Functional analysis of mitochondria-targeting Wnt-inhibitors on metabolically distinct breast cancer cell types

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Introduction

A better understanding of cancer cell metabolism can provide valuable information about the harsh tumor microenvironment, which can profoundly impact the efficacy of emerging immune therapies for solid tumors. Recent research demonstrates that at least certain cancers are heavily reliant on mitochondrial respiration, suggesting that mitochondria can be an effective therapeutic target. [1,2]

The Agilent Seahorse **XF Mito Tox assay** is a rapid and robust way to directly detect and measure mitochondrial inhibitors and uncouplers. [3,4] The drug-induced mitochondrial modulation can be quantitatively interpreted via the **Mito Tox Index (MTI)**, which enables the identification of effective drug doses for *in-vitro* applications. The further characterization of the drug effects can be achieved by Agilent **XF Electron flow assay** using cells permeabilized by the Agilent **Plasma Membrane Permeabilizer (PMP)**[5] and by the Agilent **XF Real-time ATP Rate assay.**[6]

This study investigates the impact of **Wnt-signaling modulators on mitochondrial dysfunction**. It evaluates the effects of selected drug treatments on cellular energy metabolism and cytotoxicity by comparing two distinct breast cancer cell lines: highly oxidative BT474 and glycolytic Hs578T.

Experimental

Mitochondria-targeted drug screening and evaluation workflow

MTI-based screening of mitochondrial modulator
- Inhibitor ranking
- Uncoupler ranking

→

Drug potency evaluation
- Inhibitor IC_{50}
- Uncoupler EC_{50}

→

Functional Validation
- Cellular energy metabolism
- Target function analysis

- *Screening by modulation potency represented by MTI (Mito Tox Index)*
- *Mode of action (MoA) identification: Inhibition vs. Uncoupling*
- *Improved assay throughput: Multi-file analyzer and Z' evaluation, etc.*

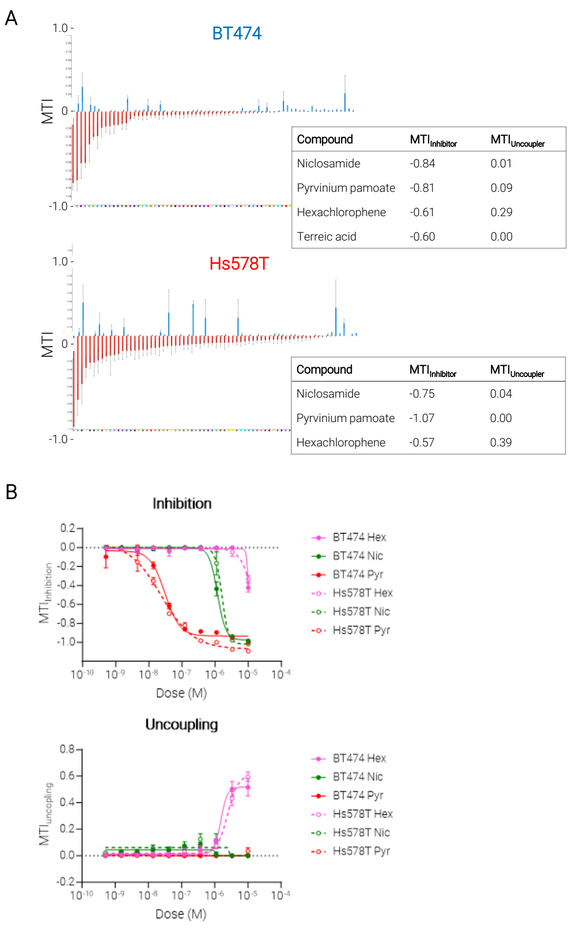
- *MTI-based dose response analysis for selected modulators*
- *Identify the optimal drug dose for the functional analysis*

- *Cellular metabolism:*
  - *Bioenergetic phenotype profiling: XF ATP Rate*
  - *Comprehensive mitochondrial functional analysis: XF Mito Stress Test, XF Substrate Oxidation Assay*
  - *Target identification: XF analysis using cells permeabilized by XF PMP and/or isolated mitochondria*
- *Target function analysis:*
  - *Proliferation and viability assays*
  - *Differentiation and activation tests*
  - *Genetic and epigenetic analysis*

Figure 1. Mitochondria-targeting drug screening and evaluation workflow.
Agilent Seahorse XF Mito Tox screening assay detects potent mitochondrial-targeting drug candidates and identifies the mode of action. The appropriate dosing information can be obtained from an MTI-based dose-response assay. The information collected from screening and dose-response analysis can then be used in further detailed functional analyses, including cellular energy metabolic profiling.

Results and Discussion

MTI-based screening and potency evaluation of Wnt modulators targeting mitochondria



Compound	Inhibition IC_{50} (μM)		Uncoupling EC_{50} (μM)	
	BT474	Hs578T	BT474	Hs578T
Niclosamide	1.40	1.96	NA	NA
Pyrvinium	0.03	0.02	NA	NA
Hexachlorophene	8.61	7.30	1.82	2.80

Figure 2. Screening and potency evaluation of mitochondrial inhibitor or uncoupler among Wnt signaling modulators
A. Highly oxidative BT474 and glycolytic Hs578T cells were treated with 10 μM Wnt signaling modulators (SCREEN-WELL Wnt Pathway, Enzo Life Sciences) for 24 hours, and the effect of the drugs on their mitochondrial activities was measured and analyzed using the Agilent Seahorse XF Mito Tox assay kit and the Agilent Seahorse XF Pro analyzer with Agilent Seahorse Analytics software, respectively. The compounds were ranked by the inhibitory impact based on the Mito Tox Index (MTI). The most potent inhibitors (MTI < -0.5) were selected, as shown in the tables above.
B. Cells were cultured in the presence of three selected Wnt modulators (**Hex**, Hexachlorophene; **Nic**, Niclosamide; **Pyr**, Pyrvinium) for 24 hours at concentrations as indicated. The dose-response pattern of MTIs for inhibitor and uncoupler were compared, and the respective IC_{50} and EC_{50} were assessed.

Molecular target characterization

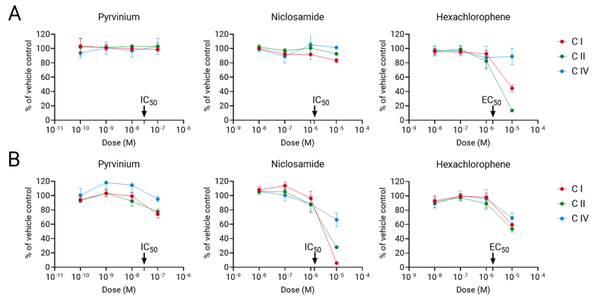


Figure 2. Mitochondrial target characterization by XF Electron flow assay[5,6]
A. BT474 cells were permeabilized by PMP and the complex specific electron flow was measured in the presence of drugs to detect acute inhibitory effect.
B. Cells were pretreated with drugs for 24 hour prior to the permeabilization and the complex activity were measured.
Indicated are IC_{50} and EC_{50} obtained by Agilent Seahorse XF Mito Tox assay (Figure 2) by arrows.

Effects on cellular energy metabolism

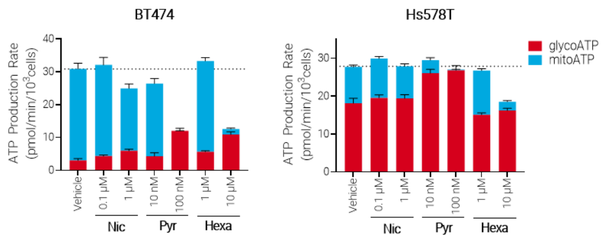
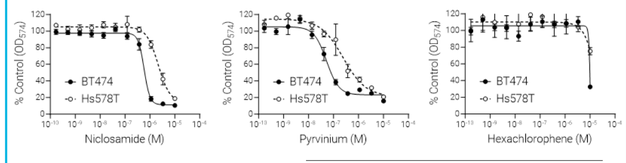


Figure 4. Changes in cellular energy metabolism induced by selected Wnt modulators.
Cells were cultured for 24 hours in the presence of the compounds at the indicated concentrations. The contribution of glycolytic and mitochondrial metabolism to the ATP production was compared.

Comparison of cancer cell killing efficacy



Compound	LC_{50} (μM)	
	BT474	Hs578T
Niclosamide	0.58	2.16
Pyrvinium	0.05	0.24
Hexachlorophene	12.2*	41.1*

**incomplete dose-response curve*

Figure 5. Differential cytotoxicity of selected Wnt modulators between metabolically distinct breast cancer cell lines.
BT474 and Hs578T cells were cultured in the presence of the compounds at the indicated concentration for 72 hours. The viable cells were stained with Crystal Violet, and the absorbance was measured at 570 nm.

Conclusions

MTI-based drug screening offers a straightforward and reliable method for identifying potent mitochondrial-targeting anticancer drugs by comparing the mode of action and potency quantitatively.
- Niclosamide and Pyrvinium were identified as the most potent mitochondrial inhibitors while Hexachlorophene was as an uncoupler among Wnt signaling modulators.
- The metabolic phenotype of the target cells did not affect the drug potency.

The molecular target of mitochondrial drugs can be specified by the **electron flow assay** using *in vitro* cultured cell lines without isolating mitochondria.
- Pyrvinium indirectly inhibited mitochondrial function ETC complex independently among the three selected Wnt modulators.
- Niclosamide targets Complex I and II indirectly.
- Hexachlorophene inhibited Complex I and II activity by uncoupling the mitochondrial activity.

The further functional validations using other **cellular energy metabolism analysis** tools in combination with a conventional **cytotoxicity assay** can provided data-enriched insights for anti-cancer drugs exploiting the metabolic vulnerability of cancer cells.
- The impact of mitochondria-targeting Wnt modulators on cellular energy metabolism showed significant difference depending on the background metabolic phenotype of the target cells, which corresponded well to the anti-cancer effect measured by *in vitro* cytotoxicity.
- BT474 cells, which rely heavily on mitochondrial respiration, are more vulnerable to ATP energy crisis induced by mitochondrial drugs compared to the more glycolytic Hs578T cells.

References

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