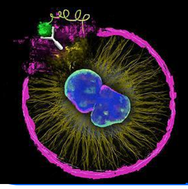
ACTIVITY OF EP-100 IN NON-HODGKIN'S LYMPHOMA – SYNERGY IN COMBINATION



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Abstract 2829

EP-100 is a targeted anti-cancer peptide comprised of Luteinizing Hormone Releasing Hormone (LHRH) fused to a membrane-disrupting lytic peptide (CLIP-71). A Phase 1 clinical trial in tumors that over-express LHRH receptors has been completed. EP-100 kills cancer cells directly via membrane disruption.

We tested a combination of EP-100 and doxorubicin in multi-drug resistant Non-Hodgkin's Lymphoma (NHL) cell lines and primary cells from refractory/relapsed NHL patients. Cells were cultured in the presence of EP-100 (0.00001-100 µM) or CLIP-71 (unconjugated lytic peptide) alone or in combination with doxorubicin (0.0000056 - 56.5 µM) and EP 100 at 0.5, 5, 50 and 500 nM. Cytotoxicity was determined by membrane integrity and cell viability assays. LHRH receptor expression was determined by flow cytometry. The effect of EP-100 on purified human recombinant p-glycoprotein (h-Pgp) pump was measured by ATPase activity.

The IC_{50} values [µM] for EP-100 alone were 0.52±0.13, 0.95±0.2, 2.8±0.5, 0.9±0.13 and values for CLIP-71 were 59±1.5, 25.6±1.7, 6.1±0.8 after 5 h of incubation for Daudi, Raji, Toledo, Hut78 cells, respectively.

EP-100 specifically killed NHL patient cells and CLIP-71 was ineffective. The IC_{50} values for EP-100 were 1.2 ± 0.1µM for cells obtained from three Mantle Cell Lymphoma patients (N=3), 2.3± 0.1 µM for Diffuse Large B Cell Lymphoma patient (N=1), 1.7± 0.3 µM for Follicular Lymphoma patients (N=4), and 1.6 ± 0.1 µM for one Waldenström Macroglobunemia patient. EP-100 or CLIP-71 did not kill B-cells from normal subjects (N=2) after 5 hour incubation. LHRH receptors were over-expressed on cell lines and patient cells.

Combination of doxorubicin and EP-100 was synergistic after 72 hour incubation and Combination Index was <1. EP-100 directly inhibited the h-Pgp ATPase activity.

These results show that specific killing of NHL cells by EP-100 is mediated via over-expression of LHRH receptors and the synergy between EP-100 and doxorubicin is due to direct inhibition of EP-100 on h-Pgp pump.

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 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. These 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. Interaction with signaling or molecular pathways is not required [3]. As a result of this technology, Esperance Pharmaceuticals is developing a new generation of targeted 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 cancer cells that express 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.

Lymphoma is the 6th leading cause of cancer death in women, 9th in men counting 70,190 cases in 2011, of which 66,360 were Non-Hodgkin's lymphoma patients. Despite new advances in treatments for Non-Hodgkin's lymphoma 19,320 patients died from recurrences in 2011 [4]. Despite various treatments used as single agent or in combination (cyclophosphamide, vincristine, doxorubicin, fludarabine, cytarabine, Rituxan®, Bexxar®, Zevalin®, Revlimid®) there remains an unmet need for treatment of NHL. Screening of NHL solid specimen showed that 94 % of tumors express LHRH receptors [5]. Tumor cells expressing LHRH receptors can be targeted by EP-100. In the current study we report data on EP-100 cytotoxicity and kinetic profiles on NHL cell lines and demonstrate the feasibility of tumor response in an *ex vivo* study from relapsed refractory NHL patients.

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. Lytic peptide have been shown to increase potency of doxorubicin in a synergistic manner [6].

The combination effect of EP-100 was tested in doxorubicin (Pgp substrate) resistant NHL cell lines *in vitro*. The mechanism of action on P-gp was evaluated on recombinant human Pgp protein. These results indicate that EP-100 is a potential treatment of NHL alone or in combination with chemotherapeutic agents.

Objectives

1. To evaluate the synergy of EP-100 and doxorubicin combination *in vitro* in multi-drug resistant Non-Hodgkins lymphoma (NHL) cell lines
2. To determine the expression of LHRH receptors in NHL cells
3. To determine the feasibility of NHL clinical study in refractory/relapsed patients: *ex vivo* studies
4. To determine the mechanism of action of EP-100 on Pgp

Materials & Methods

- **Cell lines:** Human Non-Hodgkin's lymphoma cell lines (Daudi, Raji, Toledo and HUT78), PBMC patient samples for refractory/relapsed NHL (ALL Cells).
- ***In vitro* cytotoxicity** studies were conducted in 96 well plate format (2,000 cells/well) with EP-100 or CLIP-71 for 5 h or 15, 30 minutes and 1, 4 and 24 h, Cell viability was determined using luminometric assays. (Cell Titer Glo and Cytotox Glo, Promega) Saline/Vehicle and 0.1 % Triton served as controls for 100 % viability and complete cell death.
- ***In vitro* cytotoxicity** studies for combination of EP-100 and Doxorubicin were conducted in 96 well plate format (2,000 cells/well) with single agents and in combination with EP-100. Incubations were conducted for 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 Doxorubicin 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

Kinetics and Specificity of EP-100 Cytotoxicity in NHL cell lines

EP-100 kills specifically NHL cell lines within 1 h – unconjugated CLIP-71 is not effetive

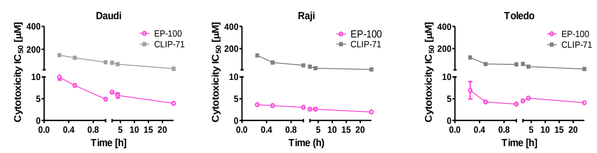

Figure 1: Kinetics of response for NHL cell lines *in vitro*. Daudi, Raji (Burkitt's lymphoma) and Toledo (Diffuse Large B Cell Lymphoma) were exposed to EP-100 and CLIP-71 (0.001, 0.01, 0.1, 1, 5, 10 and 100 µM) and cell viability was determined after 15, 30, 60 minutes and 4h and 24 h. IC_{50} values for each time point are presented for EP-100 and CLIP-71. Controls contained USP saline or 0.1 % TritonX-100™ as reference for 0 and 100 % cell death, respectively, N=8. These data demonstrate the high specificity and potency of EP-100.

Table 1: Cytotoxicity of EP-100 and CLIP-71 in NHL Cancer Cell Lines

Cell Line	EP-100 IC_{50} [µM] –5h	CLIP-71 IC_{50} [µM] –5h
Daudi (NHL, Burkitt)	0.52±0.13	35±1.5
Raji (NHL, Burkitt)	0.95±0.2	37±8.1
Toledo (DLBCL)	2.8±0.5	47±1.9
HUT78 (CTCL, Sezary)	0.9±0.13	271±28

Table 1: Cytotoxicity of EP-100 in NHL cell lines after 5 h. The IC_{50} values were in the low micromolar range except for the Toledo cell line. Unconjugated lytic peptide (CLIP-71) was not toxic with IC_{50} values of > 27 µM. These data demonstrate the high specificity and potency of EP-100.

Results ctd.

LHRH receptors expression in refractory relapsed NHL Patient samples

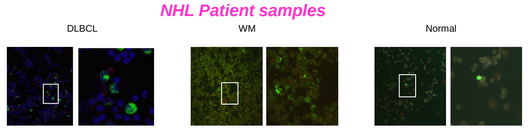

Figure 2: Peripheral blood mononuclear cell suspensions from refractory relapsed patients having Diffuse Large B-cell Lymphoma (DLBCL), Waldenströms Macroglobulinemia (WM) and a healthy donor showed labeling of cancer cells by FITC-LHRH in cancer patients.

Table 2: *Ex vivo* study in refractory relapsed NHL patient samples

Diagnosis	Prior Treatment	EP-100 IC_{50} [µM]	CLIP-71 IC_{50} [µM]
Diffuse Large B Cell Lymphoma (DLBCL)	CVP-R (CR)	2.26±0.12	18.5±1.1
Follicular Lymphoma	400 Gy (CR), R-CHOP (CR)	3.03±0.42	17.0±0.1
Follicular Lymphoma	Rituxan	0.98±0.02	34.4±0.2
Follicular Lymphoma	CVP-R (PR) XRT (CR), R+Bendamustine	1.0±0.03	25.1±0.05
Follicular Lymphoma	SR, CVP + fludarabine + vaccine (CR) Rituxan (SD) Rituxan ongoing	1.02±0.01	40.6±0.3
Waldenström's Macroglobulinemia	2-CDA, Rituxan	1.6±0.1	31.9±6.7
Mantle Cell Lymphoma	unknown	1.07±0.16	118.1±13
Mantle Cell Lymphoma	Erythropoietin Rituximab	1.3±0.2	24.3±1.7
Normal Adult	NA	>100	>100

Table 2: PBMC collections from NHL patients were treated with EP-100 or CLIP-71 for 5 h. All 8 NHL cases were sensitive to EP-100, but not to untargeted CLIP-71. In all cases cell killing was specific to LHRH receptor targeting. The IC_{50} values were in the low micromolar range for NHL cases and > 17.0 µM for CLIP-71 (unconjugated lytic peptide). Normal B-cells were not killed by either EP-100 or CLIP-71.

Table 3: Combination Studies of EP-100 and Doxorubicin in multi-drug resistant NHL Cancer Cell Lines

	Daudi		Raji		HUT78	
	[nM]	CI	[nM]	CI	[nM]	CI
EP-100	1130±43		384.5±12.9		1833±727	
Doxorubicin	126.6±12		14.2±2		82±3.2	
EP-100 (0.5 nM) + Doxorubicin	41.05±8.5	0.08	2.66±0.1	0.01	74.2±0.5	0.4
EP-100 (5 nM) + Doxorubicin	11.1±2.5	0.09	1.3±0.2	0.01	56.9±15.1	0.3
EP-100 (50 nM) + Doxorubicin	8±1.3	0.04	1.5±0.1	0.18	45.7±8	0.1
EP-100 (500nM)	1.8±0.3	0.04	1.3±0.1	0.1	24.5±4.4	0.2

Table 3: EP-100 sensitizes Doxorubicin resistant cancer cells expressing LHRH receptors resulting in potentiation of cell killing in a synergistic manner. Cell lines were incubated with increasing concentrations of Doxorubicin (0.0056-5650 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. Combinations of EP-100 and doxorubicin were highly synergistic with CI < 1.

Results ctd.

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

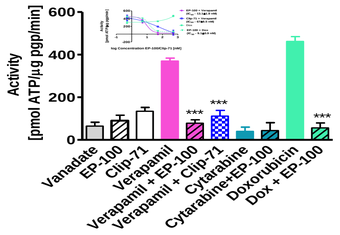

Figure 4:
Pgp ATP'ase activity on recombinant h-Pgp.
The activity of h-Pgp dependent ATP hydrolysis was determined by measurement of remaining ATP in a luminometric assay kit (Promega) (N=4). EP-100 and CLIP-71 (unconjugated lytic peptide) were added at concentrations of 0.5, 5, 50 and 500 nM. Cytarabine (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 64.5 ± 18.1 pmol ATP/µg P-gp /min and was similar to Cytarabine (40.25 ±18.3 pmol ATP/µg h-Pgp /min activity, indicating a lack of activation of h-Pgp 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 h-Pgp 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 h-Pgp/min for EP-100 in combination with Verapamil, and from 370.1±13.8 to 111.5 ± 26 pmol ATP/µg h-Pgp/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

- Combination with chemotherapy was highly synergistic
- EP-100 specifically killed NHL cells *in vitro (*within 1 hour) without killing normal cells
- NHL cells from patients samples expressed LHRH receptors
- *Ex-vivo* studies showed that EP-100 specifically kills NHL cells from relapsed/refractory patients
- EP-100 inhibited Pgp efflux pump ATPase activity
- The inhibition of Pgp ATPase activity was facilitated by the lytic portion (CLIP-71) of EP-100

Conclusion

EP-100 is a potential therapy for NHL when used alone or in combination with chemotherapeutic for multi-drug resistant NHLcancer cells expressing LHRH receptors. The combination with chemotherapeutic agents such as doxorubicin is highly synergistic. The mechanism of action of the synergy is due to the inhibition of P-gp by EP-100.

References

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