EP-100 SYNERGIZES WITH PACLITAXEL IN OVARIAN, BREAST AND PROSTATE CANCER CELL LINES

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Abstract 3715

EP-100 is a targeted anti-cancer drug comprised of Luteinizing Hormone Releasing Hormone (LHRH) fused to a lytic peptide It is currently being investigated in human clinical trials. EP-100 seeks and destroys LHRH receptor positive cancer cells via a novel mechanism of action involving direct membrane disruption. Studies were conducted to determine effects of Paclitaxel and EP-100 *in vitro* using multi-drug resistant LHRH receptor positive ovarian (OVCAR-3), breast ((ER-, Her2-, PR-) MDA-MB-231, uterine sarcoma (MES-SA-Dx5) and prostate (PC-3) and LHRH receptor negative ovarian (SKOV-3) cancer cells.

Cells were cultured in the presence of EP-100 (0.005 nM-50µM, N=8) or Paclitaxel (0.0025-500 nM, N=8) alone or in combination and incubated for 48 to 72 hours in non-constant and constant ratio formats (N=6). Viability was measured by luminometric assays. Data were analyzed as IC_{50} values using the GraphPad Prism and GraphPad Software for the Hill Equation. The synergistic effects of EP-100 and Paclitaxel were measured as Combination Index (CI) using the CompuSyn Software analysis program.

EP-100 alone killed LHRH receptor positive OVCAR-3 and MDA-MB-231cells at low micromolar concentrations after 72 h incubation (IC_{50} values 2.189 ± 0.027 µM and 2.092 ± 0.114 µM, respectively). Both cell lines showed resistance to Paclitaxel (IC_{50} values 13.6 ± 0.7 nM for OVCAR-3 and 86.2 ± 5.9 nM for MDA-MB-231). The IC_{50} values EP-100 for MES-SA-Dx5 and PC-3 were 1.419 ± 0.133 µM and 1.9 ± 0.071 µM, respectively, after 48h incubation. As expected, MES-SA-Dx5 cells were resistant to Paclitaxel (IC_{50} 94.1 ± 0.3 nM) and PC-3 cells were very sensitive with IC_{50} 5.2 ± 1.1 nM. The IC_{50} values for EP-100 and Paclitaxel in SKOV-3 cells were 10.3 ±0.36 µM and48.52 ± 4.5 nM, respectively.

The greatest synergy was observed in multi-drug resistant MES-SA-Dx5 endometrial cells. The combination of EP-100 at 50 nM and Paclitaxel at 500 nM resulted in 3620 and 6720-fold increased sensitivity when compared to Paclitaxel alone. The median CI values were 0.18, 0.05, 0.25, and 0.6 for for MES-SA-Dx5, OVCAR-3, MDA-MB-231 and PC-3, respectively indicating synergistic responses in the cell lines. No synergy was observed in the LHRH receptor negative cell line (CI=8.4).

These results indicate that a combination of EP-100 and Paclitaxel results synergistic responses in LHRH-receptor positive and multi-drug resistant cancer cells.

Background

Traditional non-targeted treatments are often associated with serious side effects, are systemically active and do not discriminate between cancer and normal cells in vital organs. Esperance Pharmaceuticals is developing a targeted Cationic Lytic Peptide (CLYP™) technology for killing cancer cells. It involves small targeted lytic peptides that seek and destroy cancer cells without harming normal cells. The peptides are linear, alpha helical, cationic and they directly interact with negatively charged membranes resulting in their disruption and cell death.

Major advantages of membrane disrupting peptides over traditional non-targeted treatments include the following: 1. Preferentially destroy negatively charged cells, such as cancer cells, (cancer cells are up to 50 times more sensitive to lytic peptides compared to normal cells [1], 2.Fast acting - causing necrosis within minutes through direct membrane interaction [2], 3. Activity is independent of intracellular uptake, 4. Activity is independent of multi-drug resistance, 5.Short half life *in vivo*, destroying cancer cells independent of proliferation, 6. No interaction with physiological pathways required [3].

As a result of this technology, Esperance Pharmaceuticals is developing a new generation of lytic peptides that bind to molecules that are over-expressed on cancer cells. Esperance's lead candidate, EP-100, tested in Phase 1 clinical trial, is now entering Phase 2 clinical trial for targeting LHRH receptors.

LHRH receptors are over-expressed in a wide range of cancers including breast, prostate, ovarian, endometrial, pancreatic, colon, renal, testicular, liver, adrenal cancers and hematological malignancies. EP-100 consists of LHRH joined to a novel 18 amino acid lytic peptide payload designated CLIP-71 to form 28 amino acid EP-100 (LHRH has 10 amino acids). Due to its small size EP-100 is manufactured by standard solid phase peptide chemistry to produce highly homogeneous and pure product that is highly water soluble. EP-100 is not immunogenic and was well tolerated in humans. Previously, lytic peptides showed potentiation of cell killing in combination with of doxorubicin [4].

P-gp, also known as MDR1and ABCB1, is a 170 kDa integral plasma membrane protein that functions as an ATP-dependent drug efflux pump. Compounds that interact with P-gp can be identified as stimulators (substrates for transport) or inhibitors of its ATPase activity.

The effect of EP-100 on P-gp dependent multi-drug resistance was tested in Paclitaxel (Pgp substrate) resistant cell lines *in vitro*. Cell lines expressing LHRH receptors (MES-SA-Dx5, OVCAR-3, PC-3, MDA-MB-231) and the LHRH receptor negative cell line SKOV-3 were compared for single agents EP-100 and Paclitaxel and in combination.

These results served as rationale for combining EP-100 with Paclitaxel in an ongoing Phase 2 clinical trial (NCT01485848).

Objectives

1. Determination of EP-100 and Paclitaxel *in vitro* responses in multi-drug resistant cancer cell lines
2. Sequential and/or continuous exposure of combination of EP-100 and Paclitaxel
3. Determination of mechanism of action for the synergistic effects, if any, of the combined drugs

Materials & Methods

- **Cell lines:** Human ovarian cancer cell lines (OVCAR-3 and SKOV-3), uterine sarcoma multi-drug resistant (MES-SA-Dx5), triple negative breast cancer (MDA-MB-231) and prostate cancer (PC-3).
- ***In vitro* cytotoxicity** studies were conducted in 96 well plate format (2,000 cells/well) with single agents (EP-100 or Paclitaxel) and in combination with EP-100. Incubations were conducted for 48 and 72 h. Cell viability was determined using luminometric assays. Saline/Vehicle and 0.1 % Triton served as controls for 100 % viability and complete cell death.
- **Data were analyzed** as IC_{50} values using the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com for the Hill Equation and GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com for statistical analyses.
- The combination effects of EP-100 and Paclitaxel were determined as Combination Index (CI) using the CompuSyn Software analysis program(Chou and Martin, CompuSyn software for drug combinations for general dose-effect analysis, ComboSyn, Inc Paramus NJ, 2007
- Combination Indices (CI of < 0.9 represent synergism, of 0.9-1.0 additive effects and > 1.0 antagonism

Results – *in vitro* Studies

EP-100 sensitizes Paclitaxel resistant cancer cells expressing LHRH receptors – highly synergistic, potentiation of cell killing

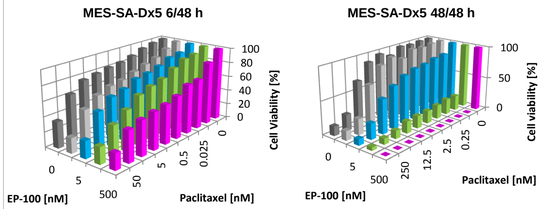

Figure 1: Short term (6h) and continuous exposure of MES-SA-Dx5 uterine sarcoma cells *in vitro* with EP-100 and Paclitaxel. Pacltiaxel in combination with EP-100 was given at doses between 0.0025-250 nM in the presence of 0.5, 5, 50 and 500 nM EP-100. IC_{50} values for single agents were 94.1 0.3 and 1419 133 nM for Paclitaxel and EP-100, respectively. Short term exposure with both drugs was less effective than long term exposure.

The potency of the combination of EP-100 and Paclitaxel is dependent on the order of administration and is highest for long term exposure

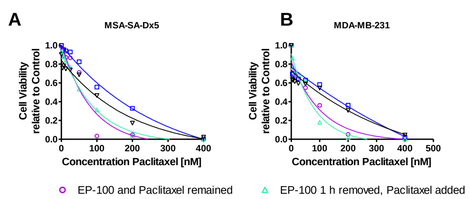

Figure 2: Continuous or sequential exposure of MES-SA-Dx5 (A) and MDA-MB-231 cell lines *in vitro* with EP-100 and Paclitaxel for 48 hours showed the maximal effect, compared to 1 h incubations with either EP-100 and Paclitaxel alone followed by 48 h incubation with the second agent. The smallest effect was seen when the supernatant was replaced by culture media after 1 hour and the cell viability determined after 48 h. Constant ratio was given 20:1.

Results ctd.

The effect of combination with EP-100 and Paclitaxel is dependent on LHRH receptors

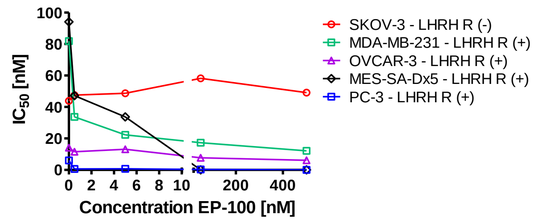

Figure 3: EP-100 sensitizes Paclitaxel resistant cancer cells expressing LHRH receptors resulting in a highly synergistic, potentiation of cell killing. Cell lines were incubated with increasing concentrations of Paclitaxel (0.0025-250 nM) in the absence or presence of EP-100 at concentrations of 0.5, 5, 50 and 500 nM. Cell viability was determined after 72 hours using a luminometric viability assay. Saline and 0.1% Triton served as controls.

Table 1: Combination Studies of EP-100 and Paclitaxel in multi-drug resistant cancer cell lines

Cell Line	Paclitaxel alone [nM]	EP-100 alone [nM]	EP-100 added [nM]	Paclitaxel alone and in combination with EP-100 [nM]	Combination Index	Fold increase in potency
SKOV-3 (72 h) (LHRH-receptor negative)	48.52 4.5	10301 361	0	48.52 4.5		
			5	45.8 1.7	NA	
			50	53.1 4.2	NA	
			500	53.8 4.3	NA	
OVCAR-3 (72 h)	13.6 0.7	2189 27.5	0	13.6 0.7		
			5	8.03 2.7	0.08	1.6
			50	5.8 1.04	0.05	2.3
			500	4.9 0.6	0.005	2.7
MDA-MB-231 (72h)	86.2 5.9	2092 114	0	86.2 5.9		
			5	22.3 0.5	0.43	4
			50	17.9 0.6	0.25	4.8
			500	15.9 0.8	0.22	5.4
MES-SA-Dx5 (48h)	94.1 0.3	1419 133		94.1 0.3		
			5	33.7 2.2	0.7	2.8
			50	0.026 0.001	0.18	3620
			500	0.014 0.02	0.001	6720
PC-3 p 32 (48 h)	5.2 1.1	1999 71	0	5.2 1.1		
			5	1.6 0.15	0.6	3.25
			50	1.3 0.2	0.7	4
			500	0.01 0.0005	0.06	520

Table 1: Continuous exposure of SKOV-3, OVCAR-3, MES-SA-Dx5, MDA-MB-231 and PC-3 cell lines *in vitro* with EP-100 and Paclitaxel for 48-72 hours with single agents EP-100 or paclitaxel and in combination at non-constant ratios. Cells were cultured in the presence of EP-100 (0.5 nM-50µM, N=8) or paclitaxel (0.0025-500 nM, N=8) alone or in combination and incubated for 48 or 72 hours. Combination indices were determined from experiments in constant ratio formats (1:11 MES-SA-Dx5, 1:333 PC-3 and 1:175 OVCAR-3) (N=6). The combination Index (CI) was determined using the CompuSyn Software analysis program. The combinations of EP-100 and Paclitaxel were highly synergistic in all cell lines that were positive for LHRH receptors – LHRH-R negative cells (SKOV-3) lacked potentiation in combination.

Results ctd.

Pgp ATP'ase Activity is caused by lytic portion of EP-100

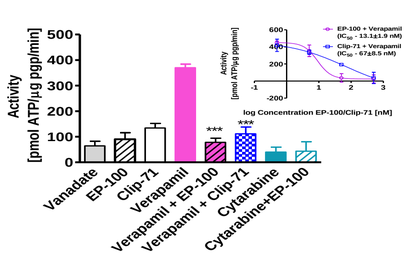

Figure 4:
Pgp ATP'ase activity on human recombinant Pgp protein.
The activity of P-gp dependent ATP hydrolysis was determined through measurement of remaining ATP in a luminometric assay (N=4). EP-100 and CLIP-71 (unconjugated lytic peptide) were added at concentrations of 0.5, 5, 50 and 500 nM. Cytarabine (500 nM) served as control for a non-Pgp substrate. Verapamil served as positive control (0.2 mM), Vanadate represented baseline activity (non-Pgp dependent).
Vanadate had a baseline activity of 64.5 ± 18.1 pmol ATP/µg P-gp /min and was similar to Cytarabine (40.25 ±18.3 pmol ATP/µg P-gp /min), indicating a lack of activation of P-gp ATPase. P-gp ATPase activity was for Verapamil (0.2 mM) 370.1±13.8 pmol ATP/µg P-gp/min. EP-100 alone and CLIP-71 alone had ATPase activities comparable to baseline values, indicating neither of the lytic peptide conjugate or lytic peptide activated P-gp ATPase.
The combination of Verapamil (0.2 mM) with EP-100 or CLIP-71 reduced the P-gp ATPase activity by a factor of 4-5 from 370.1±13.8 to 78.25±16 pmol ATP/µg P-gp/min for EP-100 in combination with Verapamil, and from 370.1±13.8 to 111.5 ± 26 pmol ATP/µg P-gp/min for CLIP-71. Cytarabine alone and in combination with EP-100 was without effect on P-gp ATPase activity. Determination of the inhibitory concentration of EP-100 and CLIP-71 after ATPase activation through Verapamil expressed as IC_{50} were 9.1±0.9 nM for EP-100 and 67±8.5 nM for CLIP-71.

Summary

- EP-100 combination with Paclitaxel was highly synergistic in drug resistant cancer cells
- Potency was increased up to 5,000 fold compared to the single agent Paclitaxel
- The effect was specific for cells that express LHRH receptors
- The potency of the combination of EP-100 and Paclitaxel was dependent on the sequence of administration
- The effects of EP-100 was mediated via inhibition of P-gp efflux pump
- The inhibition of P-gp ATPase activity was facilitated by the lytic portion (CLIP-71) of EP-100

Conclusion

The combination of EP-100 and Paclitaxel is highly synergistic in cancer cells that express LHRH receptors. These results provide rationale for the combination of EP-100 with Paclitaxel as potential treatment of ovarian, breast, prostate cancers.

References

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