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The main products of cyclophosphamide bioactivation exert a cardiotoxic effect at clinical important concentrations in AC16 cardiac cells

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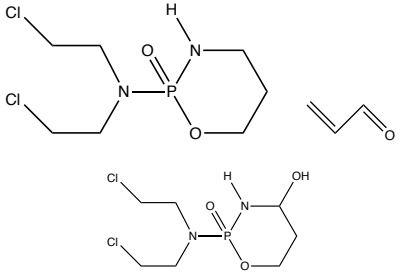
Abstract: Cyclophosphamide is used against lymphomas, solid tumors, namely breast, ovarian, bone and soft tissue tumors, in bone marrow transplant conditioning regimens and also in the treatment of autoimmune diseases. Despite its broad use, the application of cyclophosphamide is dose limited by its cardiotoxic effects, which have been linked to its intricate bioactivation process. In this study, we evaluated the cytotoxicity of cyclophosphamide (100 to 10000 µM) and two of its main metabolites, 4-hydroxycyclophosphamide (1 to 25 µM) and acrolein (5 to 100 µM) in AC16 cells, a human cardiomyocyte cell line. Furthermore, metabolomic evaluation was conducted in proliferative and differentiated cells after their incubation for 24h with subtoxic concentrations LC_{05} of cyclophosphamide, 4-hydroxycyclophosphamide and acrolein.

Keywords: Cyclophosphamide; Metabolism; Chemotherapy; Cardiotoxicity

Graphical Abstract

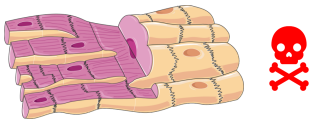
Cyclophosphamide and metabolites

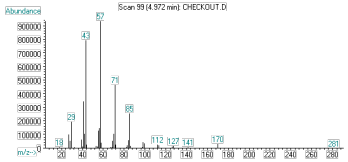


AC16 human cardiomyocytes

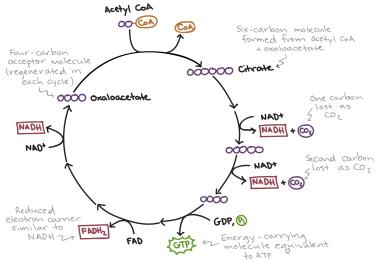
Cell Viability Assays

Cellular Damage Evaluation



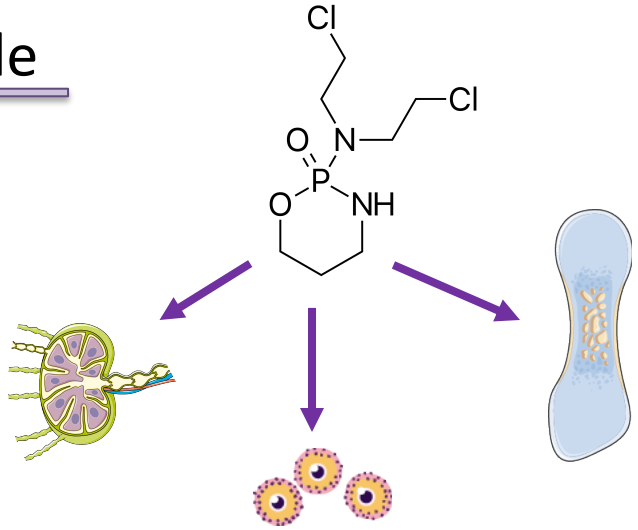


GC-MS



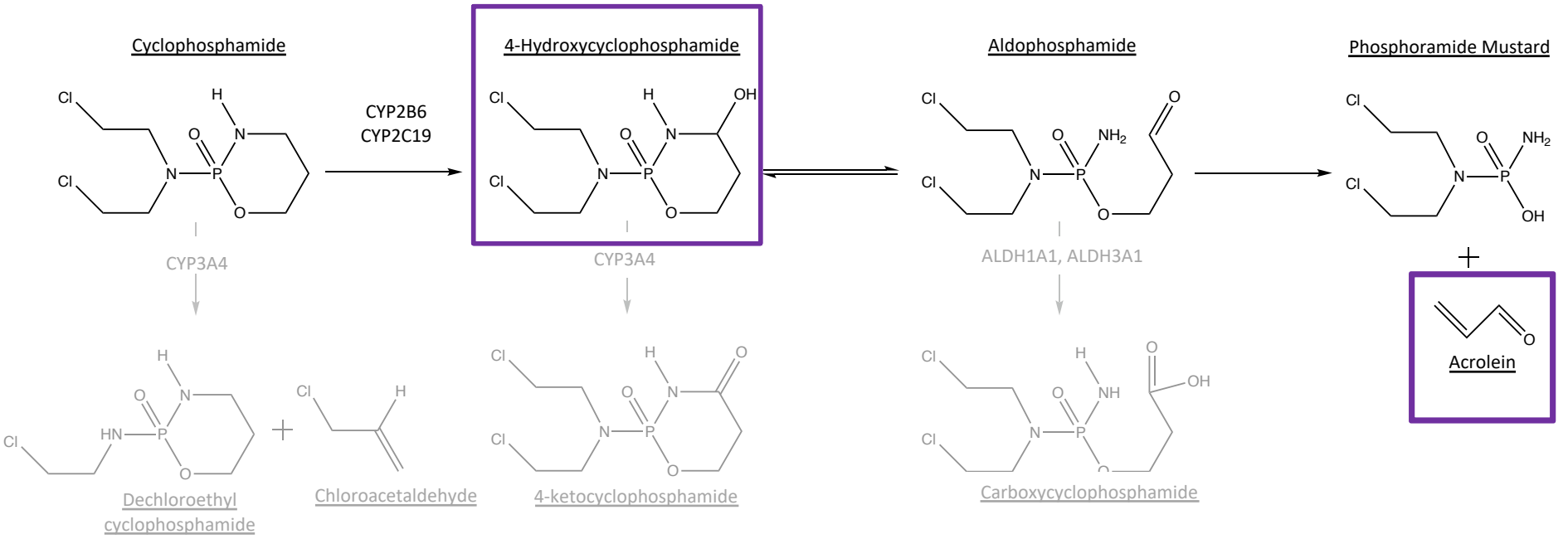
Metabolic Profiling

Cyclophosphamide



- Prodrug
- Extensively metabolized to active metabolites
- Treatment of lymphomas, solid tumors namely breast and ovarian and bone marrow transplants regimens
- Dose limiting effect: **cardiotoxicity**

The role of metabolism



Main Objectives

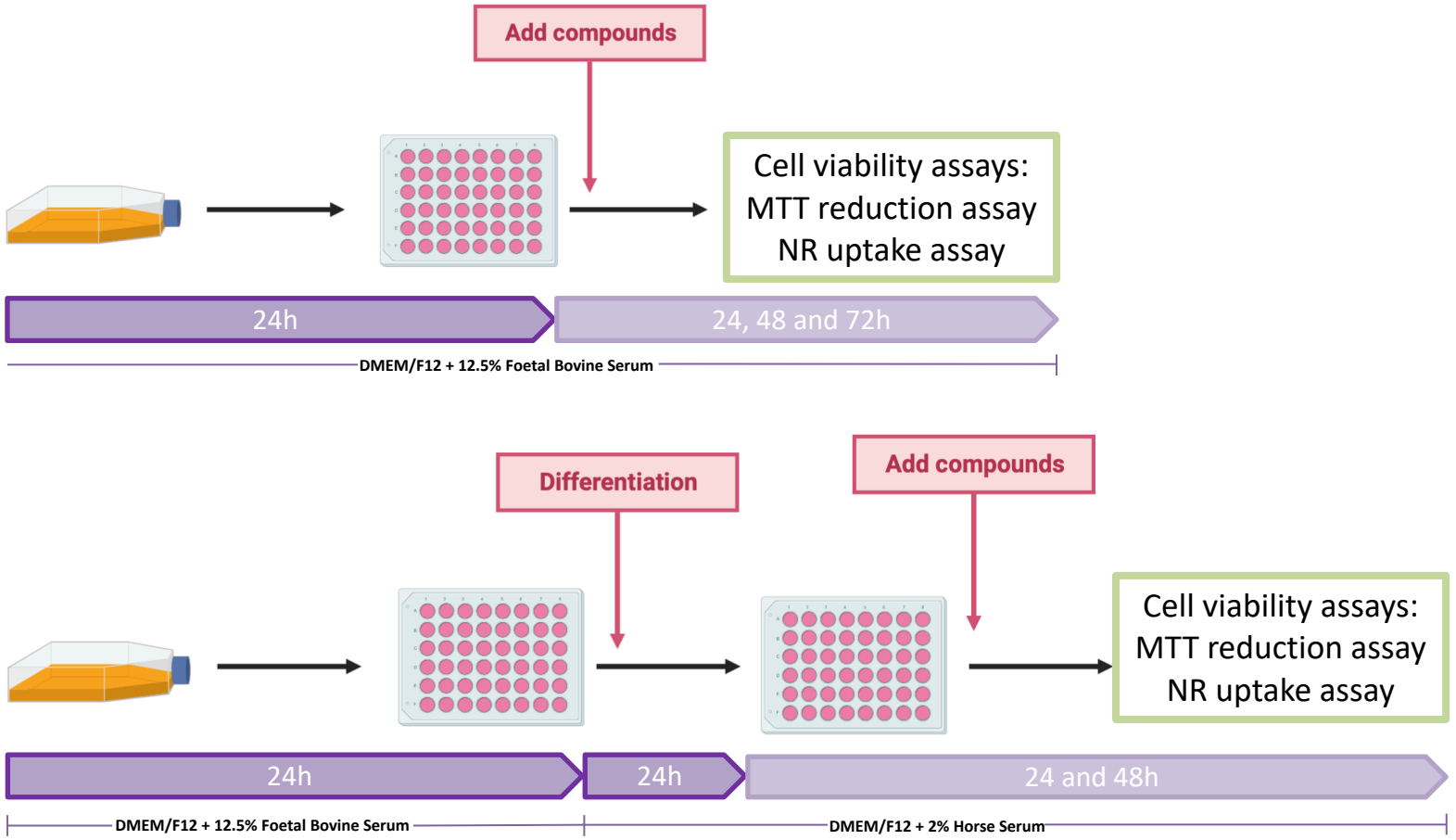
Determine the cytotoxicity of cyclophosphamide and its main toxic active metabolites in differentiated and proliferative AC16 human cardiac cells

Metabolic profiling of differentiated and proliferative AC16 cells incubated with subtoxic (LC_{05}) doses of cyclophosphamide and toxic active metabolites

Experimental Design: Cytotoxicity assays

- Concentration-response curve of cyclophosphamide and two toxic active metabolites – 4-hydroxycyclophosphamide and acrolein
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as indicator of mitochondrial activity
- Neutral Red (NR) uptake assay as indicator of lysosomal integrity
- Two cellular states used: proliferative and differentiated

Experimental Design: Cytotoxicity assays



Experimental Design: Metabolic Profilling

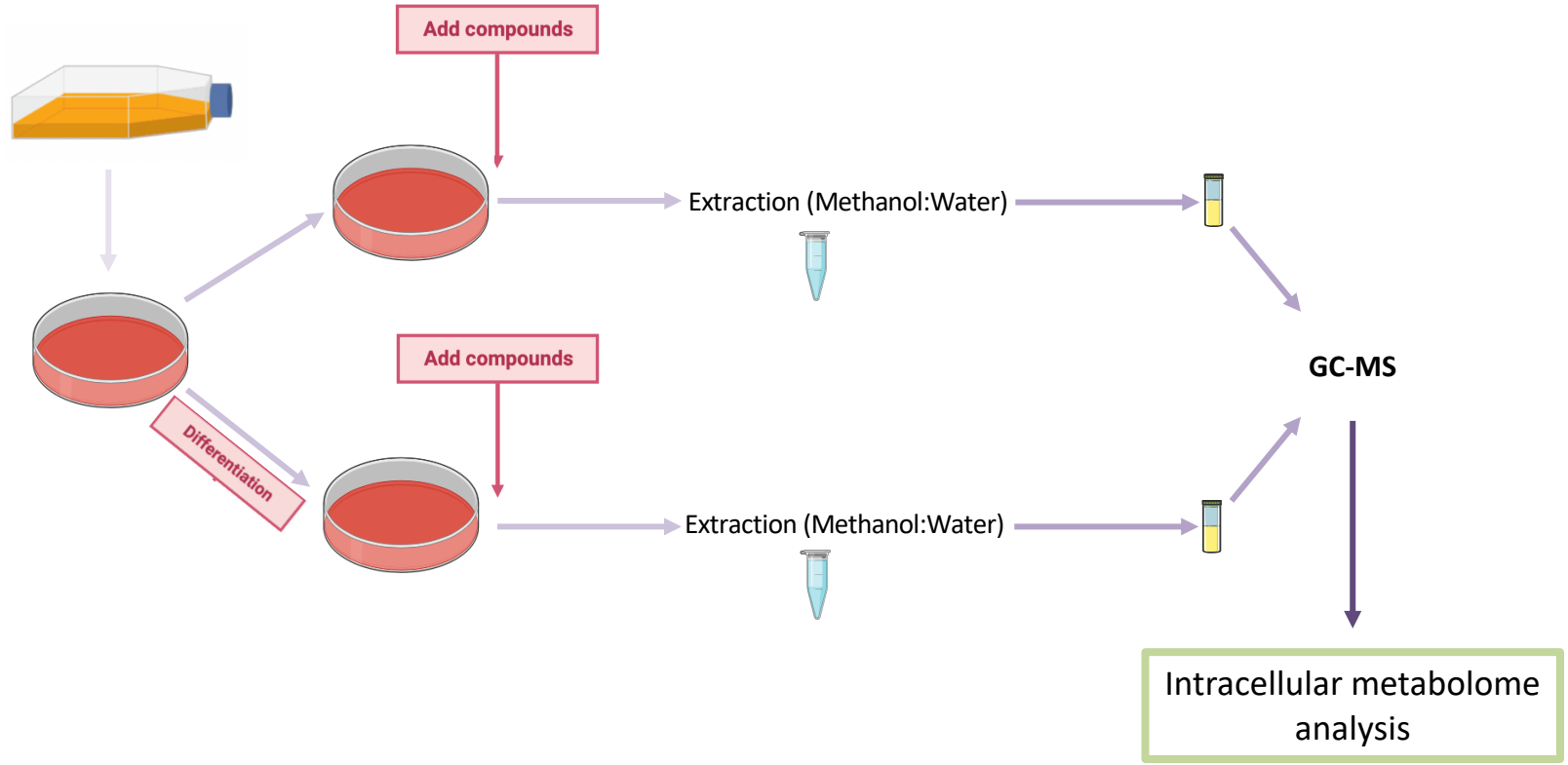
- Profiling of the metabolome of AC16 cells, either proliferative and differentiated, incubated with cyclophosphamide, 4-hydroxycylophosphamide and acrolein

- Analysis of intracellular metabolome

- PLS-DA (presented graphics): supervised regression of the separation of two classes

- If $Q^2>0.05$ and $p<0.05$, the model has a robust separation of the two groups

Experimental Design: Metabolic Profilling



Results: Cytotoxicity Assays

Cytotoxicity Assays

MTT Reduction Assay										
	Differentiated					Proliferative				
Cyclophosphamide (µM)	1 000	2 500	5 000	7 500	10 000	1 000	2 500	5 000	7 500	10 000
24h	-	-	++++	++++	++++	-	-	+++	++++	++++
48h	-	++++	++++	++++	++++	-	-	++++	++++	++++
72h						-	++	++++	++++	++++

NR Uptake Assay										
	Differentiated					Proliferative				
Cyclophosphamide (µM)	1 000	2 500	5 000	7 500	10 000	1 000	2 500	5 000	7 500	10 000
24h	-	-	-	++	++++	-	-	++++	++++	++++
48h	-	-	-	++++	++++	-	-	-	++++	++++
72h						-	-	-	++++	++++

Results expressed vs control (PBS -/- incubated)

Cytotoxicity Assays

MTT Reduction Assay										
	Differentiated					Proliferative				
4-Hydroxycyclophosphamide (µM)	DMSO 0.05%	1	5	15	25	DMSO 0.05%	1	5	15	25
24h	-	-	++++	++++	++++	-	-	-	++++	++++
48h	-	-	++++	++++	++++	-	-	++++	++++	++++
72h						-	++	++++	++++	++++

NR Uptake Assay										
	Differentiated					Proliferative				
4-Hydroxycyclophosphamide (µM)	DMSO 0.05%	1	5	15	25	DMSO 0.05%	1	5	15	25
24h	-	-	-	++++	++++	-	-	-	++++	++++
48h	-	-	++++	++++	++++	-	-	+++	++++	++++
72h						-	-	++++	++++	++++

Results expressed vs control (PBS -/- incubated)

Cytotoxicity Assays

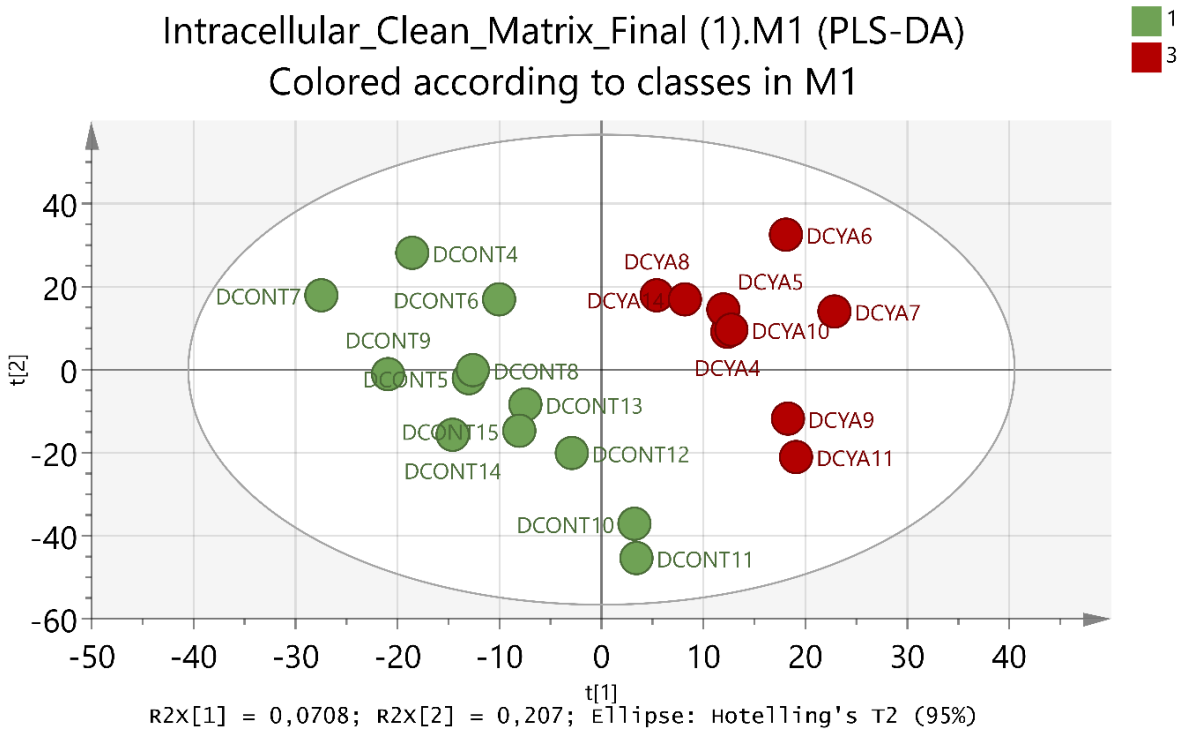
MTT Reduction Assay										
	Differentiated					Proliferative				
Acrolein (µM)	15	25	35	50	100	15	25	35	50	100
24h	++++	++++	++++	++++	++++	-	+++	-	++++	++++
48h	++++	++++	++++	++++	++++	-	++++	+	++++	++++
72h						-	+++	++++	++++	++++

NR Uptake Assay										
	Differentiated					Proliferative				
Acrolein (µM)	15	25	35	50	100	15	25	35	50	100
24h	-	++++	++++	++++	++++	-	++++	++++	++++	++++
48h	-	++++	++++	++++	++++	-	-	-	++++	++++
72h						-	-	-	++++	++++

Results expressed vs control (PBS -/- incubated)

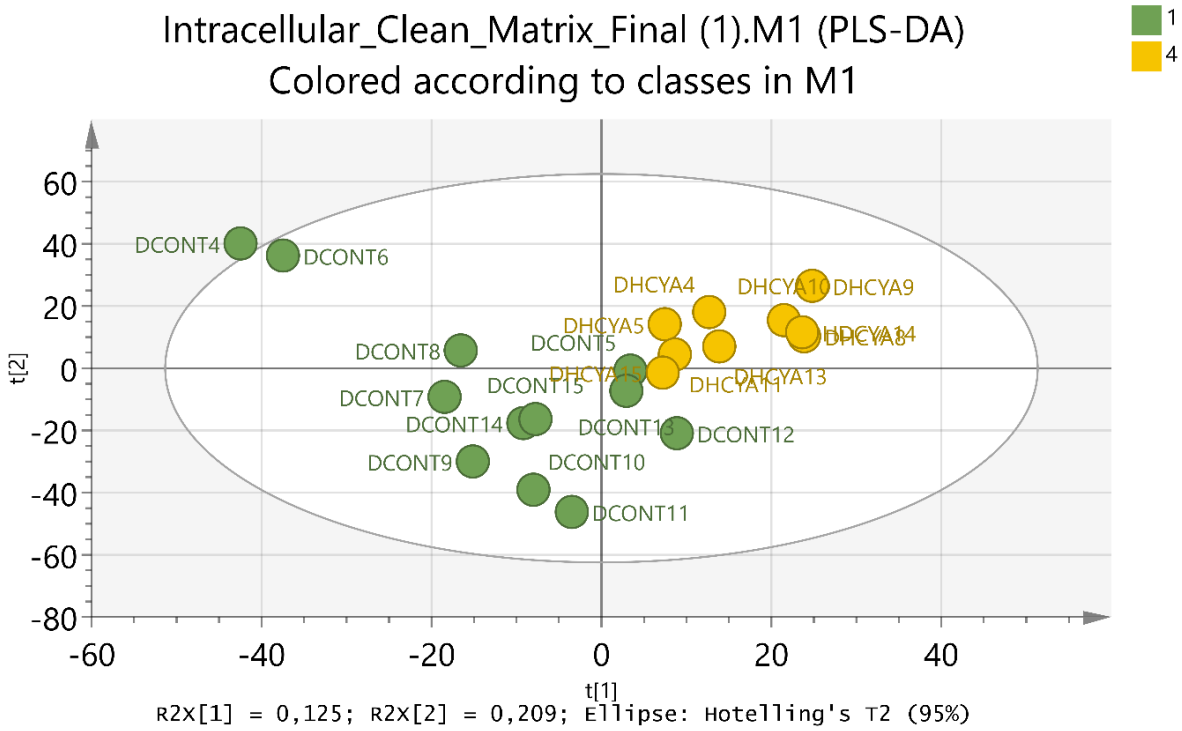
Results: Metabolic Profilling

Differentiated AC16 cells
Control vs Cyclophosphamide



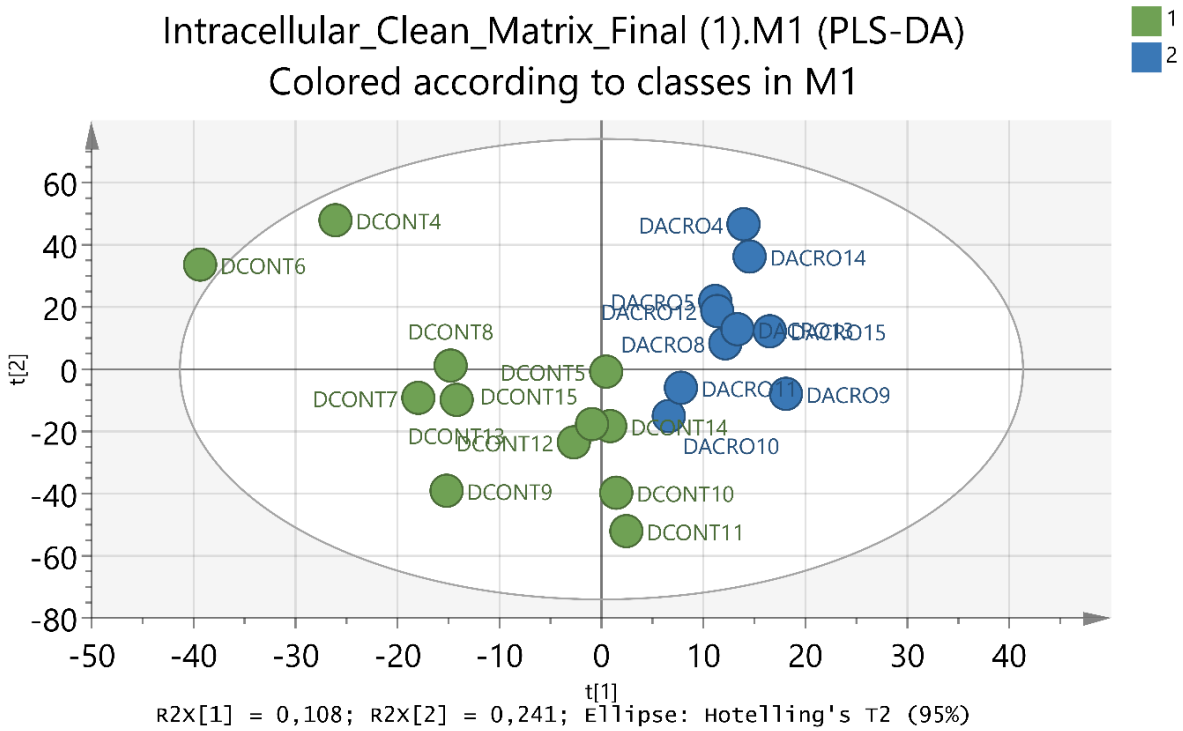
Q2<0.500; p>0.05

Differentiated AC16 cells
Control vs 4-Hydroxycyclophosphamide



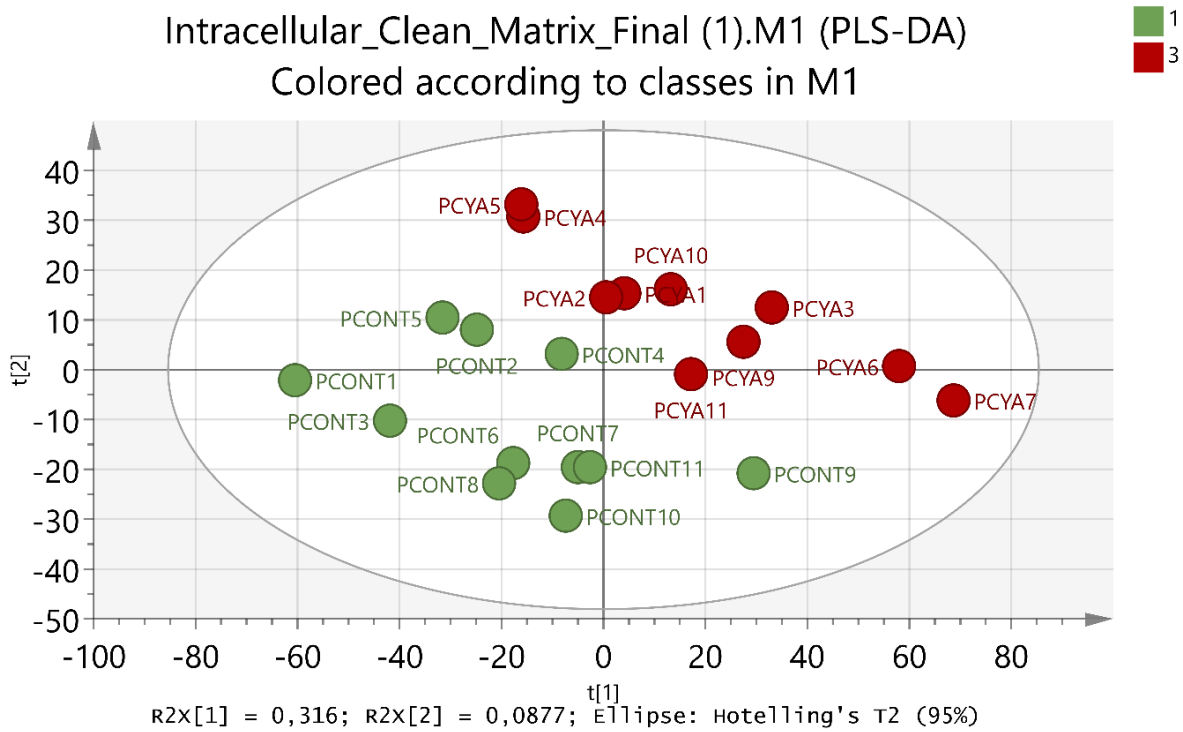
Q2<0.500; p>0.05

Differentiated AC16 cells
Control vs Acrolein



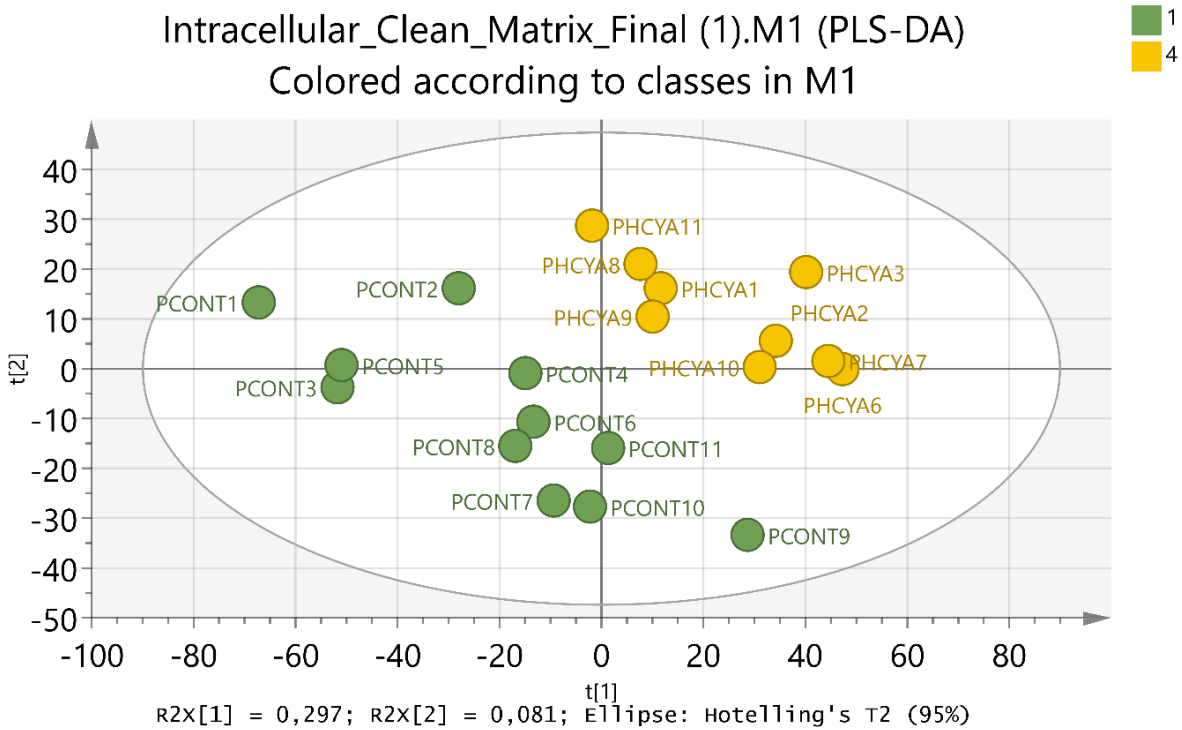
Q2<0.500; p>0.05

Proliferative AC16 cells
Control vs Cyclophosphamide



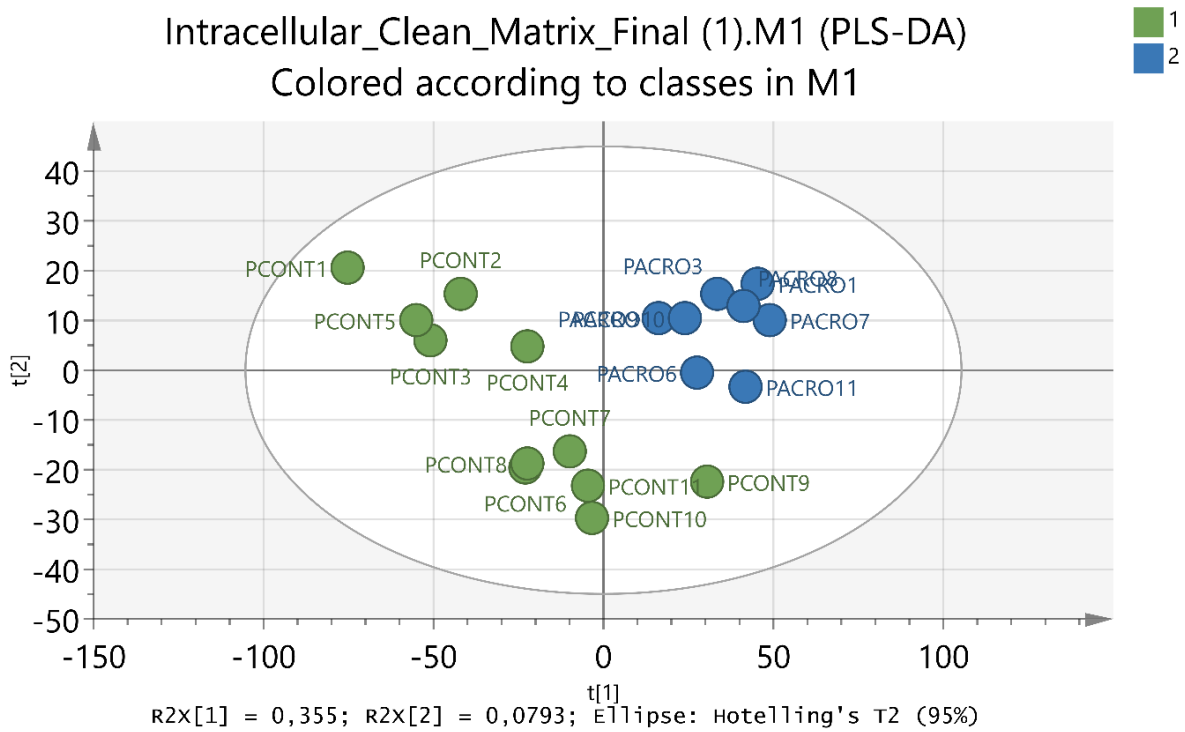
Q2<0.500; p>0.05

Proliferative AC16 cells
Control vs 4-Hydroxycyclophosphamide



Q2>0.500; p<0.05

Proliferative AC16 cells
Control vs Acrolein



Q2>0.500; p<0.05

Discussion

	Plasma concentration levels	Lowest Cytotoxic Concentration - Differentiated	Lowest Cytotoxic Concentration - Proliferative
Cyclophosphamide	Up to 250 µM[#]	2 500 µM	2 500 µM
4-Hydroxycyclophosphamide	1 to 10 µM[#]	1 µM	5 µM
Acrolein	1 to 10 µM[#]	25 µM	15 µM

[#]De Jonge M. E., Huitema A. D. R., Rodenhuis S., Beijnen J. H., Clinical Pharmacokinetics of Cyclophosphamide, Clinical Pharmacokinetics, 44(11), 2005, 1135-1164

Discussion

	Differentiated Cells	
	Q^2	*p*
Control vs **Cyclophosphamide**	**<0.500**	**>0.05**
Control vs **4-Hydroxycyclophosphamide**	**<0.500**	**>0.05**
Control vs **Acrolein**	**<0.500**	**>0.05**

Discussion

Proliferative cells		
	Q^2	*p*
Control vs **Cyclophosphamide**	**<0.500**	**>0.05**
Control vs **4-Hydroxycyclophosphamide**	**>0.500**	**<0.05**
Control vs **Acrolein**	**>0.500**	**<0.05**

Conclusions

- Cyclophosphamide is cytotoxic at relatively high concentrations
- 4-Hydrocyclophosphamide and acrolein are cytotoxic at clinically relevant concentrations
- In AC16 proliferative cells, the metabolites cause a marked distinct metabolic pattern while cyclophosphamide does not
- Robust separation of the intracellular results in control proliferative AC16 cells vs metabolites but not in the differentiated cells

Acknowledgements

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