Synergistic Effect of a Mesothelin Targeted Thorium-227 Conjugate in Combination with DNA Damage Response Inhibitors in Ovarian Cancer Xenograft Models

Authors

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ABSTRACT

Targeted thorium conjugates (TTCs) represent a new class of therapeutic radiopharmaceuticals for the targeted alpha therapy. They are comprised of the alpha emitter thorium-227 complexed to a 3,2-hydroxypyridinone (3,2-HOPO) chelator conjugated to a tumor targeting monoclonal antibody. The high energy and short range of the alpha particles induce anti-tumor activity, driven by the induction of complex DNA double strand breaks. We hypothesized that blocking the DNA damage response (DDR) pathway should further sensitize cancer cells by inhibiting DNA repair, thereby increasing the response to TTCs. Methods: This study reports the evaluation of the mesothelin targeted thorium-227 conjugate (MSLN-TTC; BAY 2287411) in combination with DDR inhibitors, each of them blocking different DDR pathway enzymes. Mesothelin is a validated cancer target known to be overexpressed in mesothelioma, ovarian, lung, breast and pancreatic cancer with low expression in normal tissue. In vitro cytotoxicity experiments were performed on cancer cell lines by combining the MSLN-TTC with inhibitors of Ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), DNA-dependent protein kinase (DNA-PK), and Poly [ADP-ribose] polymerase 1/2 (PARP1/2). Further, we evaluated the anti-tumor efficacy of the MSLN-TTC in combination with DDR inhibitors in human ovarian cancer xenograft models. Results: Synergistic activity was observed in vitro for all tested inhibitors when combined with MSLN-TTC. ATRi and PARPi appeared to induce the strongest increase in potency. Further, in vivo anti-tumor efficacy of the MSLN-TTC in combination with ATRi or PARPi was investigated in the OVCAR-3 and OVCAR-8 xenograft models in nude mice, demonstrating synergistic anti-tumor activity for the ATRi combination at
doses demonstrated to be non-efficacious when administered as monotherapy.

**Conclusion:** The presented data support the mechanism-based rationale for combining the MSLN-TTC with DDR inhibitors as new treatment strategies in MSLN-positive ovarian cancer.
INTRODUCTION

Targeted alpha therapy represents a promising modality whereby the high energy (5-9 MeV) and short range (50-100 µm) of the alpha-particle can be exploited, together with a targeting moiety, to specifically irradiate a tumor while sparing normal tissue (1,2). Targeted thorium-227 conjugates (TTCs) comprise the alpha particle emitter thorium-227 complexed to a 3,2-hydroxypyridinone (3,2-HOPO) chelator covalently attached to a tumor-targeting antibody (3-6). Thorium-227 has a half-life of 18.7 days and the decay chain deposits five high-energy alpha and two beta particles (7). The dense ionization track of the alpha particle induces complex DNA damage both directly and indirectly via generation of reactive oxygen species (1,8). In this study, we explore treatment strategies utilizing a mesothelin-targeted thorium-227 conjugate (MSLN-TTC; BAY 2287411) in combination with DNA damage response (DDR) inhibitors. Mesothelin (MSLN) is a 40 kDa membrane-anchored glycoprotein that is frequently overexpressed in cancer types including mesothelioma, ovarian, lung and pancreatic cancers (9). In contrast, MSLN has limited expression in nonmalignant tissue and is therefore considered a suitable antigen for targeted therapies (10-12). We have previously demonstrated that MSLN-TTC induces specific anti-tumor activity. This agent is currently in clinical phase I indicated for mesothelioma and ovarian cancer (NCT03507452).

Efficient DNA damage repair is a contributing factor to poor prognosis and treatment outcome following radiotherapy (13,14). We therefore hypothesized that DDR pathways also contribute to the efficacy following treatment with MSLN-TTC. This study investigated in vitro and in vivo combinations of inhibitors of the key mediators of DDR,
including Ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), DNA-dependent protein kinase (DNA-PK), and Poly [ADP-ribose] polymerase 1/2 (PARP1/2) (15) with MSLN-TTC.

MATERIALS AND METHODS

Cells

OVCAR-3, OVCAR-8 from American Type Culture Collection; NCI-H226 from National Institute of Health; Capan-2, HT29 from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH. All cell lines authenticated using PCR fingerprinting. Cells maintained at 37°C, 5% CO₂. Capan-2 and NCI-H226 cultured in Roswell Park Memorial Institute (RPMI) medium. HT29-MSLN in RPMI with 1 % sodium bicarbonate and 2 % hygromycin, OVCAR-3 in RPMI with 10 μg/ml insulin (bovine) and 2 mmol/L glutamine and OVCAR-8 in DMEM/F12. Culture medium was supplemented with 10 % FBS, 1 % penicillin/streptomycin for all cell lines. HT29 cells transfection with human Mesothelin as described previously (10).
Compounds

BAY 1895344 (2-[(3R)-3-methylmorpholin-4-yl]-4-(1-methyl-1H-pyrazol-5-yl)-8-(1H-pyrazol-5-yl)-1,7-naphthyridine) (WO2016020320), PARPi (olaparib), ATMi (AZD0156) and DNA-PKi (VX-984) were synthesized at Bayer AG.

Preparation of the MSLN-TTC

The 3,2-HOPO chelator was conjugated to the MSLN and isotype control antibodies and labeled with thorium-227 as previously described (4,16). Antibody-chelator conjugate formulations were incubated with 0.5 – 2.5 MBq thorium-227 at room temperature for 60 min. Radiochemical purity (RCP), defined as the amount of thorium-227 bound to the MSLN-TTC, was determined by instant thin-layer chromatography (iTLC).

In Vitro Experiments

OVCAR-3 cells seeded in 12 (2 ml/well) or 6-well plates (4 ml/well) 24 hours before treatment with MSLN-TTC (1/10 kBq/ml) and ATRi (10 nM) or PARPi (0.5 µM). Due to the half-life of TTC (18.7 d) the cells were exposed to treatment for three days before fixation with 70 % ethanol, followed by staining and detection with Guava Easycyte 8HT. Cell cycle analysis determined by staining with PI/RNase (Cat#F10979, Thermo Fisher), double strand breaks (DSBs) with γH2A.X antibody (Alexa Fluor®-647 Conjugate, Cat# 9720, Cell Signaling Technology) and apoptotic cells with cleaved-caspase-3 antibody (Cat#9669 Alexa Fluor®-488 Conjugate, Cell Signaling Technology). The γH2A.X-positive/ cleaved-caspase-3-negative cells were collected for the determination of DSBs, in order to exclude apoptotic DNA cleavage from the
measurements. Viability was determined after five days exposure by CellTiter Glo (CTG) 2.0 (Cat#G9243, Promega). Data analysis was performed using FlowJo software (version 10) and GraphPad Prism software (version 7).

Combination experiments were conducted in 384-well plates (30 µL/well, 30 000 cells/ml for Capan-2, NCI-H226 and OVCAR-3, 100 000 cells/ml for HT29-MSLN). After 24 hours cells were treated with a titration of MSLN-TTC and DDRi’s using a D300e digital dispenser (Tecan) in the following combination ratios with MSLN-TTC (C1) and the DDRi (C2): 1xC1, 0.9xC1+0.1xC2, 0.8xC1+0.2xC2, 0.7xC1+0.3xC2, 0.6xC1+0.4xC2, 0.5xC1+0.5xC2, 0.4xC1+0.6xC2, 0.3xC1+0.7xC2, 0.2xC1+0.8xC2, 0.1xC1+0.9xC2 and 1xC2. MSLN-TTC was titrated in the range 0.001-5 kBq/ml for HT-29-MSLN and 0.01-50 kBq/ml for Capan-2, OVCAR-3/8 and NCI-H226, at a specific activity of 40 kBq ²²⁷Th/µg antibody chelator conjugate for HT29-MSLN, Capan-2 and OVCAR-3/8 and 20 kBq ²²⁷Th/µg for NCI-H226. DDR inhibitors were dissolved in dimethyl sulfoxide and titrated in the range 0.002-10 µM for ATRi (BAY 1895344), ATMi (AZD0156) or DNA-PKi (VX-984), 0.01-50 µM for PARPi (olaparib/AZD2281). After incubation (HT29-MSLN: 5 days; OVCAR-3/8, NCI-H226 and Capan-2; 7 days) viability was determined using CTG according manufacturer’s protocol. Note: optimization for each cell line was required to account for variations in doubling time, receptor density, internalization rates, and radiosensitivity. As a result specific activity, total activity and incubation time vary between the cell lines. IC⁵₀-isobolograms were generated by plotting IC⁵₀ values from MSLN-TTC/ DDRi on x- and y-axis, respectively. The combination index (CI) was determined according to the median-effect model of Chou-
Telalay (17), with CI<0.8 defined as synergistic effect, 0.8≤CI≤1.2 defined as additive effect and CI>1.2 defined as antagonistic effect.

**In Vivo Efficacy**

Experimental protocols were approved by the National Animal Research Authority and conducted according to Federation of European Laboratory Animal Science Association and Directive 2010/63/EU of European Parliament regulations. All animals received an intraperitoneal injection of murine IgG2a antibody (200 µg/animal; UPC10; Sigma) 16-24 hours prior treatment to block nonspecific spleen uptake (18). The OVCAR-3-bearing mice were supplemented with 17-beta estradiol either in drinking water (4 mg/L, average daily dose of 1 mg/kg, Sigma-Aldrich, Cat#, E8875) or subcutaneous implantation of pellets (1.7 mg/pellet, time release: 90 days, Innovative Research of America, Cat# SE-121). The tumor growth and the body weights were measured by caliper every second or third day. Animals were sacrificed by cervical dislocation upon reaching the humane endpoint (tumor volume ≥ 1.500 mm³; body weight loss ≥ 20%).

Five million OVCAR-3 cells in 0.1 ml PBS were inoculated subcutaneously into mice (female, 4-6 weeks old HsdCpb: Athymic nude Foxn1nu, Department for Comparative Medicine, Oslo, Norway, first generation of animals obtained from Harlan, Amsterdam, The Netherlands). At an average tumor area of 25-35 mm², the mice (n=10) received a single intravenous (i.v.) injection of MSLN-TTC (100, 250 or 500 kBq/kg, 0.14 mg/kg), isotype-control (250 kBq/kg, 0.14 mg/kg), non-radioactive MSLN antibody-chelator conjugate (0.14 mg/kg) or vehicle. One group was treated with MSLN-
TTC (100 kBq/kg, 0.14 mg/kg) and ATRi (40 mg/kg in 60% polyethylene glycol 400/10% ethanol/30% water, 2QD, 3 days on/4 days off, 4 weeks) or PARPi (50 mg/kg in PBS supplemented with 10% 2-HPβCD, daily, 4 weeks). At the study endpoint, tumors treated with MSLN-TTC (500 kBq/kg) and vehicle groups were stained for γH2A.X using rabbit anti-γH2A.X antibody (Milipore, MABE205) with BrightVision rabbit/HRP (Immunologic, DPVR110HRP) incubated for 30 minutes followed by 3,3′-Diaminobenzidine for 5 minutes.

Three million OVCAR-8 cells in 0.1 ml matrigel were inoculated subcutaneously into mice (female, 4-6 weeks old CB-17/Scid coincidence mice, Janvier, Paris, France). At an average tumor area of 30-40 mm², the mice (n=10) received three i.v. injections of MSLN-TTC (200 kBq/kg, 0.14 mg/kg, day 1, 22 and 43) and ATRi (40 mg/kg 2QD, 2 days on/5 days off, seven weeks). Blood samples were collected at end-point of study and analyzed with Hemavet (HV950, Drew Scientific, Inc., Dallas, USA).

To evaluate the cooperativity of combination treatment the expected additivity was calculated according to the Bliss model (19): C=A+B-A*B; wherein C is the expected T/C of the combination of drug A and drug B if they act additive, A is T/C of drug A, B is T/C of drug B. Excess >10% over the expected additive effect is assumed to indicate synergism, <10% of the expected additive effect is assumed antagonism.
Statistics

Statistical significance was evaluated using GraphPad Prism software (version 7.0) applying student t-test and one-way ANOVA followed by Tukey’s test.

RESULTS

Preparation and Characterization of MSLN-TTC

Radiolabeling was effected by incubation of thorium-227 with the antibody conjugate at ambient temperatures (3,4,6). The RCP was determined by iTLC for each experiment and was consistently ≥ 95 %. The binding affinity was not impaired by conjugation or radiolabeling (Supplemental Figs. 1 and 2).

Synergistic Effect of MSLN-TTC and DDR Inhibitors In Vitro

In vitro cytotoxicity experiments were performed on cell lines of different tissue origin and MSLN expression (see table 1). The effect of the combination was evaluated by isobolograms as exemplified in Fig. 1 for the OVCAR-3 cell line and Supplemental Figs. 3-6. Data analysis according to the median-effect model of Chou-Telalay gave combination indexes indicating synergistic, additive and antagonistic effects (17). All DDR inhibitors demonstrated synergy in combination with MSLN-TTC in the OVCAR-3 cell line (table 1). Furthermore, the ATRi induced strongest synergy in combination with MSLN-TTC in all tested cell lines. In contrast, the synergistic effect was not as well pronounced for ATMi or DNA-PKi, which induced both additive and antagonistic effects.
ATRi and PARPi Potentiate MSLN-TTC Activity by Suppressing DNA Damage Repair

Based on the robust synergy observed in vitro we investigated the mechanism of combination treatment in the OVCAR-3 model to determine whether the enhanced effect correlated with inhibition of DNA damage repair.

DSBs and cell cycle arrest in G2 or M phase was observed evidenced by increased γH2A.X and accumulation of cells with 4N DNA content respectively in cells treated with a single sub-lethal dose of MSLN-TTC. When combined with a non-efficacious dose of ATRi (10 nM), the percentage of cells entering the G2/M cell cycle phase was reduced. In contrast the number of γH2A.X positive cells increased (Figs. 2A and 2B; Supplemental Table 1) correlating with a higher level of apoptosis and decrease in cell viability, demonstrating the potency of the combination (Supplemental Figs. 7A and 7B; Supplemental Table 1). When MSLN-TTC was combined with a non-efficacious dose of PARPi (0.5 µM) we observed an increase in cell cycle arrest and γH2A.X (Figs. 2C and 2D; Supplemental Table 2). This translated to a modest increase in potency of MSLN-TTC as determined by viability assay and increase in apoptosis (Supplemental Figs. 7C and 7D; Supplemental Table 2), although to a lesser extent than in the combination with ATRi.

Tumor Growth Inhibition of MSLN-TTC in the OVCAR-3 Ovarian Cancer Xenograft Model

The in vivo efficacy was determined by measuring changes in tumor area after administration of a single dose of MSLN-TTC at 100, 250 and 500 kBq/kg. An isotype
control (250 kBq/kg) was included for comparison. Statistically significant tumor growth inhibition compared to vehicle was achieved for the MSLN-TTC at 250 and 500 kBq/kg (p< 0.0001) the highest dose resulting in a more pronounced reduction in tumor size (Fig. 3A). No statistical significance was observed for the dose of 100 kBq/kg of MSLN-TTC or for the isotype control versus the vehicle control group. IHC analysis of $\gamma$H2A.X on tumor tissue demonstrated induction of a higher level of DSBs compared to vehicle control after treatment with MSLN-TTC (500 kBq/kg) (Fig. 3B).

**Enhanced Potency of MSLN-TTC in Combination with ATRi or PARPi in OVCAR-3 and OVCAR-8**

Based on the observed synergistic effect of MSLN-TTC in combination with ATRi and PARPi *in vitro*, we further evaluated the *in vivo* efficacy of both combinations in an OVCAR-3 xenograft model. Since a single dose of 250 kBq/kg was highly efficacious as a monotherapy, we selected the lower dose of 100 kBq/kg for the combination study. ATRi was dosed one week after MSLN-TTC at 40 mg/kg 2QD, three days on/four days off, for four weeks and PARPi at 50 mg/kg daily for four weeks based on internal and published data respectively (20). Results from biodistribution studies show that TTCs typically accumulate in tumors over four-seven days and have extensive retention (4,5). Given the 18.7 days half-life of thorium-227, the absorbed dose to tumor is delivered over several weeks. Thus, DDR inhibitors are administered over a four week period as more DNA damage is induced over time by the TTC.

The ATRi combination enhanced the potency of the MSLN-TTC with significant tumor growth inhibition compared to the vehicle control (P< 0.0001) and monotherapy
groups (p<0.01) (Fig. 4A). Furthermore, the combination effect was determined to be synergistic based on the Bliss model (19). Although the PARPi combination was efficacious compared to the vehicle group, significance was not achieved when compared to the respective MSLN-TTC monotherapy, the combination effect was determined to be additive (Fig. 4B). Both dosing schedules were well tolerated as evidenced by the stable body weights in all groups tested (Supplemental Fig. 8).

We further explored the combination of MSLN-TTC and ATRi in the OVCAR-8 ovarian cancer xenograft model, which had previously demonstrated in vitro synergy from the ATRi combination (Supplemental Fig. 6). Due to lower receptor-level and a more rapid growth rate the dosing schedule was changed to 3 x 200 kBq/kg MSLN-TTC and ATRi (40 mg/kg, 2QD, 2 days on/ 5 days off). There was a significant effect of monotherapy with MSLN-TTC or ATRi compared to vehicle treated control in the OVCAR-8 model (P<0.0001). The combination induced a significantly enhanced anti-tumor effect as compared to the monotherapy (p< 0.001) (Fig. 5A) which was synergistic. No significant change in body weight was observed in any of the treatment groups (Supplemental Fig. 8). However, a significant reduction in white blood cells (WBCs) (P<0.001) and platelets (P<0.001) was observed for MSLN-TTC monotherapy and the ATRi combination (Fig. 5B) in comparison to vehicle control. The reduction in WBCs and platelets was comparable for MSLN-TTC and the combination, indicating no combined toxicity effect at the timepoint evaluated.
Several clinical and preclinical studies support the synergistic effects of combining targeted drugs with radiation therapy for the treatment of solid tumors (21). Drug combinations have the potential to sensitize tumor cells to ionizing radiation, enhancing the therapeutic effect while minimizing side effects and damage to normal tissue. We report herein the pre-clinical evaluation of a combination of inhibitors of DNA damage repair with systemic targeted alpha therapy. As the mode of action of targeted alpha therapy is based on the induction of complex DNA damage, we postulated that combinations with DDR inhibitors may induce synergistic effect. Alpha particles are considered to be highly cytotoxic due to extensive induction of DSBs and the development of radio-resistance has not been reported for alpha particle therapy (22,23). In contrast, low linear energy transfer (LET) particles or rays, induce sublethal damage such as single strand breaks (SSBs), which cells have a higher capacity to repair by e.g. excision repair mechanisms (24). In the present study, a mesothelin specific antibody-chelator conjugate was prepared using the fully human anti-mesothelin antibody anetumab. The antibody binds specifically to MSLN with high affinity (K_D of 14 nM) and induces efficient internalization on MSLN expressing cell lines. We initially screened for synergistic effects of MSLN-TTC and DDR inhibitors in viability assays. The MSLN-TTC was shown to synergize with all DDR inhibitors tested (ATMi, ATRi, DNA-PKi and PARPi) while a more pronounced effect was observed for ATRi and PARPi. Mechanistic studies evaluating the ATRi combination on OVCAR-3 cells revealed a significantly higher proportion of cells continuing through the cell cycle into G1/S phase than for the MSLN-TTC monotherapy (p < 0.0001). The reduced level of
cell cycle arrest for the ATRi combination can be explained by the blockage of the ATR kinase at the G2 cell cycle checkpoint (25). It also appeared that this combination resulted in a higher level of DSBs as evidenced by γ-H2A.X staining. Furthermore, the potency of the combination was reflected in an increase in apoptosis and reduction in cell viability. In contrast, the PARPi combination appeared to increase cell cycle arrest and γ-H2A.X staining was less pronounced, as was induction of apoptosis markers and cell viability. The cell cycle data therefore appear to reflect key mechanistic differences between ATR, involved in cell cycle arrest and DNA DSB repair and PARP, involved in single strand repair.

Ovarian cancer is an indication with unmet medical need and is a preferred indication for the MSLN-TTC clinical study. Based on the strong in vitro synergy on the ovarian cancer cell line OVCAR-3, we chose this model for in vivo evaluation of the combinations of MSLN-TTC with the PARPi and the ATRi. First, a dose response study was performed in OVCAR-3 tumor bearing mice treated with a single dose of 100, 250 or 500 kBq/kg of MSLN-TTC. Significant inhibition of tumor growth was measured at 250 and 500 kBq/ kg, while 100 kBq/kg gave no measurable effect on growth inhibition compared to vehicle control. Non-significant efficacy was observed for the isotype control at 250 kBq/kg, which is likely to arise from non-target mediated enhanced permeability and retention effect (26). IHC analysis of γH2A.X indicated higher levels of DSBs in the 500 kBq/ kg group compared to vehicle controls further supporting the validity of testing the DDR inhibitor combination in this model at lower doses.

When MSLN-TTC was combined with the PARPi at a single dose of 100 kBq/kg, statistical significance was achieved when compared to the vehicle control (P<0.001).
However, the PARPi combination was not significantly different to the 100 kBq/kg monotherapy treatment. This lack of in vivo synergy may serve to highlight that inhibition of SSB repair, mediated by PARP, has a less pronounced effect than targeting DSB repair and cell cycle checkpoint, mediated by ATR, in the response to alpha-radiation induced DNA damage. In contrast, MSLN-TTC combined with ATRi (40 mg/kg) at a single dose of 100 kBq/kg, resulted in a more pronounced tumor growth inhibition in comparison to the combination with PARPi. The combination induced significant (p<0.001) tumor growth inhibition, the level of this effect being equivalent to the single dose of 250 kBq/kg of MSLN-TTC, and was determined to be strongly synergistic by the Bliss additivity model. This is in alignment with our observations from the in vitro studies and further supports the hypothesis that blockage of the DNA double strand break repair machinery results in stronger therapeutic efficacy. Further studies need to be performed in order to investigate the effect of alternative dosing schedules.

A second human ovarian cancer xenograft model using the OVCAR-8 cell line was selected for further evaluation of the ATRi combination. This model had a lower level of receptors and a more rapid growth rate. The dosing regimen was therefore changed to 3 x 200 kBq/kg administered as monotherapy or in combination with ATRi (40 mg/kg 2QD, 2 days on/ 5 days off). Each single agent (MSLN-TTC or ATRi) induced a significant tumor growth inhibition (p<0.0001) compared to vehicle. However, the combination resulted in a significant increase of tumor growth inhibition compared to both monotherapies (p<0.001), demonstrating the synergistic activity. All doses were well tolerated as evidenced by no critical body weight loss. Hematological analysis
showed comparable values of MSLN-TTC monotreatment and the combination with ATRi, indicating that there was no increased toxicity from the combination treatment.

In summary, these findings demonstrate that the combination effect appears to be DDR pathway dependent. The ATRi has been reported to induce synthetic lethality in tumors with defects in G1 cell cycle checkpoint, including mutations in ATM and TP53 (25). Interestingly, the monotherapy with ATRi was more efficacious in the OVCAR-8 model (ATM\textsuperscript{mut}/ TP53\textsuperscript{mut}), inducing a significant tumor growth inhibition in comparison to the OVCAR-3 model (TP53\textsuperscript{mut}) (27). The mutations may have contributed to the enhanced efficacy of the ATRi treatment in the OVCAR-8 model as ATM defects has been describe to make cells more dependent on ATR. However, as these models are not sufficiently comparable a broader assessment of the therapeutic relevance of targeting specific mutations in relation to synergy in pre-clinical models will be the subject of future work. It also remains to be explored if the synergy of MSLN-TTC/PARPi combinations can be enhanced in tumors characterized with BRCA1/2 defects, as the PARP/BRCA combination is well described to induce synthetic lethality (28).

CONCLUSION

The presented study supports the rationale for combining the MSLN-TTCs with DDR inhibitors based on their individual mode of action as a new strategy for treating ovarian cancer patients characterized by overexpression of mesothelin.
ACKNOWLEDGEMENTS

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KEY POINTS

**Question:** As the mechanism of action of MSLN-TTC is primarily linked to induction of DNA damage we hypothesized that combinations with DNA damage response (DDR) inhibitors would induce synergistic effect and improved treatment outcome at lower doses.

**Pertinent finding:** *In vitro* evaluation demonstrated synergistic effect of MSLN-TTC in combination with inhibitors of ATR, ATM, DNA-PK and PARP. In addition, *in vivo* studies demonstrated significantly enhanced anti-tumor efficacy and synergy of MSLN-TTC and ATRi combination in ovarian cancer xenografts at dose levels shown to be non-eficacious when administered as monotherapies.

**Implication for patient care:** The presented study supports the rationale for combining the MSLN-TTCs with DDR inhibitors based on their individual mode of action as a new strategy for treating ovarian cancer patients characterized by overexpression of mesothelin.
REFERENCES


Figure 1. Synergistic effect of MSLN-TTC and DDRis on ovarian cancer cell line OVCAR-3. IC<sub>50</sub>-isobolograms with MSLN-TTC and A) ATMi AZD0156, B) ATRi BAY 1895344, C) DNA-PKi VX-984 and D) PARPi olaparib on OVCAR-3 cells. Combination index (CI) (mean, n=3) was determined according to the median-effect model of Chou-Telalay, CI<0.8 defined as synergistic effect, 0.8<CI>1.2 defined as additive effect and CI>1.2 defined as antagonistic effect.
Figure 2. *In vitro* mechanistic experiments from MSLN-TTC +/- ATRi BAY 1895344 or PARPi olaparib on OVCAR-3. A) Cell cycle analysis and B) $\gamma$-H2AX determined after treatment with MSLN-TTC (10 kBq/ml) and ATRi (10 nM) for three days. C) Cell cycle analysis and D) $\gamma$-H2AX determined after treatment with MSLN-TTC (1 kBq/ml) and PARPi (0.5 µM) for three days. Error bars represent SD of mean, n=3.
Figure 3. *In vivo* characterization of monotherapy with MSLN-TTC in OVCAR-3 xenograft model. A) Tumor size determined after a single dose (100, 250 or 500 kBq/kg, 0.14 mg/kg, i.v.) of MSLN-TTC, isotype control (250 kBq/kg, 0.14 mg/kg, i.v.) or vehicle control. Statistical analysis was performed using one-way ANOVA followed by Tukey’s test. ****, P < 0.0001. B) IHC of $\gamma$H2A.X on tumors after a single dose (500 kBq/kg, 0.14 mg/kg, i.v.) of MSLN-TTC or vehicle control.
Figure 4. *In vivo* efficacy of MSLN-TTC in combination with ATRi or PARPi in the OVCAR-3 xenograft model. A) Tumor size determined after a single dose of MSLN-TTC (100 kBq/kg, 0.14 mg/kg, i.v.) and ATRi (40 mg/kg 2QD, 3 days on/ 4 days off, 4 weeks) and B) Tumor size determined after a single dose of MSLN-TTC (100 kBq/kg, 0.14 mg/kg, i.v.) and PARPi (50 mg/kg QD, 4 weeks). Statistical analysis was performed using one-way ANOVA followed by Tukey’s test. **, P < 0.01; *** , P < 0.001; ****, P < 0.0001. T/C: treatment-to-control ratio
Figure 5. *In vivo* efficacy of MSLN-TTC in combination with ATRi in the OVCAR-8 xenograft model. A) Tumor size and B) WBCs and platelets determined at the endpoint of study after three (i.v.) injections of MSLN-TTC (200 kBq/kg, 0.14 mg/kg) and ATRi (40 mg/kg 2QD, 2 days/5 days off). Statistical analysis was performed using one-way ANOVA followed by Tukey’s test. ***, P < 0.001, ****, P < 0.0001, T/C: treatment-to-control ratio.
Table 1. *In vitro* characterization of MSLN-TTC and DDRi’s

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<th>Cell line, cancer type</th>
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</table>

IC₅₀, half maximal inhibitory concentration; CI, combination index, ND: not determined; CI-data presented as mean ±SD, n=3, * in combination with MSLN-TTC. (add) = additive, (ant) = antagonistic, (s) = synergistic.
SUPPORTING DATA

Figure 1: FACS Analysis Comparing Binding Potency of MSLN-antibody Conjugate

Cells were seeded in 96 well plates (100 000 cells, 100 µl) and incubated with a titration of 0.0006-100 µg/ml of anti-MSLN antibody, MSLN-antibody conjugate and isotype control for one hour at 4°C, followed by incubation with 100 µl anti-human IgG-PE (Cat# 409304, biolegend) for one hour at 4°C. The mean fluorescence intensity (MFI) was calculated using GraphPad Prims software version 7.0 and was plotted against the protein concentration. The mAbs/cell was determined making a standard curve using beads from Quantibrite (BD biosciences).

Figure 1. FACS analysis on different cell lines, comparing binding potency of naked MSLN antibody with MSLN-antibody conjugate. An isotype control conjugate was included to demonstrate specificity. Data were fitted using Graph Pad Prism software, EC50 values are presented in the table. Binding to A) HT29-MSLN cells; B) Ovcar-3 cells; C) NCI-H226 cells
For ELISA, recombinant human MSLN was coated to 96-well plates (1 µg/mL; NUNC/Maxisorp). Wells were blocked with 3 % BSA in PBS. Cold MSLN-antibody conjugate, an isotype control antibody and the radiolabeled MSLN-TTC (7 MBq/10mg, stored for 72 hours) were titrated (1:3; 100 µg/mL) on the MSLN coated ELISA plate. Unbound samples were washed off and bound samples were visualized using horseradish peroxidase labeled goat anti-human lambda antibody (Southern Biotech) followed by visualization with the peroxidase substrate ABTS (Life Technologies). The absorbance was measured at 405 nm in a plate reader (Perkin Elmer). EC₅₀ values were calculated using GraphPad Prism Software.

**Figure 2. ELISA on recombinant human MSLN.** Binding affinity of the radiolabeled MSLN-TTC (7 MBq/ 10 mg) is compared against the antibody-chelator conjugate and isotype control after 72 hours incubation, demonstrating no change in binding affinity.
Figure 3. Isobologram generated from CAPAN-2 cell line treated with MSLN-TTC in combination with DDR inhibitors. Cell viability was determined by use of CellTiterGlo. The IC₅₀-isobolograms were generated by plotting the actual IC₅₀ values of MSLN-TTC and DDRi along the x- and y-axis, respectively.
Figure 4: Isobologram generated from HT29-MSLN Cell Line Treated with MSLN-TTC in Combination with DDR Inhibitors.

Cell viability was determined by use of CellTiterGlo. The IC_{50}-isobolograms were generated by plotting the actual IC_{50} values of MSLN-TTC and DDRi along the x- and y-axis, respectively.
Figure 5. Isobologram generated from NCI-H226 cell line treated with MSLN-TTC in combination with DDR inhibitors. Cell viability was determined by use of CellTiterGlo. The IC\textsubscript{50} isobolograms were generated by plotting the actual IC\textsubscript{50} values of MSLN-TTC and DDRi along the x- and y-axis, respectively.
Figure 6: Isobologram generated from OVCAR-8 Cell Line Treated with MSLN-TTC in Combination with ATR Inhibitor.

Figure 6. Isobologram OVCAR-8 treated with MSLN-TTC in combination with ATRi. Cell viability was determined by use of CellTiterGlo. The IC₅₀-isobolograms were generated by plotting the actual IC₅₀ values of MSLN-TTC and DDRi along the x- and y-axis, respectively.
Figure 7. *In Vitro* Experiments from MSLN-TTC +/- ATRi BAY 1895344 or PARPi olaparib on OVCAR-3.

A-B) Apoptosis and viability determination after combination treatment with MSLN-TTC (10 kBq/ml) and BAY 1895344 (10 nM). C-D) Apoptosis and viability determination after combination treatment with MSLN-TTC (1 kBq/ml) and olaparib (0.5 µM).
Supplemental table 1 and 2: Mean Values ± SD of Mechanistic Markers

Table 1: Mean Values ± SD of Mechanistic Markers MSLN-TTC + ATRi

<table>
<thead>
<tr>
<th>Marker</th>
<th>CTR</th>
<th>MSLN-TTC</th>
<th>ATRi</th>
<th>MSLN-TTC + ATRi</th>
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</thead>
<tbody>
<tr>
<td>DSB (γH2A.X)</td>
<td>5.3 ± 1.0</td>
<td>26.3 ± 2.1</td>
<td>6.3 ± 0.5</td>
<td>43.3 ± 1.8</td>
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<tr>
<td>Apoptosis (Cleaved Caspase-3)</td>
<td>0.4 ± 0.3</td>
<td>12.45 ± 3.0</td>
<td>0.3 ± 0.3</td>
<td>23.0 ± 4.5</td>
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<tr>
<td>Viability (ATP)</td>
<td>100 ± 0.5</td>
<td>81.1 ± 5.5</td>
<td>94.5 ± 2.4</td>
<td>25.3 ± 2.4</td>
</tr>
</tbody>
</table>

Table 2: Mean Values ± SD of Mechanistic Markers MSLN-TTC + olaparib

<table>
<thead>
<tr>
<th>Marker</th>
<th>CTR</th>
<th>MSLN-TTC</th>
<th>olaparib</th>
<th>MSLN-TTC + olaparib</th>
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</thead>
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<tr>
<td>DSB (γH2A.X)</td>
<td>4.6 ± 1.2</td>
<td>29.6 ± 0.7</td>
<td>10.8 ± 0.4</td>
<td>35.8 ± 3.5</td>
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<tr>
<td>Apoptosis (Cleaved Caspase-3)</td>
<td>3.5 ± 0.8</td>
<td>8.7 ± 1.0</td>
<td>4.9 ± 0.4</td>
<td>13.1 ± 2.1</td>
</tr>
<tr>
<td>Viability (ATP)</td>
<td>100 ± 1.5</td>
<td>76.9 ± 0.2</td>
<td>99.8 ± 0.8</td>
<td>57.8 ± 1.3</td>
</tr>
</tbody>
</table>
Figure 8: Body Weights Determined after Treatment with MSLN-TTC in Combination with ATRi BAY 1895344 or PARPi olaparib.

Figure 8. Body weights determined after treatment with MSLN-TTC in combination with ATRi BAY 1895344 or PARPi olaparib. A) Body weight of OVCAR-3 xenograft bearing mice determined after a single dose administration of MSLN-TTC (100 kBq/kg, 0.14 mg/kg, i.v.) and ATRi (40 mg/kg 2QD, 3 days on/ 4 days off, 4 weeks), B) Body weight of OVCAR-3 xenograft bearing mice determined after a single dose administration of MSLN-TTC (100 kBq/kg, 0.14 mg/kg, i.v.) and olaparib (50 mg/kg QD for 4 weeks), C) Body weight of OVCAR-8 xenograft bearing mice determined after three intravenous (i.v.) injections of MSLN-TTC (200 kBq/kg, 0.14 mg/kg, day 1, 22 and 43) and BAY 1895344 (40 mg/kg 2QD, 2 days/5 days off, 7 weeks).