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Messa a punto di un Nuovo Approccio Metodologico (NAM) per gli inibitori dell'enzima 4-idrossifenilpiruvato diossigenasi (HPPDi)

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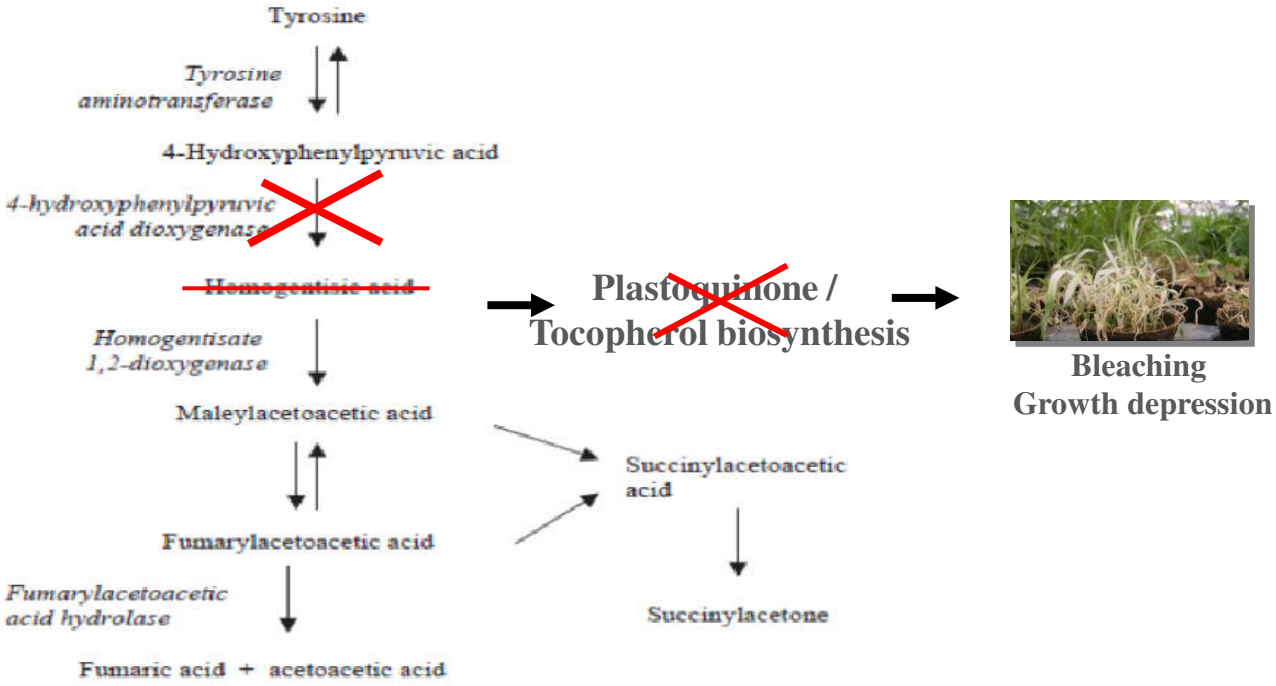
Developing a NAM for HPPD inhibitors

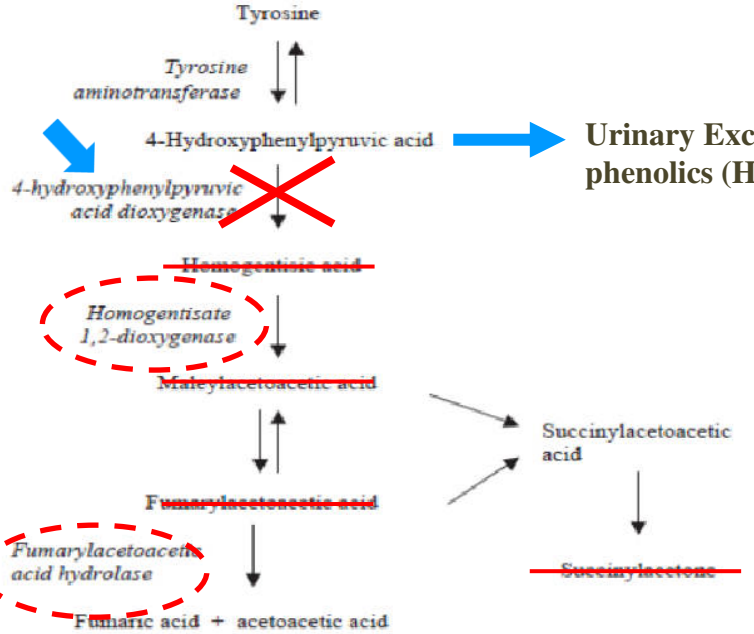
- The mode of action is known
- The toxic endpoints are identified
- The *in vivo* database supporting the MoA is reliable and robust
- The relevance to humans is known

HPPDi: Mode of action in plants



HPPDi: Therapeutic use for genetic disorder humans

Nitisinone (NTBC)



Alkaptonuria
NTBC prevents homogentisic acid accumulation

Type 1 tyrosinaemia
NTBC prevents formation of succinyl acetate

= enzymatic deficiency

HPPD-Inhibitors: Toxic effects

- Increased systemic concentrations of tyrosine (tyrosinaemia)
- The increase is dose-related.
- The same dose of HPPDi induced different levels of tyrosinaemia in laboratory animals:
  - The rat is the most sensitive species.
- Ocular lesions (corneal opacity) observed in rats and with less frequency in dogs when tyrosinaemia is above 800-1000 nmol/mL.

Species	Rat	Dog	Rabbit	Mouse
Ocular lesions toxicity studies (10 HPPDi)	Consistently seen at very low doses already inducing high tyrosinaemia	Sporadically observed	Not observed	Not observed
Maximum level of tyrosine (nmol/mL) when HPPD fully inhibited	2673 Lock *et al* 1996	1814 Roberts 2006 Lock *et al* 2006	1480 Lock *et al* 2006	1154 Dearden 2006 Lock *et al* 2000

Species sensitivity – TAT hypothesis

- When HPPD is inhibited, Tyrosine Aminotransferase (TAT), the first enzyme of the carbolic pathway, becomes the relevant enzyme that regulates tyrosine catabolism.
- TAT activity is different across species
- Species with higher TAT activity might better regulate tyrosinaemia levels and more promptly excrete 4-HydroxyPhenylPyruvic Acid (HPPA) via the urine

New data from *in vitro* studies using more accurate methodology have been developed and validated in 5 species by CropLife Europe (CLE)

TAT basal activity in cytosol

Liver TAT specific activity in three pools of liver cytosols (n=6 subjects) from Human, C57Bl6 mouse, New Zealand rabbit, Beagle dog, Wistar rat

0.5mg/ml cytosolic protein conc	Human	Mouse	Rabbit	Dog	Rat
Pool 1	8.25±0.35	3.29±0.37	3.95±0.56	0.423±0.046	1.36±0.12
Pool 2	7.68±0.36	4.33±0.16	/	0.715±0.047	1.68±0.18
Pool 3	6.34±0.44	4.46±0.34	/	0.612±0.089	1.26±0.17

(Botham *J. et al.* 2023, Archives of Toxicology, Species differences and human relevance of the toxicity of 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors and a new approach method in vitro for investigation | SpringerLink)

TAT activity in hepatocytes

Basal and after 4- hour incubation with and without NTBC

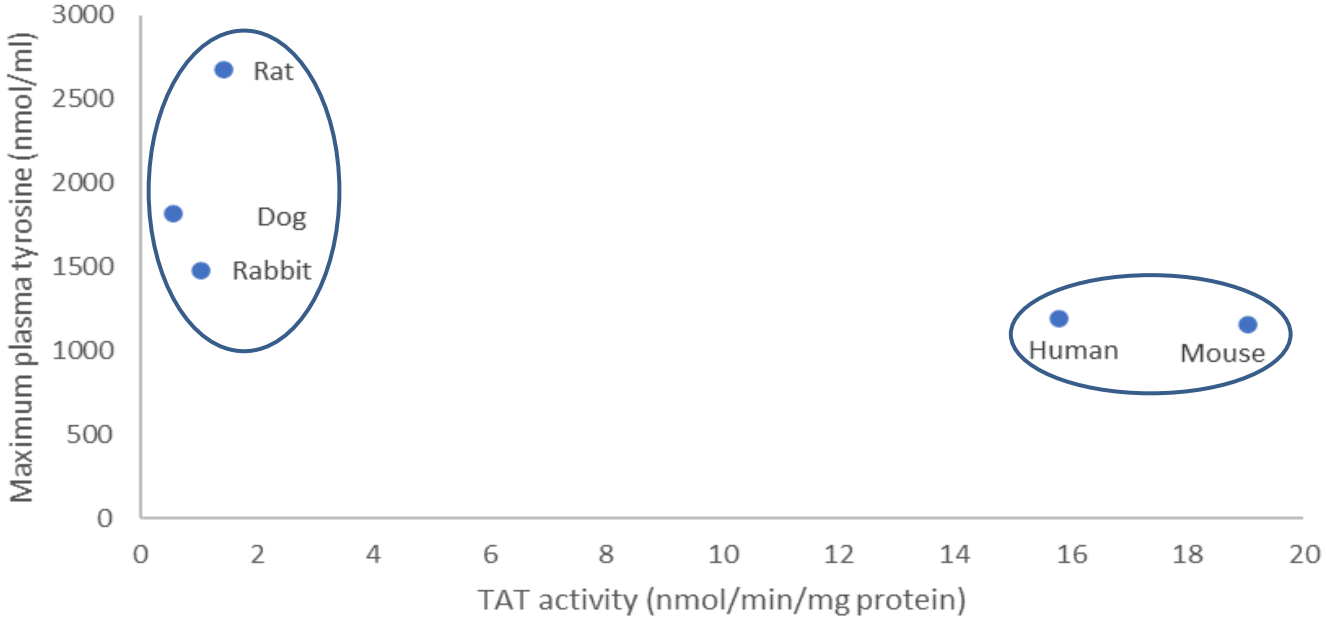
Species/strain**	Treatment	HPPA (nmol/min/mg protein) ±SD	HPPA (nmol/min/mg protein) minus vehicle control
Human	Vehicle	1.9 ±0.56	**16**
	NTBC (100uM)	18 ±6.9*	
CD-1 mouse	Vehicle	4.4 ±1.33	**19**
	NTBC (100uM)	23 ±5.5*	
New Zealand rabbit	Vehicle	0.34 ±0.115	**1.0**
	NTBC (100uM)	1.4 ±0.22*	
Beagle dog	Vehicle	0.70 ±0.146	**0.56**
	NTBC (100uM)	1.3 ±0.29	
Sprague-Dawley rat	Vehicle	1.6 ±0.52	**1.4**
	NTBC (100uM)	3.0 ±1.34*	

*Significantly different from the appropriate vehicle control (0.1%v/v DMSO) as a result of one-way analysis of variance. SD=Standard deviation (study repeated 3 times independently)
** hepatocytes taken from males with the exception of humans (males and females)

(Botham *J. et al.* 2023)

Comparison *in vitro* TAT activity with *in vivo* Tyrosinaemia

Maximal tyrosinaemia (NTBC) & TAT Rate in Primary Hepatocytes across Species



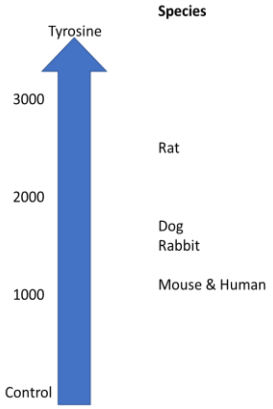
(Botham *J. et al.* 2023)

TAT hypothesis Conclusions

- Rats, dogs and rabbits have significantly lower TAT activity than mice and humans
- The TAT activity is inversely related to the maximal tyrosinaemia experienced in all species tested when HPPD is inhibited
- TAT activity measured in hepatocytes better correlate to in vivo tyrosinaemia than TAT measured in cytosols

Maximal tyrosinaemia with complete HPPD inhibition:

Rat > Dog & Rabbit > Mouse & Human



(Botham *J. et al.* 2023)

Why this work represents a NAM for HPPDi?

- TAT activity is the critical endpoint for tyrosine-related toxicity induced by HPPDi in mammals.
- A reliable and reproducible method has been developed to measure and TAT *in vitro* using various HPPDi inhibitors.
- In *vitro* measurements show a clear correlation between high TAT activity and low *in vivo* tyrosinaemia-related toxicity.

In vitro measurements of hepatic TAT can replace _in vivo_ measurements of tyrosinaemia

Regulatory considerations

- The rat is the most sensitive species to tyrosinaemia related effects.
- There are more than 200 regulatory toxicity and mechanistic studies supportive of the extreme sensitivity of the rat to HPPD inhibitors, which is attributed to the low TAT activity for this species.
- This approach in measuring TAT activity supports that species such as the mouse to be more relevant for predicting effects in humans.

The rat should not be considered an appropriate model for assessing tyrosine-related effects of HPPD inhibitors in humans*

* (US EPA memorandum , 2020 Regulations.gov)

Contributors

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