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Abstract

Cellular efflux as mediated by members of the ATP binding cassette family, MRP/ABCC1, is believed to be responsible for cross resistance to a number of cancer chemotherapeutic drugs such as the anthracyclines and the vinca alkaloids. Although a number of compounds have been reported to inhibit MRP-1, none appear to be sufficiently potent or selective to merit clinical testing. The aim of the present study was to conduct a high throughput screening campaign using the NIH MLSCN Small Molecule Repository of 85,200 compounds to identify novel MRP-1 inhibitors. The screening strategy involved the multidrug resistant human lung tumor cell line H69AR to measure the effects of library compounds on cell viability in the presence and absence of a subtoxic dose of doxorubicin.

From the primary screen and follow-up dose-response confirmation, 29 compounds were identified as active. The hits were next evaluated in a series of follow-up assays to determine their ability to left shift the dose-response curve to doxorubicin and to inhibit the efflux of calcein-AM, a known MRP-1 substrate. The results revealed a novel 5-quinolone scaffold that was common to a number of active compounds. For example, one compound in this series decreased the IC₅₀ value to doxorubicin by 13-fold and inhibited calcein-AM efflux with an IC₅₀ of 1.8 μM. Follow-up synthesis, screening, and selectivity profiling efforts are in progress to define structure-activity relationships for the development of a pharmacophore model to aid in the design of novel highly potent and selective MRP-1 inhibitors.

Objectives

- Identify compounds capable of increasing sensitivity of multidrug resistant H69AR cells to doxorubicin using a compound library available to the NIH Molecular Libraries Screening Center Network.
- Select for compounds that reduce viability to less than or equal to 40% of controls in the presence of doxorubicin while being non-cytotoxic (viability ≥ 75% of controls) when administered in the absence of doxorubicin.
- After performing secondary screening to confirm hits from the initial screen, select for compounds that have a reasonable IC₅₀ (e.g. ≤10 μM) to warrant further testing.
- Screen selected compounds using a calcein-AM efflux assay to determine functional inhibition of MRP-1 mediated efflux.

HTS Methods

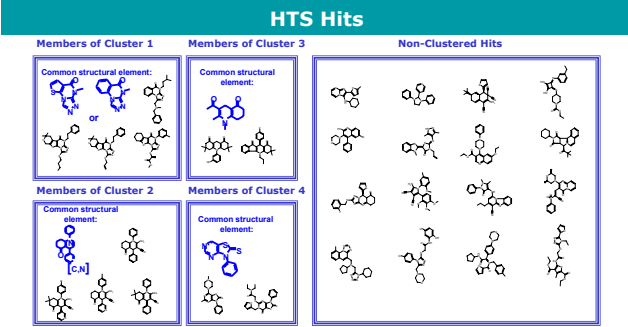
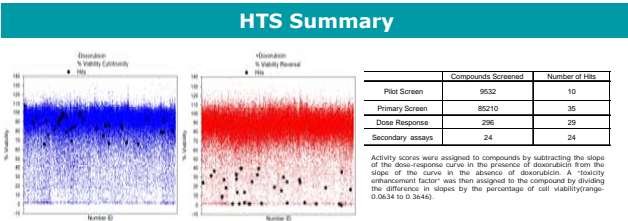
Cell Culture: H69AR (doxorubicin) cells were obtained from ATCC and subcultured every 3-4 days in RPMI-1640 + 4.5 g/L glucose + 10 mM HEPES pH 7.4 + 10% FBS + 1 mM glutamine (complete growth medium), and incubated at 37°C in 5% CO₂ and humidified at subconfluent density.
H69AR cells were subcultured every 3-4 days in RPMI-1640 + 2.0 g/L glucose + 2.3 mM glutamine, and incubated at 37°C in 5% CO₂.

HTS Screening Strategy: A flowchart showing the screening process from library compounds to hit identification and validation.

Primary Screen: 29 compounds were identified as active.

Secondary Screen: 29 compounds were identified as active.

Validation Assay: At the end of the treatment period, assay plates were removed from the incubator and equilibrated to room temperature. An equal volume of Cell Titer Glo reagent was added and the plates were incubated for 10 minutes. After incubation, assay plates were analyzed with a Perkin Elmer Envision microplate reader with a read time of 1.0 second in luminescence mode.



Secondary Screening Methods

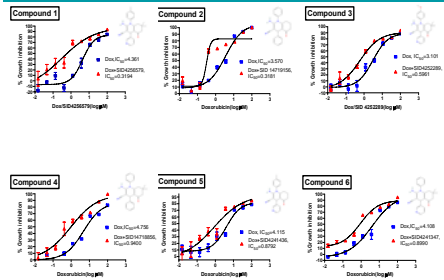
Doxorubicin Sensitivity Assay: Plating: One hundred μL of cells were dispensed (5000 cells per 100 μL complete growth medium) with 100 μM penicillin and 100 μg/ml streptomycin. Plates were incubated at room temperature for 30-40 minutes prior to placing in incubator for 18-24 hrs.

Dosing: Compounds were diluted in complete growth medium to prepare a 4X dosing solution. A non-cytotoxic 10 μM dose of each compound was used. 50 μL of diluted compound (1/4 of total culture volume) was applied to the cell plate followed 4 hrs later by 50 μL of 3X serial dilutions of doxorubicin (100 μM top dose). Cells were placed in the incubator for 72 hrs.

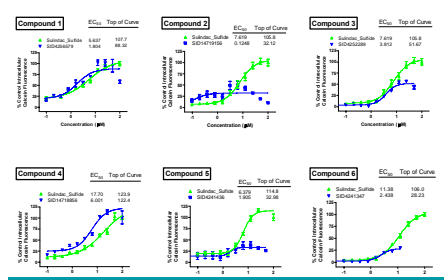
Cell Viability: At the end of the treatment period, assay plates were removed from the incubator and equilibrated to room temperature. An equal volume of Cell Titer Glo reagent was added and the plates were incubated for 10 minutes. After incubation, assay plates were analyzed with a Perkin Elmer Envision microplate reader with a read time of 1.0 second in luminescence mode.

MRP-1 Efflux Assay: H69AR cells were plated in cover glass bottom 96-well plates and allowed to acclimatize overnight. On the assay day, cells were incubated for 3-6 hrs with a dilution series of several agents followed by a 20 min incubation with a calcein-AM MRP-1 substrate. At the end of the loading period, free calcein-AM was washed away with PBS. Cellular fluorescence was analyzed using a Fluorimetric Image Scanning Cytometer. Salindac sulfide was used as a positive control based on confirmation (Panel A above) of the observation by Duffy and Cyrus (Eular) (Cancer, 1998) (p. 1250-0) that salindac sulfide inhibits MRP-1 mediated efflux. In the presence of increasing concentrations of salindac sulfide, cellular fluorescence of calcein-AM (0-10 μM) increases (Panel B). Increased cellular accumulation of calcein-AM in the presence of an efflux inhibitor increases the measured fluorescence due to prolonged exposure of the calcein-AM substrate to intracellular esterase (Panel C).

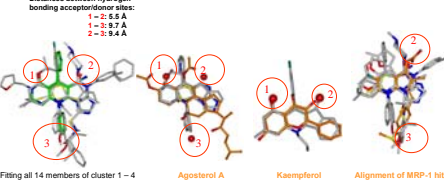
Doxorubicin Sensitivity Assay



MRP-1 Efflux Assay



Pharmacophore Modeling



Secondary Screening Results

Compound	Structure	Fold Increase in Sensitivity	EC ₅₀ Efflux Assay (μM)	% Viability @ 10 μM
1		13	1.8	84.8
2		11	0.12	58.5
3		5	3.8	75.3
4		5	0.6	73.7
5		5	1.9	96.5
6		5	2.4	80.5

Summary and Conclusions

- High throughput screening and confirmatory dose response assays identified 29 compounds with low cytotoxicity yet having the ability to increase sensitivity to doxorubicin in a multidrug resistant cell line, H69AR.
- Clustering of hits identified 6 compounds based around the 5-quinolone scaffold with one compound, SID 4265679 able to left shift the doxorubicin dose response curve 13-fold and inhibit efflux (IC₅₀ of 1.8 μM).
- Pharmacophore modeling found overlap of ring structures of similar shape as well as overlap of hydrogen bonding groups with known MRP-1 inhibitors agosterol A, kaempferol, and salindac sulfide.
- Further chemical synthesis and *in vivo* testing is ongoing to refine necessary substituents for activity.
- Future testing will confirm MRP-1 specificity using vesicle transport of LTC₃ and the measurement of cellular glutathione levels.
- In vivo* efficacy testing is planned once a lead compound is identified.

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