Supporting Information for:

Peptide-Chlorambucil Conjugates Combat Pgp-Dependent Efflux

Sonali B. Fonseca,[a] Shana O. Kelley[a,b=]

[a]Department of Pharmaceutical Sciences, Faculty of Pharmacy, [b]Department of Biochemistry, Faculty of Medicine, Toronto, Ontario, Canada

* [=]Correspondence should be addressed to: shana.kelley@utoronto.ca
Phone: +1-416-978-8641, Fax: +1-416 978-2979

Experimental Procedures:

Cell Culturing Conditions. A2780 wild-type and adriamycin resistant cells were cultured in RPMI 1640 (Invitrogen, Carlsbad) supplemented with 10% (v/v) FBS at 37 °C with 5% CO_2. Resistant cells were treated with 10 μM adriamycin every 10 passages to maintain high Pgp expression.

Peptide Synthesis & Characterization. Solid–phase synthesis was performed on Rink amide MBHA resin (0.7 mmol/g, 100-200 mesh) (NovaBiochem) using a Prelude Protein Technologies peptide synthesizer as described previously.[1] Peptides were synthesized on a 25 μmol or 50 μmol scale. Thiazole orange (*to*) was synthesized as described previously[2] and coupled to peptides using HBTU (4 eq, Protein Technologies, Tucson), HBTU = *O*-(benzotriazol-l-yl)-*N,N,N',N'*-tetramethyl-uronium hexafluorophosphate), and DIPEA (8 eq, Sigma-Aldrich, St. Louis), DIPEA=*N,N*-diisopropylethylamine) in *N,N*- dimethyl formamide (DMF) overnight. Chlorambucil (Sigma-Aldrich, St. Louis) was coupled to peptides using HBTU (4 eq) and DIPEA (4 eq) in DMF. The N-terminus of unlabeled peptides was capped using acetic anhydride, pyridine and DCM (1:5:10, Sigma). Peptides were deprotected and cleaved from the resin using TFA:triisopropylsilane:H_2O (95:2.5:2.5) and precipitated in cold ether. All peptides were purified to >95% purity by RP-HPLC on a C18 column with a

H_2O/MeCN gradient in 0.1% TFA and identity confirmed by electrospray ionization mass spectroscopy. Peptides containing chlorambucil were immediately flash frozen in liquid nitrogen post purification and lyophilized to dryness. Thiazole orange labelled peptides were quantified at 500 nm using an extinction coefficient of 63,000 M^{-1} cm^{-1}. Chlorambucil conjugated peptides were quantified at 258 nm using the chlorambucil extinction coefficient of 15200 M^{-1} cm^{-1}.[3] The thiazole orange extinction coefficient was used for conjugates where both the fluorescent dye and chlorambucil were present.

Confocal Microscopy. Cells were seeded in 8 well µ–slides (iBidi, Germany) at a density of 25,000 cells per well one day prior to experiments. MEM alpha without phenol red (Invitrogen, Carlsbad) was used for these experiments. Adriamycin (20 µM, Sigma, St. Louis) or MitoTracker 633 (150 nM, Invitrogen, Carlsbad) in the presence or absence of 5 µM peptide-Cbl conjugate was added to cells for 20 min. For conjugate localization experiments, fluorescently labelled peptide-Cbl conjugates (5 µM) were added to the wells for 5 min and then replaced with fresh media for 25 min. For higher concentrations of conjugate i and ii, the same protocol was followed and cells were treated with 5 µM, 10 µM, 15 µM and 20 µM of peptide-Cbl conjugate. Cells were then washed twice and imaged using an inverted Zeiss LSM 510 confocal microscope.

Peptide Uptake. Cells were seeded at 50,000 cells per well in triplicate in a 24-well plate one day prior to experiments. Cells were incubated with 5 µM chlorambucil, peptide, peptide-Cbl conjugate or cyclosporine A in Opti-MEM (Invitrogen, Carlsbad) for 40 min and then adriamycin (100 µM) was added to the wells for 20 min. For higher concentrations of conjugate i and ii, cells were treated with 5 µM, 10 µM, 15 µM and 20 µM and the above method was followed. For analysis of conjugate efficacies, the above method was followed except that 200 µM adriamycin and 8 µM actinomycin D was used. For time course experiments cells were incubated as above with 200 µM adriamycin for 1 h or 8 h. Cells were then washed with PBS, trypsinized and analyzed on FACSCanto

(BD, Franklin Lakes) to determine relative intracellular substrate concentrations. Statistical analysis was done using Graphpad Prism Software (Graphpad, La Jolla). (Note: for the time course experiments, the FACSCanto detector voltage for adriamycin was lowered compared to other experiments. This was done to ensure that the 8 h adriamycin uptake in the presence of cyclosporine was within the quantifiable range. However, the same voltage setting was used for the 1h and 8h experiments to allow these uptake values to be directly compared).

Coimmunoprecipitation of Biotin-F_xr3kCbl and Pgp. A2780 AdrR cells were treated with 5 μM Biotin-Fxr3kCbl for 1 h in Opti-MEM (Invitrogen, Carlsbad). Cells were then scraped with a rubber policeman and lysed in 1% digitonin for 20 min at 4°C. Debris was spun down at 5,000 rpm, 5 min, 4°C and the supernatant was incubated with streptavidin magnetic beads (New England Biolabs, Ipswich) for 45 min at 4°C. Beads were then washed 3 times with 0.1% digitonin and boiled with Laemmli sample buffer for 5 min. Samples were resolved in 6% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% milk for 1 h and incubated with anti-Pgp (1:250, Abcam) overnight. The membrane was then washed and incubated with a 1:5,000 donkey anti-mouse IgG-HRP secondary antibody for 1 h prior to ECL chemiluminescence detection (GE Amersham, Baie d'Urfe).

Analysis of Toxicity. Cells were seeded in 96-well flat bottom tissue culture plates (Starstedt, Germany) at a density of 12,000 cells per well one day prior to experiments. The culture medium was removed and cells were washed. Peptide and cyclosporine A incubations (5 μM) in Opti-MEM (Invitrogen, Carlsbad) were conducted for 1 h followed by addition of 200 μM adriamycin for 1 h, 8 h and 24 h. Cellular viability was analyzed using the CCK-8 viability dye (Dojindo, Rockville) at an absorbance of 450 nm. Statistical analysis was done using Graphpad Prism Software (Graphpad, La Jolla).

Supporting Data:

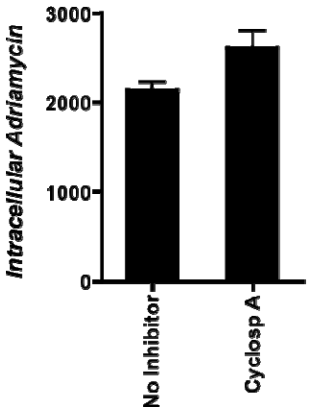

Figure S1. Basal intracellular levels of Adriamycin with and without cyclosporine A from Figure 1c. These are the basal adriamycin uptake levels that correspond to the intracellular staining in Figure 1c .

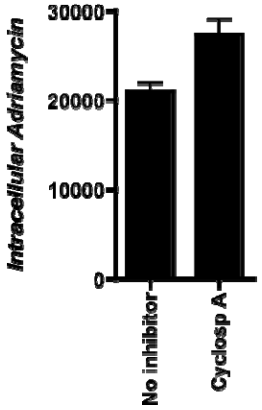

Figure S2. Basal intracellular levels of Adriamycin with and without cyclosporine A from Figure 2b. These are the basal adriamycin uptake levels that correspond to the intracellular staining in Figure 2b .

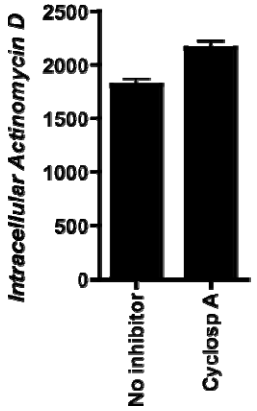

Figure S3. Basal intracellular levels of Actinomycin D with and without cyclosporine A from Figure 2c. These are the actinomycin D uptake levels that correspond to the intracellular staining in Figure 2c.

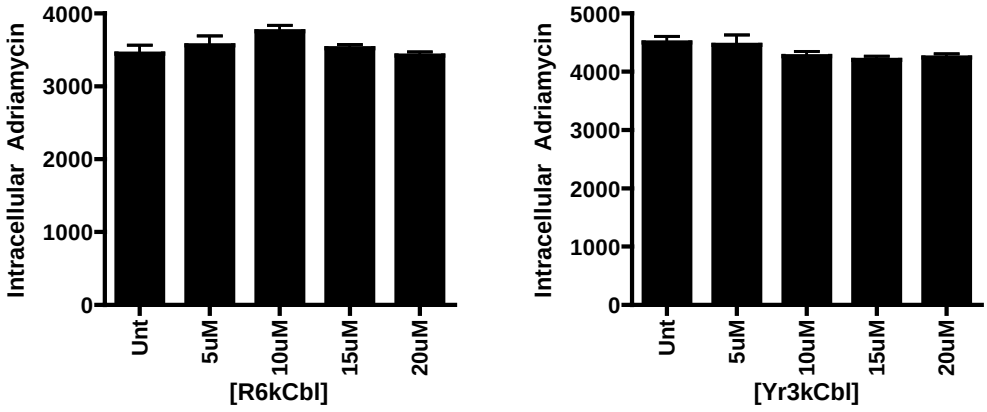

Figure S4. Intracellular levels of adriamycin in the presence of increasing concentrations of R6kCbl (conjugate i) and Yr3kCbl (conjugate ii). A2780 AdrR cells were pre-incubated with conjugates at 5 μM, 10 μM, 15 μM and 20 μM. Uptake of adriamycin was then measured using flow cytometry. Even at higher concentrations, no increase in adriamycin uptake was noted with either peptide-Cbl conjugate compared to untreated cells. Mean values are plotted, n=3, error bars are s.e.m.

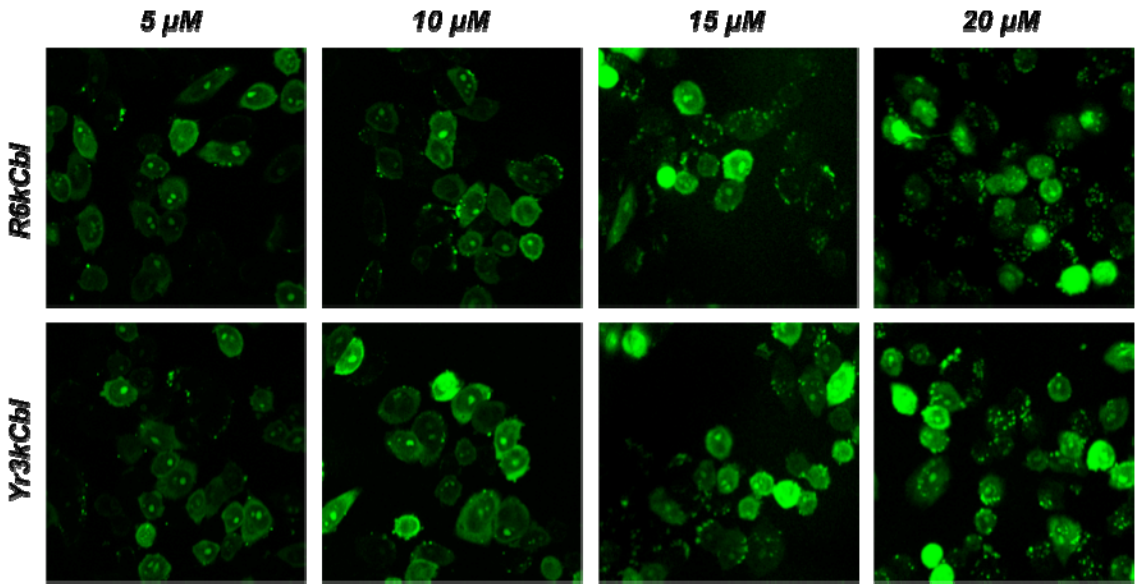

Figure S5. Intracellular localization of increasing concentrations of R6kCbl and Yr3kCbl. Peptide-Cbl conjugates at 5 μM, 10 μM, 15 μM and 20 μM were incubated with live cells and visualized with confocal microscopy. Even at higher concentrations, membrane staining of these peptide-conjugates was not seen.

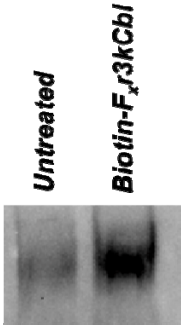

Figure S6. Co-immunoprecipitation of Biotin-F_xr3kCbl and Pgp. To provide evidence for the direct interaction between the peptide-Cbl conjugate and the Pgp efflux pump, a co-immunoprecipitation was performed as described above. The complex was pulled down using streptavidin magnetic beads and immunoblotting for Pgp was performed. The stronger signal in the cells treated with Biotin-F_xr3kCbl provides support for a direct interaction between the peptide-Cbl conjugate and Pgp pumps.

Table 1. List of peptide-Cbl conjugates and retention times on a C18 column with acetonitrile/water gradient.

Conjugate	Sequence	% MeCN
i	R6kCbl	39%
ii	Yr3kCbl	40%
iii	F_Nr3kCbl	38%
iv	Fr3kCbl	44%
v	F_Xr3kCbl	49%
vi	F_Xk3kCbl	49%

Table 2. Cytotoxicity of various Pgp inhibitors in A2780AdrR cells.

Compound	% Cytotoxicity
Cyclosporine A	15%
F_Nr3kCbl	10%
Fr3kCbl	10%
F_Xr3kCbl	25%
F_Xk3kCbl	25%

References:

1. Horton, K. L.; Stewart, K. M.; Fonseca, S. B.; Guo, Q.; Kelley, S. O. *Chem Biol* **2008,** *15*, 375.
2. Carreon, J. R.; Stewart, K. M.; Mahon, K. P., Jr.; Shin, S.; Kelley, S. O. *Bioorg Med Chem Lett* **2007,** *17*, 5182.
3. Cullis, P. M.; Green, R. E.; Malone, M. E.; Merson-Davies, L.; Weaver, R. *Biochem Soc Trans* **1994,** *22*, 402S.