Resistance to TRAIL-induced apoptosis in ovarian cancer cell lines is overcome by co-treatment with cytotoxic drugs

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Abstract

Background. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand, is a recently identified cytokine that preferentially kills transformed cells while sparing most normal cells.

Methods. We investigated the ability of TRAIL alone and TRAIL in combination with cytotoxic drugs to induce apoptosis in six ovarian cancer cell lines. To get some insight into the resistance to TRAIL, the expression of TRAIL receptors and selected downstream signaling elements was determined.

Results. TRAIL induced significant apoptosis (up to 80%) in three out of six ovarian cancer cell lines (MZ-26, CaOV-3, ES-2). In A2780 and A2780ADR cells, resistance to TRAIL-induced apoptosis correlated with their lack of DR4-expression. MZ-15 cells, which expressed the processed form of FLIPL, p43 (FADD-like IL-1β-converting enzyme (FLICE)-like inhibitory protein (FLIP)), and FLIPS, were resistant to TRAIL in spite of the presence of DR4. When TRAIL-resistant cell lines were co-incubated with routinely used cytotoxic agents, TRAIL exerted a synergistic effect leading to apoptosis rates unachievable by incubation with cytotoxic agents alone.

Conclusion. The ability of TRAIL to induce apoptosis in ovarian cancer cells as well as to potentiate the activity of chemotherapeutic agents even in cell lines that are resistant to TRAIL-induced cytotoxicity is a powerful promise in the fight against this deadly disease.

Keywords: Ovarian cancer; Apoptosis; TRAIL resistance; Chemotherapy

Introduction

Due to the lack of efficient screening methods, ovarian cancer is usually diagnosed at late stages, and therefore, the majority of patients require additional treatment after the surgical removal of tumors. After an initial response to chemotherapy, most patients ultimately develop resistance, and substances to enhance and support the activity of cytotoxic drugs are desperately needed.

Several members of the tumor necrosis factor family such as tumor necrosis factor alpha (TNFα), Fas ligand (FasL), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) have been shown to induce apoptosis in susceptible cells (for review, see Refs. [1–3]). Unlike FasL and TNFα, TRAIL induces apoptosis preferentially in malignant cells while sparing normal cells. Apoptosis is induced by binding of TRAIL to its pro-apoptotic receptors DR4 or DR5, while binding to its soluble receptor osteoprotegerin (OPG) or to decoy receptors 1 and 2 (DcR1, DcR2), which lack a functional death domain, does not lead to cell death. Ligand binding to DR4 and DR5 causes receptor oligomerization and formation of a death inducing signaling complex (DISC) further downstream, which leads to the recruitment and
activation of caspase 8. The FADD-like IL-1β-converting enzyme (FLICE) inhibitory protein (FLIP) is an intracellular inhibitor of caspase 8 that binds to the DISC and potentially inhibits apoptosis mediated by all death ligands including TRAIL [4].

In the present study, we have evaluated the activity of TRAIL in a panel of ovarian cancer cell lines in terms of induction of apoptosis and expression of TRAIL-receptors DR4, DR5, DcR1, and DcR2 as well as of apoptosis-modulating proteins caspase 8, FLIP, and OPG. In addition, we have examined the cytotoxic effect of TRAIL in combination with the cytotoxic drugs most commonly administered in ovarian cancer.

Materials and methods

Cell lines

Epithelial ovarian cancer cell lines ES-2 (CRL-1978) and CaOV-3 (HTB-75) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), and cell lines A2780 and A2780ADR from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK). Through cooperation with the Department of Gynecology of the Medical School, Vienna, Austria, we received cell lines MZ-15 and MZ-26. All cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and penicillin/streptomycin.

The normal human chondrocytes were purchased from PromoCell bioscience alive GmbH (Heidelberg, Germany). Both benign cell lines were cultured in the cell type specific appropriate medium supplied by PromoCell bioscience alive GmbH. Experiments with primary culture cells were performed within two to four passages.

TRAIL-, paclitaxel-, topotecan-, carboplatin-, and adriamycin-mediated cytotoxicity and apoptosis

Recombinant human TRAIL was purchased from CHEMICON International (Temecula, CA, USA). Cells were seeded in 6-well plates at a density of 1 × 10^6 cells/well and allowed to adhere to the plate overnight. To assess the tumoricidal activity of TRAIL, recombinant human TRAIL was added at final concentrations of 100, 300, or 1000 ng/ml, and cells were incubated for additional 6, 12, 24, and 48 h. Apoptosis was measured by DNA fragmentation assay as described below.

To investigate the effect of TRAIL in the presence of paclitaxel, doxorubicin, topotecan, or carboplatin, cells were plated as described above and co-incubated with TRAIL (final concentrations: 100, 300, and 1000 ng/ml) and doxorubicin (final concentrations: 0.5, 5, and 50 μM; Pharmacia and Upjohn, Kalamazoo, MI, USA), paclitaxel (final concentrations: 0.5, 5, and 50 μM; Bristol-Myers Squibb Co., Princeton, NJ, USA), topotecan (0.1, 1, and 10 μg/ml; GlaxoSmithKline, Uxbridge, Middlesex, UK) or carboplatin (10, 100, and 1000 μg/ml; Bristol-Myers Squibb, New York, NY, USA) for 6, 12, 24, and 48 h. Apoptosis was assessed by DNA fragmentation assay as described below.

DNA fragmentation assay

Using the Apo-Direct™ Kit (Phoenix Flow Systems, San Diego, CA, USA), the 3’ OH termini in DNA breaks were measured by attaching fluorescent-tagged deoxyuridine triphosphate nucleotides, FITC-dUTP, in a reaction catalyzed by terminal deoxynucleotidyl transferase (TdT), and the amount of incorporated fluorescein was detected by flow cytometry. After incubation, treated and untreated cells were harvested, washed twice in phosphate-buffered saline (PBS), and fixed in 1% (w/v) paraformaldehyde in PBS (pH 7.2) for 15 min on ice. After two more washing steps, cells were resuspended in ice-cold 70% ethanol and stored at −20°C until further use. According to the manufacturer’s instructions, cells were washed twice in wash buffer, resuspended in 50 μl staining solution (10 μl reaction buffer, 0.75 μl TdT, 8 μl FITC-dUTP, and 32 μl distilled water) and incubated for 1 h at 37°C. After one more washing step, cells were resuspended in 1 ml propium iodine PI/RNase solution and incubated in the dark for 30 min at room temperature. Subsequently, cell samples were analyzed by flow cytometry on a FACScan (Becton Dickinson, CA, USA).

RT-PCR

The expression of DR4, DR5, DcR1, DcR2, FLIP, OPG, and caspase 8 was investigated by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from cell lines by standard procedures and digested with DNase to remove DNA contamination. First strand cDNA was generated from total RNA by incubating 2 μg of total RNA with oligo(dT)_12-18 and 200 units SuperScript II RNAase H Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) at 42°C for 50 min. PCR reaction was performed with 5 μl of cDNA template in a final volume of 25 μl containing all four dNTPs (2 μl 10 mM dNTP Mix), 2.5 μl 10x PCR buffer, 0.2 μl Taq DNA polymerase (5 U/μl), and 1 μl (10 pmol) of each primer. PCR conditions were as follows: 5 min denaturation at 94°C followed by 35 cycles, 30 s/94°C; 30/60°C; 30 s/72°C. The PCR-amplified products were run on a 1% agarose gel containing ethidium bromide and were visualized under ultraviolet light. Amplification of β-actin served as a positive control.
The sequences of specific primers used in this experiment were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR4.for</td>
<td>ATGGCGCCACCACCACGCTAG</td>
<td>1407</td>
</tr>
<tr>
<td>DR4.rev</td>
<td>TCACTCCAAGGACAGGCCAGA</td>
<td>181</td>
</tr>
<tr>
<td>OPG.for</td>
<td>GTGACGAGTGTCTATACTGCA</td>
<td>486</td>
</tr>
<tr>
<td>FLIP.for</td>
<td>AATTCAAGGCTCAGTCGTTCGTCTCAC</td>
<td>226</td>
</tr>
<tr>
<td>DcR1.for</td>
<td>ACCTTAAAGTCGTGCAGTCATGCTCAT</td>
<td>205</td>
</tr>
<tr>
<td>DcR1.rev</td>
<td>TTCTTNGCTGCTTCTCTCTTC</td>
<td>463</td>
</tr>
<tr>
<td>DR5.for</td>
<td>GGGAGCCGCTCATGAGGAAGTTGG</td>
<td>181</td>
</tr>
<tr>
<td>DR5.rev</td>
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<td>181</td>
</tr>
<tr>
<td>DcR2.for</td>
<td>CTTTTCCGGCGGCGTTCATGCTCCTTC</td>
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</tr>
<tr>
<td>DcR2.rev</td>
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<td>1407</td>
</tr>
<tr>
<td>Caspase 8.for</td>
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<td>426</td>
</tr>
<tr>
<td>Caspase 8.rev</td>
<td>CTTTGACATCTGCTGCTCG</td>
<td>426</td>
</tr>
<tr>
<td>FLIP.for</td>
<td>AATTCAAGGCTCAGAAGCGA</td>
<td>226</td>
</tr>
<tr>
<td>FLIP.rev</td>
<td>GCCGACGACCTGTTCTGCAGTCTGTGTA</td>
<td>226</td>
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<tr>
<td>β-ACTIN for</td>
<td>ATGGATGATGATATCGCCGCG</td>
<td>1127</td>
</tr>
</tbody>
</table>

Western blots

Protein expression of DR4, DR5, DcR1, DcR2, FLIP, and caspase 8 was determined by Western blot analysis. Cells were lysed in 1 ml of RIPA buffer (10 mM TRIS–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na-Deoxycholate, 0.1% SDS) supplemented with Protease Inhibitor Cocktail Tablets (Roche Applied Science, Mannheim, Germany). The lysates were incubated at 4°C for 10 min and centrifuged at 15,000 rpm for 10 min. Protein concentration was determined using Protein Assay ESL (Roche Applied Science) according to the manufacturer’s instructions. Equal amounts of protein were separated on 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following primary antibodies were used: rabbit polyclonal anti-DR4 (NT) (ProSciInc, Poway, CA, USA); goat polyclonal anti-TRAIL-R2/DR5/TNFRSF10B (R&D Systems, Minneapolis, MN, USA); mouse monoclonal anti-caspase 8 (FLICE) Ab-1 (clone 8CSP01) (NeoMarkers Fremont, CA, USA); goat polyclonal anti-FLIP (C-19), goat polyclonal anti-FLIP (F-20), and goat polyclonal anti-Actin (I-19) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA). Secondary antibodies: anti-goat IgG peroxidase conjugate (Sigma-Aldrich, Vienna, Austria), anti-mouse (NA 931) and antirabbit (NA 934) IgG HRP-linked (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein determination was done by Protein Assay ESC (Roche Diagnostics, IN, USA). In case of quantitative experiments 40μg of total protein was loaded and loading control performed by β-actin staining. We used ChemiGlow™ (Alpha Innotech, San Leandro, CA, USA) to detect chemifluorescence on Western Blots.

Statistical analysis

The data were expressed as mean ± standard deviation calculated from three separate experiments, each performed in triplicate.

Data analysis for combination treatment

Synergism was determined by calculating the combination index (CI) using the median effect analysis for fixed drug dose combinations [5–8].

\[
CI_x = \frac{(D_1)_x}{(D_1)_a} + \frac{(D_2)_x}{(D_2)_a} + \alpha \left( \frac{(D_1)_a}{(D_1)_a} \right) + \left( \frac{(D_2)_a}{(D_2)_a} \right)
\]

where CI_x is the CI value for x% effect. \((D_1)_x\) and \((D_2)_x\) are the doses of agents 1 and 2 required to exert x% effect alone, whereas \((D_1)_a\) and \((D_2)_a\) are the doses of agents 1 and 2 that elicit the same x% effect in combination with the other agent, respectively. α describes the mode of interaction: \(α = 0\) for mutually exclusive (similar modes of action), \(α = 1\) for mutually nonexclusive drugs (independent modes of action). CI = 1 indicates additivity, CI < 1 synergism, and CI > 1 antagonism.

Results

TRAIL-induced apoptosis in MZ-26, ES-2, and CaOV-3 cells

Six ovarian cancer cell lines as well as two benign cell lines, human chondrocytes, and human umbilical cord endothelial cells (HUVEC) were incubated with three different concentrations of TRAIL in triplicate experiments. TRAIL sensitivity was defined as >25% apoptosis at 24 h after the initiation of incubation with TRAIL, as compared to control cells and quantitated by DNA fragmentation analysis. Three of the tested cell lines (MZ-26, ES-2, and CaOV-3) were sensitive to the cytotoxic effects of TRAIL, whereas the other three (A2780, A2780ADR, and MZ-15) showed less than 10% (9.75, 4.61, 4.86, respectively) apoptosis even upon incubation with the highest concentration of TRAIL (1000 ng/ml) (Fig. 1).

For all three apoptosis-sensitive (MZ-26, ES2, and CaOV3) cell lines, a clear dose- and time-dependent correlation between TRAIL concentration and the degree of apoptosis induction was observed with up to 80% (MZ-26), 50% (ES2), and 49% (CaOV3) apoptotic cells after 24 h of incubation with 1000 ng/ml TRAIL, respectively (Fig. 1).

The tested benign cell lines HUVEC and human chondrocytes were resistant to TRAIL after any incubation time and at any final TRAIL concentration.
**TRAIL receptor expression and resistance to TRAIL**

Expression of TRAIL-receptors DR4, DR5, DcR1, and DcR2 was analyzed by RT-PCR and Western blotting (Figs. 2 and 3). TRAIL-sensitive cell lines MZ-26, ES2, and CaOV3 showed DR4 expression, whereas DR4 was undetectable in the TRAIL resistant cell lines A2780 and A2780ADR. This observation could not be confirmed in MZ-15 cells that expressed DR4, but were resistant to TRAIL.

DR5 expression as well as expression of decoy receptors DcR1 and DcR2 was seen in all cell lines and thus failed to predict for TRAIL-resistance.

**Expression of genes regulating TRAIL-induced apoptosis**

All ovarian cancer cell lines expressed caspase 8, which was described to be crucial in the TRAIL signaling cascade (Fig. 2). Likewise, all ovarian cancer cell lines expressed mRNA of the caspase activation inhibitor FLIP at a comparable level when tested by RT-PCR (Fig. 2). On the protein level, a different picture was observed. Western Blotting revealed that protein expression of the short (c-FLIP(S)) and long (c-FLIP(L)) splice variants of FLIP varied between cell lines (Fig. 3): FLIP<sub>S</sub> was only expressed by the TRAIL-resistant cell line MZ-15. In the same cell line, a 43-kDa cleavage product of FLIP<sub>L</sub> was detected.

Transcripts of the soluble TRAIL receptor OPG were detected in MZ-15, ES-2, and CAOV-3 cells, but not in the cell lines MZ-26, A2780, and A2780ADR (Fig. 2).

**Cytotoxic agents and TRAIL act synergistically in cell lines resistant to TRAIL alone**

To determine whether TRAIL resistance could be overcome by co-incubation of ovarian cancer cell lines with...
cytotoxic agents and TRAIL, TRAIL-resistant cell lines A2780, A2780 ADR, and MZ-15 cells were treated either with paclitaxel (0.5, 5, and 50 \( \mu \text{M} \)), topotecan (0.1, 1, and 10 \( \mu \text{g/ml} \)), doxorubicin (0.5, 5, and 50 \( \mu \text{M} \)), and carboplatin (10, 100, and 1000 \( \mu \text{g/ml} \)) alone or concomitantly with 100, 300, and 1000 ng/ml TRAIL for 6, 12, 24, and 48 h. Considerable dose- and time-dependent apoptosis induction by cytotoxic agents was shown in the three TRAIL-resistant cell lines, with maximum apoptosis seen after 24 h (Fig. 4). Only A2780ADR cells were completely resistant to doxorubicin treatment as expected because they were derived from A2780 by selecting for cells resistant to this drug. In all three TRAIL-resistant ovarian cancer cell lines, co-incubation of TRAIL 300 ng/ml with the individual therapeutics was able to overcome TRAIL-resistance, leading to a strong synergic response (Fig. 4). Only A2780ADR cells were completely resistant to doxorubicin treatment as expected because they were derived from A2780 by selecting for cells resistant to this drug.

In all three TRAIL-resistant ovarian cancer cell lines, co-incubation of TRAIL 300 ng/ml with the individual therapeutics was able to overcome TRAIL-resistance, leading to a strong synergic response (Fig. 4). No further increase of apoptosis was observed by using higher doses of TRAIL or extending incubation time to more than 24 h. Interestingly, the complete resistance of A2780ADR cells to doxorubicin could not be overcome by co-incubation with TRAIL and vice versa.

Discussion

Resistance to TRAIL appears to be a multifaceted phenomenon, and little conclusive evidence about its mechanism is available. In an attempt to gain some insight into the possible mechanism of apoptosis resistance observed in three of the tested ovarian cancer cell lines, we extended our experiments to the study of expression of TRAIL receptors and selected downstream signaling elements of potential functional significance.

All cell lines sensitive to TRAIL-induced apoptosis were positive for DR4 expression, whereas we were unable to detect DR4 in the TRAIL-resistant cell lines A2780 and A2780ADR. These findings are in accordance with accumulating evidence that missing or low DR4 expression correlates with TRAIL resistance [9,10]. Moreover, reconstitution of functional DR4 restored sensitivity in a TRAIL-resistant nasopharyngeal cancer cell line with a homozygously deleted DR4 gene [11]. Yet, MZ-15 cells expressed DR4 but were resistant to TRAIL, indicating different mechanisms of resistance. DR5 expression was seen in all cell lines, and so was DcR1 and DcR2 mRNA, indicating no correlation of their presence with TRAIL sensitivity or resistance.

Binding of TRAIL to its pro-apoptotic receptors turns on a signaling cascade that leads to the formation of the DISC, containing the FAS-associated death domain protein (FADD), as well as other still unknown proteins [3].

Caspase 8 binds to the DISC and is thereby processed into the active form. It was proposed that undetectable or reduced expression of caspase 8 could explain the resistance to TRAIL-induced apoptosis [12]. We tested our cell lines for caspase 8 expression and could not find any significant difference between TRAIL-resistant and TRAIL-sensitive cell lines (Fig. 2).

FLIP functions as an anti-apoptotic molecule by blocking apoptosis induced by death receptors and exists in a long (FLIP\(_L\)) Mr 55,000 and a short (FLIP\(_S\)) Mr 28,000 isoform [4]. Overexpression of either FLIP isoform has been reported to provide protection against TRAIL-induced apoptosis in different cell types [13–16]. When probed with FLIP\(_S\) antibody, only the TRAIL-resistant cell line MZ-15 was positive. In the same cell line, the FLIP\(_L\) antibody detected a 43-kDa cleavage product of FLIP\(_L\). The presence of this 43-kDa cleavage product of FLIP\(_L\) in MZ-15 cells presumably leads to prevention of caspase 8 activation and subsequently to TRAIL resistance like it.
has been demonstrated earlier for Fas-mediated apoptosis in neuroblastoma cells [13]. To our knowledge, a correlation between this 43-kDa cleavage product of FLIPL and resistance to TRAIL induced apoptosis in a cancer cell line has never been demonstrated before. A2780ADR was the only cell line that expressed the unprocessed FLIPL isoform. Whether this phenomenon contributes to the TRAIL resistance of the A2780ADR cell line is not clear because this particular cell line also lacks the expression of TRAIL receptor DR4.

Transcripts of the soluble TRAIL receptor OPG were detected in MZ-15, ES-2, and CAOV-3 cells, but not in the cell lines MZ-26, A2780, and A2780ADR. OPG is physiologically involved in the regulation of bone density and thus expectedly did not influence the response to TRAIL [17].

Apoptosis induced by TRAIL has recently been investigated in several ovarian cancer cell lines and has resulted in contradicting observations. These discrepancies are possibly due to different TRAIL preparations used, because it is well
known that various preparations of TRAIL cause different toxicities at least in normal cells (reviewed in 2). For example, the cell line SKOV-3 that was sensitive to TRAIL in two studies, which used recombinant human gluthatione-S-transferase (GST)-TRAIL [18,19], was resistant in the next that used a recombinant TRAIL-protein without any fusion to heterologous sequences [20].

To investigate whether TRAIL resistance could be overcome by the addition of a cytotoxic agent, MZ-15, A2780, and A2780ADR were co-incubated with TRAIL and paclitaxel, topotecan, doxorubicin, or carboplatin, which are commonly used chemotherapeutic agents in the treatment of ovarian cancer. A strong synergic response was observed with either combination. The mechanism of the synergic effect between TRAIL and the chemotherapeutic agent is either the upregulation of pro-apoptotic molecules or the downregulation of anti-apoptotic molecules [21] and will hardly be associated with the change of a single molecule. This is once more demonstrated in our model, where the resistance to TRAIL is based on two different mechanisms, which could both be overcome by a variety of clinically used cytotoxic agents having different modes of action and targets.

While one priority of us and other groups is to elucidate the mechanism by which sensitivity to TRAIL is regulated, we think that one has to avoid a simplistic approach as demonstrated by the following experiment.

By adding doxorubicin to A2780 cells in a gene profiling experiment using Affymetrix GeneChip Human Genome U133A, compromised of more than 22,000 probe sets, 791 (3.55%) transcripts represented on the gene chip showed a higher expression compared to the control. For 315 transcripts, a three times higher expression was observed and 476 probes were present after doxorubicin treatment compared to their absence in the control. As from gene ontology, an estimated 11% of the probes are related to cell death regulation, we can demonstrate with this experiment that a considerable higher amount of genes is involved when TRAIL and a cytotoxic agent are co-incubated (unpublished data) than one might expect from the study of selected molecules.

To summarize, TRAIL had a synergic effect in almost all combinations with cytotoxic drugs, even though TRAIL by itself was unable to induce cell death in A2780, A2780ADR, and MZ-15 ovarian cancer cell lines. This phenomenon was independent from the underlying mechanism of TRAIL resistance as well as from the cytotoxic agent used. In the MZ-15 cell line, TRAIL resistance is probably based on the expression of the processed form of FLIP_L, p43 (FLIP) and FLIP_S, in A2780 as well as in A2780ADR cells on the absence of DR4 expression. Interestingly, in one cell line, which had developed complete resistance to a chemotherapeutic agent (A2780ADR to doxorubicin), the addition of TRAIL could not compromise this resistance.

Most recently, the expression of TRAIL in ovarian cancer tissue was linked to the prognosis of the disease implicating an important role of the TRAIL pathway in ovarian cancer [22]. While detailed clinical and mechanistic studies are definitely needed to get more insight into the contribution of the TRAIL apoptotic pathway to ovarian cancer biology and the multifaceted phenomenon of TRAIL resistance, our data and the safety of TRAIL administered in vivo to athymic mice locoregionally [23] suggest that TRAIL-based tumor therapy may be an efficient strategy in the treatment of ovarian cancer, where tumor spread is restricted mostly to the peritoneal cavity and where therapeutics can be applied in relatively high concentrations locoregionally.

Acknowledgments

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References

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