10^4$/200 μL/well) with AdAMP (final concentrations: 25, 50, 100 and 200 μM) and TPCK (final concentrations: 8, 16, 32 and 64 μM), alone or in combination, for 24 h. In further experiments Infliximab at the concentration of 1, 10 or 100 μg/mL was added to the wells with AdAMP and TPCK and the cells were incubated in the same way. At the end of each incubation medium was removed and each well was washed with 200 μL of phosphate buffered saline (PBS). 50 μL of 0.1% water solution of crystal violet was added to each well for 10 minutes at room temperature. Then the plates were washed in distillated water. Crystals of violet were dissolved by adding 200 μL of 1% solution of sodium dodecyl sulfate (SDS) and shaking the plates. The plates were read on an ELISA reader (SLT-Labinstruments, Salzburg, Austria) using a 550 nm filter.

The means and standard deviations were determined for triplicate samples. The cytotoxic effect was expressed as

the relative viability and was calculated as follows: relative viability = [(experimental absorbance - background absorbance)/(absorbance of vehicle-treated cells — background absorbance) ×100.

### Statistical analysis

Statistical evaluations were performed with Statistica 7.0 software (StatSoft, Poland). Comparisons between groups of continuous outcomes were performed by Student's t-test after testing for normal distribution by the Kolmogorov-Smirnov test. $P < .05$ was considered significant.

Chou-Talalay analysis was performed to evaluate the strength of interaction between the drugs [23]. The resulting combination index (CI) was calculated. CI < 0.9 was considered to be synergism. Dose reduction index (DRI) — a measure of how many folds the dose of each drug in a synergistic combination may be reduced — was calculated using Calcusyn software.

## RESULTS

### AdAMP and TPCK alone are cytotoxic to CAOV-1

To evaluate the cytotoxic effect of AdAMP and TPCK we incubated CAOV-1 cell line in 96-well plates as described above for 24 hours and assessed cell culture viability in crystal violet assay. AdAMP was not cytotoxic to CAOV-1 line in the concentration of 25, 50 and 100 μM. The cytotoxic effect of AdAMP was statistically significant at the concentration of 200 μM ($P < 0.01$) (Fig. 1A).

16, 32, 64 and 128 μM TPCK was cytotoxic to CAOV-1 line. TPCK at the concentration of 8 μM had no effect on the cell line viability (Fig. 1B).

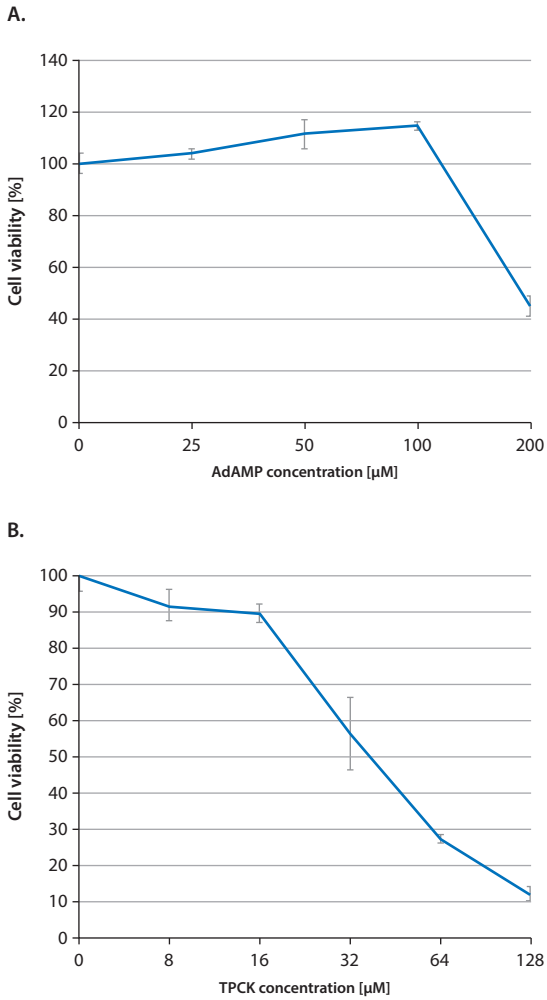


**Figure 1.** Cytotoxic effect of AdAMP (**A**) and TPCK (**B**) on CAOV-1 cell line. Values are mean ± SD. *P < 0.05, #P < 0.01, ^P< 0.001

### Combinations of AdAMP and TPCK synergistically inhibit the growth of CAOV-1 cells in vitro

To determine if effectiveness of these two agents could be enhanced by applying them together we tested 25, 50, 100 and 200 μM AdAMP combined with 16, 32, 64 and 128 μM TPCK. AdAMP potentialized cytotoxic activity of TPCK in the following concentrations: 50 μM AdAMP + 64 μM TPCK, 100 μM AdAMP + 32–64 μM TPCK, 200 μM AdAMP + 8–128 μM TPCK. Only the combination of 200 μM AdAMP and 32 μM TPCK resulted in the synergistic interaction (CI = 0.598) (Fig. 3). Dose Reduction Index (DRI) for that combination of drugs was 1.946 for AdAMP and 11.883 for TPCK.

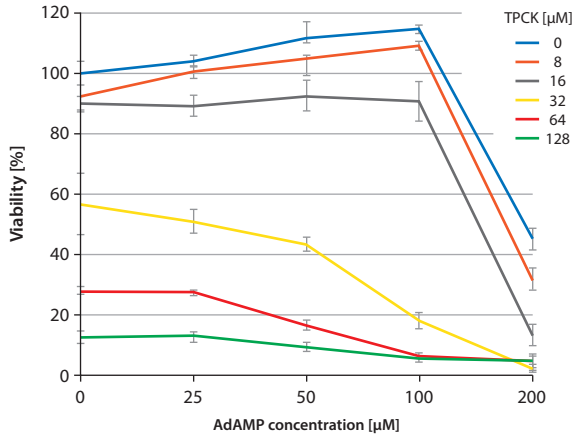


**Figure 2.** Cytotoxic effect of AdAMP and TPCK on CAOV-1 cell line. Values are mean ± SD. P value presents differences in series between cells non-stimulated and stimulated by AdAMP. *P < 0.001, #P < 0.0001

### TNF blocking by Infliximab does not reverse the synergistic effect of AdAMP and TPCK

To answer the question if potentialization/synergy in cytotoxic activity of AdAMP and TPCK is the result of enhanced TNF secretion associated with NF-κB pathway inhibition we blocked TNF with Infliximab in the concentration of 1, 10 or 100 mg/L. As Infliximab was cytotoxic to CAOV-1 cell line in

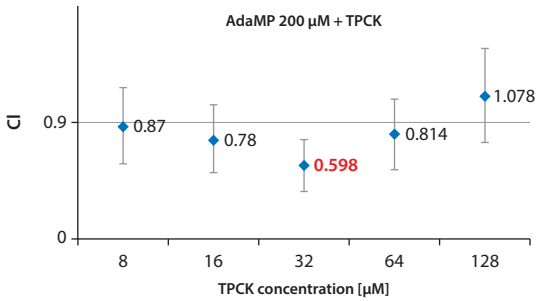


**Figure 3.** Cytotoxic interactions of AdAMP and TPCK. CI — combination index, CI was calculated from cytotoxic activity of drug combination. CI + 1SD < 0.9 is considered to be synergism

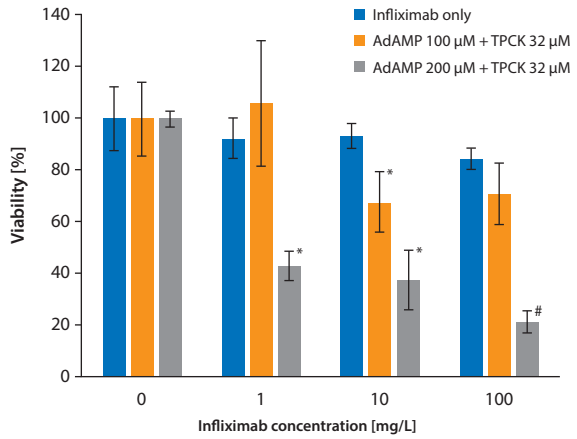


**Figure 4.** Influence of Infliximab on combined AdAMP and TPCK cytotoxicity in CAOV-1 cell line. Values are mean ± SD. P value presents differences between cells incubated only with Infliximab or with Infliximab and AdAMP and TPCK. *P < 0.01, #P < 0.001

the concentration of 100mg/L (relative viability 84.6 ± 4.0%, P < 0.0005) cellular viability was compared between the group incubated only with Infliximab or with Infliximab and AdAMP and TPCK. Infliximab did not reverse cytotoxic activity neither in 200 µM AdAMP and 32 µM TPCK nor in 100 µM AdAMP and 32 µM TPCK. 1, 10, 100 mg/L of Infliximab even enhanced cytotoxicity of 200 µM AdAMP and 32 µM TPCK and 10 mg/L of Infliximab had similar effect when added to 100 µM AdAMP and 32 µM TPCK (Fig. 4).

## DISCUSSION

A combination chemotherapy based on adjuvant mechanisms of action of drugs has become the standard approach in the treatment of ovarian cancer. In target therapy the complementary response to different agents usually includes interfering with different stages of cell signaling what in turn results in augmenting or silencing the desired reaction [24].

The study of Pazhang et al. is an example of a combination target therapy with NF-kB inhibitor [25]. The author showed synergistic effects of cytotoxic activity of NF-kB inhibitor, celastrol, and X-linked inhibitor of apoptosis protein (XIAP) inhibitor, embelin used in combination in an acute myeloid leukemia cell line, HL-60. He explained that the synergy of the two agents may be due to cross-talk between NF-κB and XIAP pathway of signal transduction.

In the present study we demonstrated that a NF-kB inhibitor, N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is an active agent cytotoxic to CAOV-1 ovarian cell line. Its cytotoxic activity can be augmented by 2-(1-adamantylamino)-6-methylpyridine (AdAMP) in some concentrations. It is noteworthy that AdAMP potentialized cytotoxic activity of TPCK not only in the concentration in which AdAMP had cytotoxic activity alone (200 µM) but also in the concentration of 50–100 µM in which it was not cytotoxic to CAOV-1 cell line when applied as the only agent. We hypothesized that it could be at least partly due to the enhanced autocrine secretion of TNF associated with NF-kB pathway inhibition. This idea seemed to be supported by our previous study where we demonstrated a stimulation of TNF secretion by CAOV-1 cell line in the concentrations of 0.1 to less than 100 µM [12]. The real potential of CAOV-1 to secrete TNF in higher concentration of AdAMP is unknown as it is confounded by decreased viability of ovarian cancer cells.

The synergistic effect of AdAMP and TPCK defined according to Chou and Talalay [23] was observed only in the combination of 200 AdAMP AdAMP and 32 µM TPCK. However, it is necessary to emphasize that synergy can only be calculated if both drugs have cytotoxic activity. So, the CI had no application in the AdAMP concentrations of less than 200 µM that were "non-toxic" concentrations alone.

The experimental model with Infliximab added to AdAMP and TPCK showed that neither the synergy between AdAMP and TPCK nor the augmentation of cytotoxic activity of TPCK by AdAMP can be entirely explained by TNF-dependent apoptosis. Blocking TNF by Infliximab did not reverse the cytotoxic effect of AdAMP and TPCK what can suggest that TNF-induced-apoptosis with NF-κB pathway inhibition does not have be the dominating mechanism of action in this situation. On the other hand, Infliximab binds both soluble and membranous TNF and activates in vitro antibody- and complement-dependent cellular cytotoxicity by its Fc portion [26]. Lugering et al. showed that TNF inhibition by Infliximab causes apoptosis in T-cells through the activation of caspase-8, -9, -3 and the increased transcription of the pro-apoptotic proteins Bax and Bak [27, 28]. It is not proved that this mechanism of action of Infliximab can be observed in ovarian cancer cells but we are aware that the potential of Infliximab to stimulate apoptosis could conceal the real action of AdAMP and TPCK in our model. In light of these investigations the influence of Infliximab both in cytotoxic and non-cytotoxic concentrations on the viability of CAOV-1 cell line treated with AdAMP and TPCK observed in our study can indirectly suggest that AdAMP and TPCK

interact through the TNF signaling pathway. Unfortunately, multidirectional and dose-dependent TNF action makes it difficult to unambiguously prove one specific pathway of TNF signaling [29].

## CONCLUSIONS

We conclude that 2-(1-adamantylamino)-6-methylpyridine and N-a-tosyl-L-phenylalanine chloromethyl ketone are agents active against ovarian cancer cells and applied together reveal synergy or potentialization of cytotoxic activity. The molecular mechanism of that interactions remains unclear, however, it is probable that they act through TNF signaling.

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