GENERATION OF PARKIN, DJ-1, AND PINK1 EXPRESSING pGIPz shRNAmir PLASMIDS IN HUMAN SH-SY5Y CELLS

Name	Gene locus	Gene	Inheritance
PARK1/4	4q21	α-Synuclein	Autosomal dominant/synaptic vesicle
PARK2	6q	Parkin	Autosomal recessive/synaptic-everywhere
PARK3	2p	?	Autosomal dominant-
PARK5	4p	UCH-L1	Dominant (rare-one family)
PARK6	1p	PINK1	Autosomal recessive/mitochondria
PARK7	1p	DJ-1	Autosomal recessive/mito-
PARK8	12p	LRRK2	Autosomal dominant
PARK9	1p	ATP13A2/ lysosomal ATPase	Autosomal recessive/lysosomal
PARK10	1p	?	?
PARK11	2q	?	?
PARK12	Xq21-q25	?	?
PARK13	2p12	HTRA2, serine protease	Identified previously by yeast-two hybrid interaction with presenilin 1-an AD gene

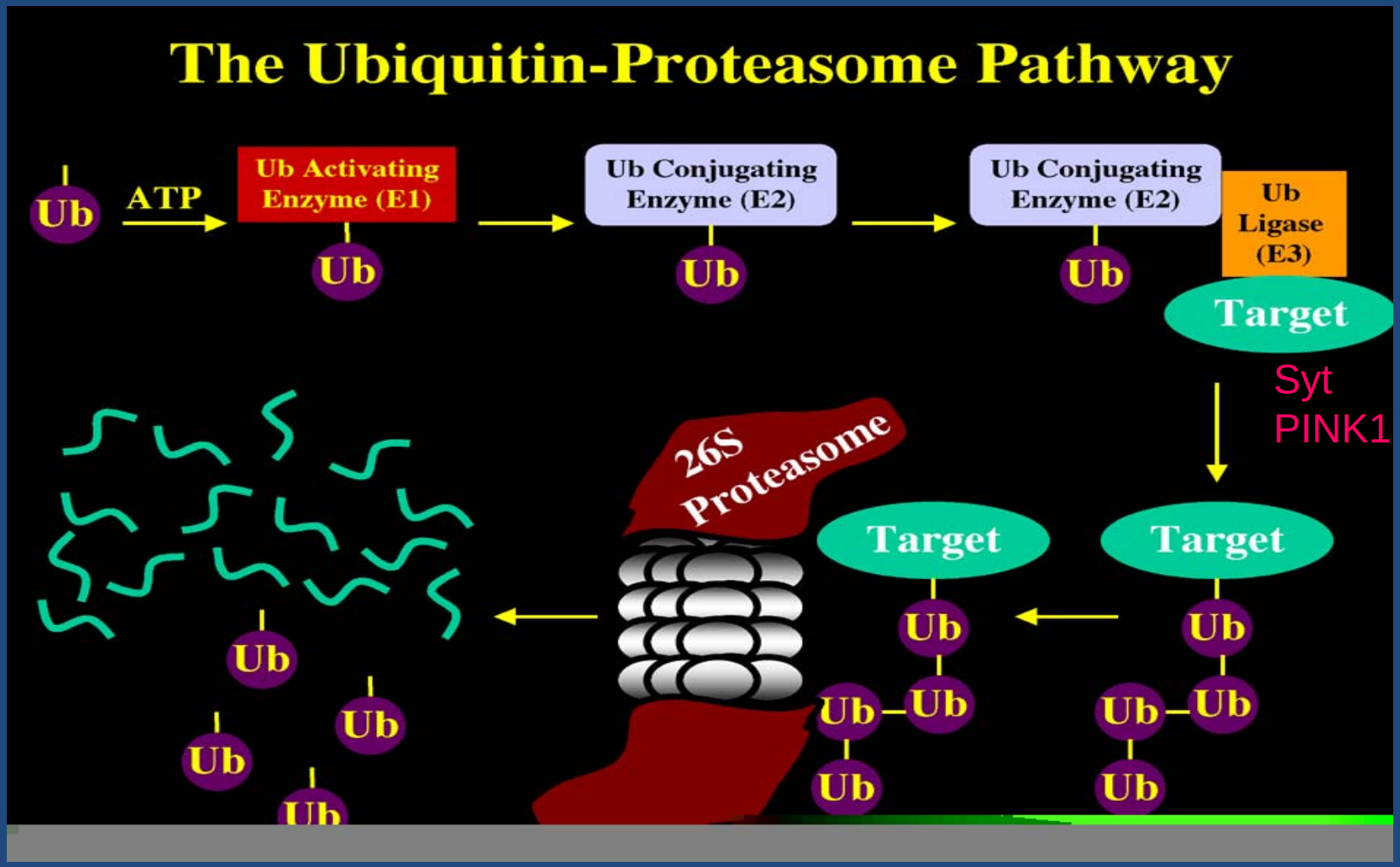
PARK2 autosomal recessive juvenile parkinsonism (AR-JP) and parkin

- E3 ubiquitin ligase and localized predominantly in the cytosol, ER, and some associates with the cytoplasmic surface of the outer mitochondrial membrane..
- In addition to its proteasome-dependent function (K48 polyubiquitination), parkin ubiquitin ligase activity can have proteasome-independent activity (monoubiquitination and K68 linked polyubiquitination) (Matsuda et al, JBC 2006, Hampe (Brice)—Hum Mol Genet 2006, Doss-Pepe et al, JBC, 2005)/
- The monubiquitination and K68-linked polyubiquitination modification can influence cellular processes such as signal transduction, transcriptional regulation, and protein and membrane trafficking without promoting substrate degradation (Mukhopadhyay and Riezman, Science 2007).

Animal Models:

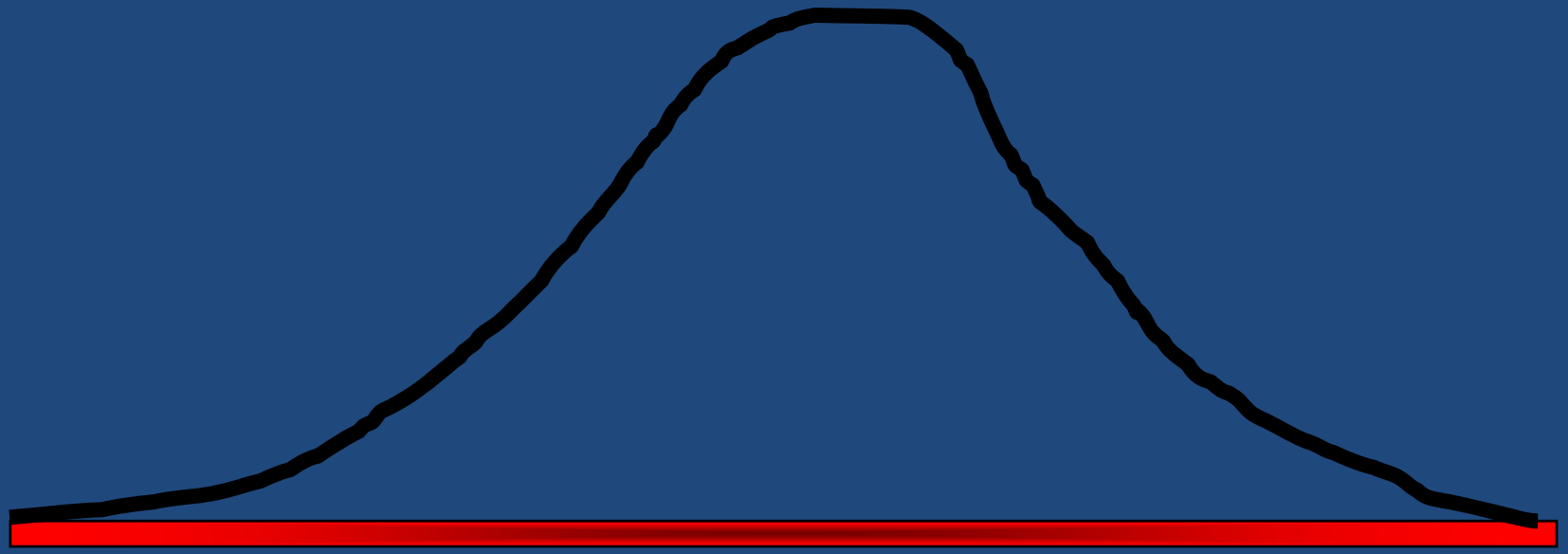
- Parkin KO (exon-3 KO) moderate defect in dopamine transmission, no dopaminergic neuron degeneration.
- Some interactors increased in Parkin KO mice.
- Parkin loss-of-function mutants in Drosophila showed dramatic mitochondrial defects swollen mitochondria having severely fragmented cristae-in high energy tissues-such as flight muscle, flight muscles ultimately die showing features of apoptosis (several labs-including Ming Guo-UCLA).
- Although parkin null mice did not show mitochondrial defects, but showed reduced mitochondrial respiratory activity.

Parkin is a ubiquitin-ligating (E3) enzyme



Parkin-PINK1-HtrA2/Omi
Results in:
Chaperone/protease function
in the mitochondria.

Etiology of PD

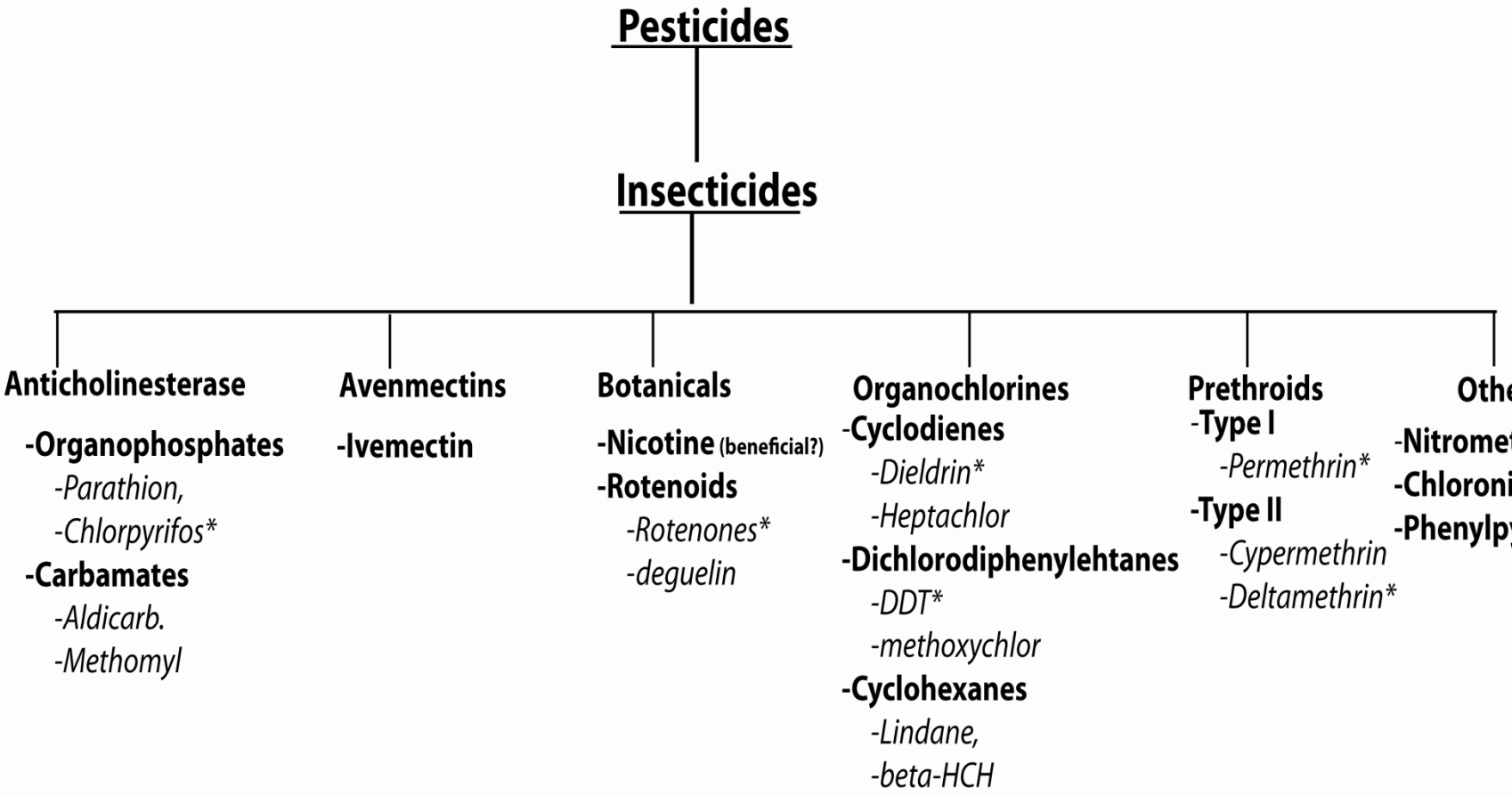


Genetic
α-synuclein
Parkin
PINK1
DJ-1
LRRK2

Modifiers (?)
DNA polymorphism
Nurr 1, BDNF
(frequently reported, but not many are unequivalently associated)
Glutathion Transferase

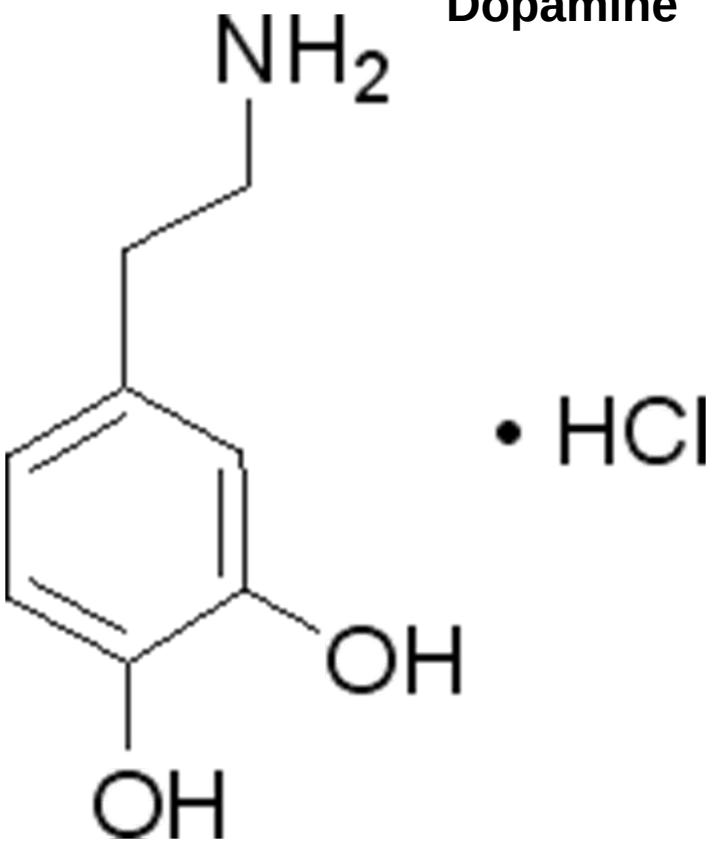
Environmental
MPTP
rotenon and
paraquat
pesticide/herbicide

Sporadic PD

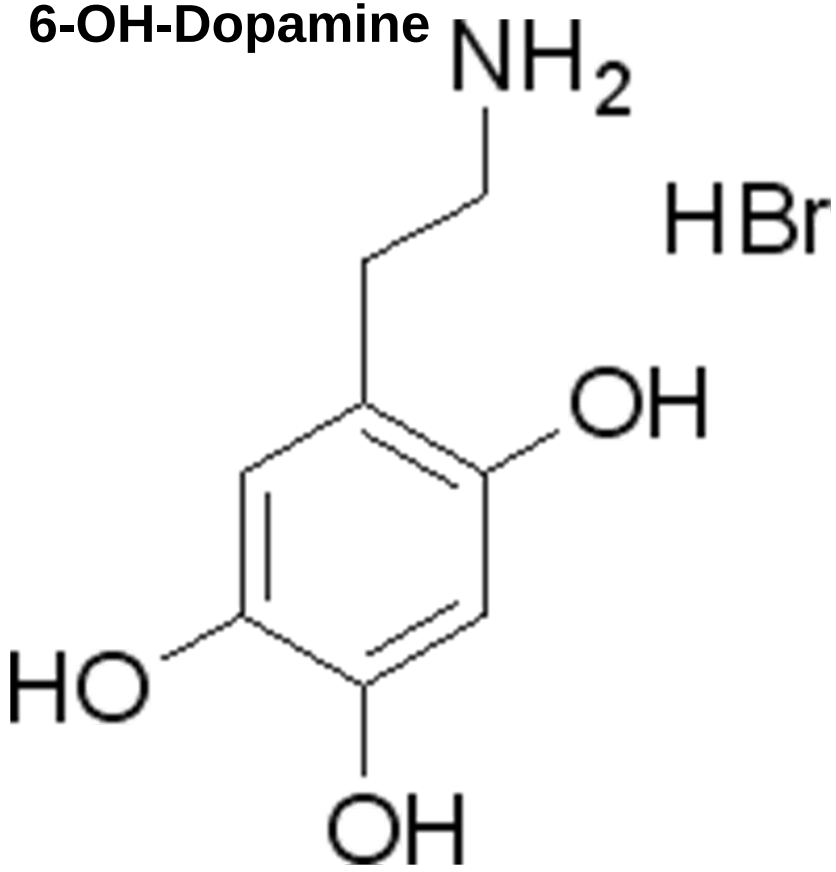

Pesticides
Insecticides
Anticholinesterase
-Organophosphates
-Parathion,
-Chlorpyrifos*
-Carbamates
-Aldicarb.
-Methomyl
Avenmectins
-Ivemectin
Botanicals
-Nicotine (beneficial?)
-Rotenoids
-Rotenones*
-deguelin
Organochlorines
-Cyclodienes
-Dieldrin*
-Heptachlor
-Dichlorodiphenylehtanes
-DDT*
-methoxychlor
-Cyclohexanes
-Lindane,
-beta-HCH
Prethroids
-Type I
-Permethrin*
-Type II
-Cypermethrin
-Deltamethrin*
Others
-Nitromethylne
-Chloronicotinyl
-Phenylpyrazole

Dopamine hydrochloride

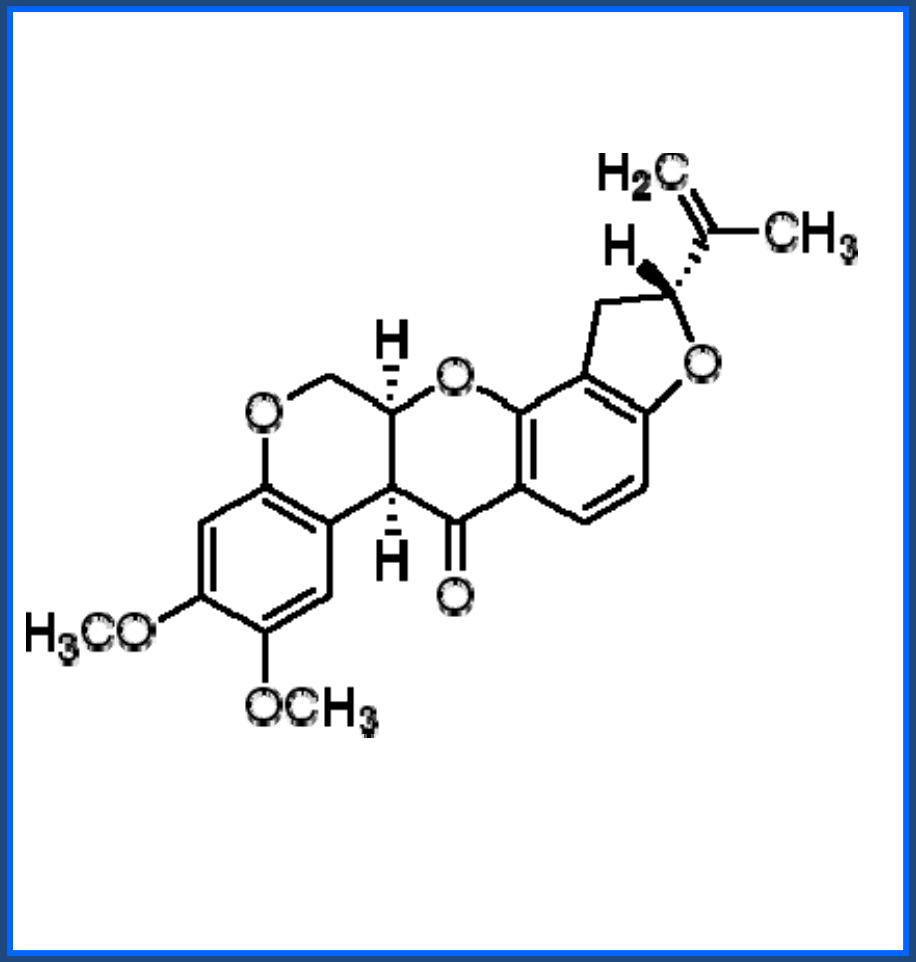
Dopamine



6-OH-Dopamine

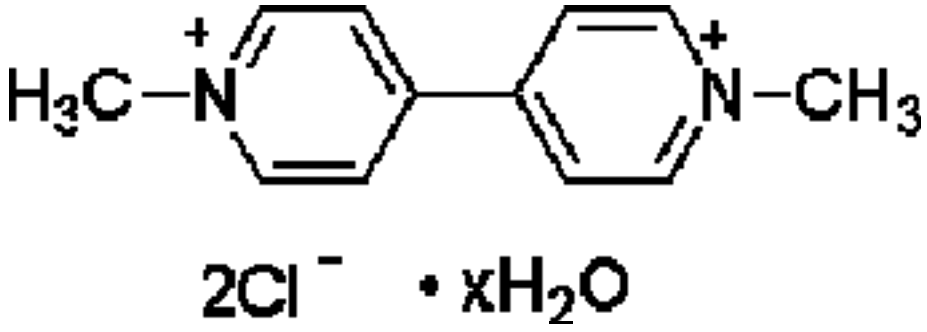


Rotenone

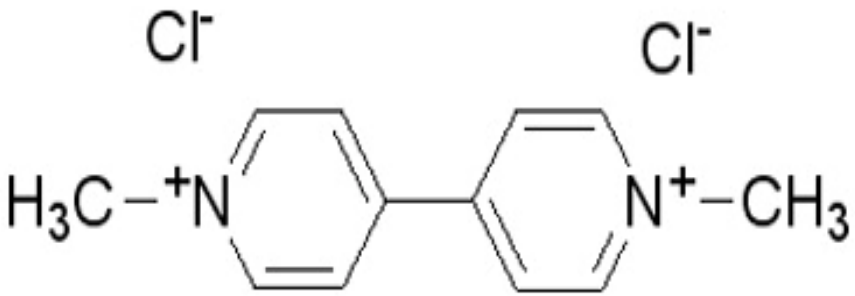


1. is a plant derived pesticide, induces cell destruction by inhibiting mitochondrial respiration at the level of complex I.
2. is a useful reagent for mimicking the biochemical lesions of PD, both in vivo and in vitro (Betarbet et al., 2000; Alam and Schmidt, 2002).

Methyl viologen dichloride



Paraquat chloride

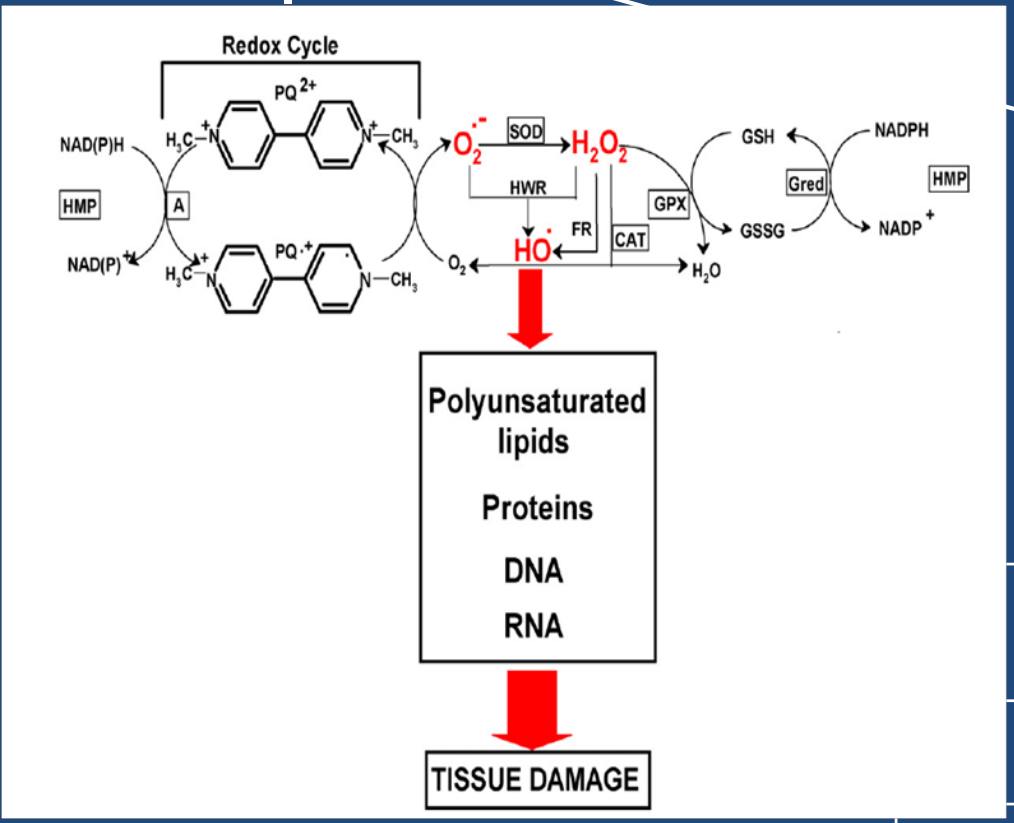


Methyl viologen/ paraquat chloride

1. Structure similar to a known DA neurotoxin: MPP+ (1-methyl-4-phenyl-pyridine).
2. A widely used herbicide.
3. Occupational Exposure to pesticide/herbicide can be a risk factor for PD (Hertzman *et al.,* 1990; Liou *et al.,* 1997)
4. Caused apoptosis and autophage in SH-SY5Y cells.
5. Caused mitochondrial defects, reduced DA synthesis, inhibit complex I (DA neurons highly sensitive to complex I inhibition).
6. Induces Alpha Synuclein up regulation and aggregation,

Paraquat toxicity mechanism

NADH-Mitochondrial complex I
NADPH cytochrome P450 reductase



Paraquat_PQ

NADPH/NADH

Monocation free radical, PQ*

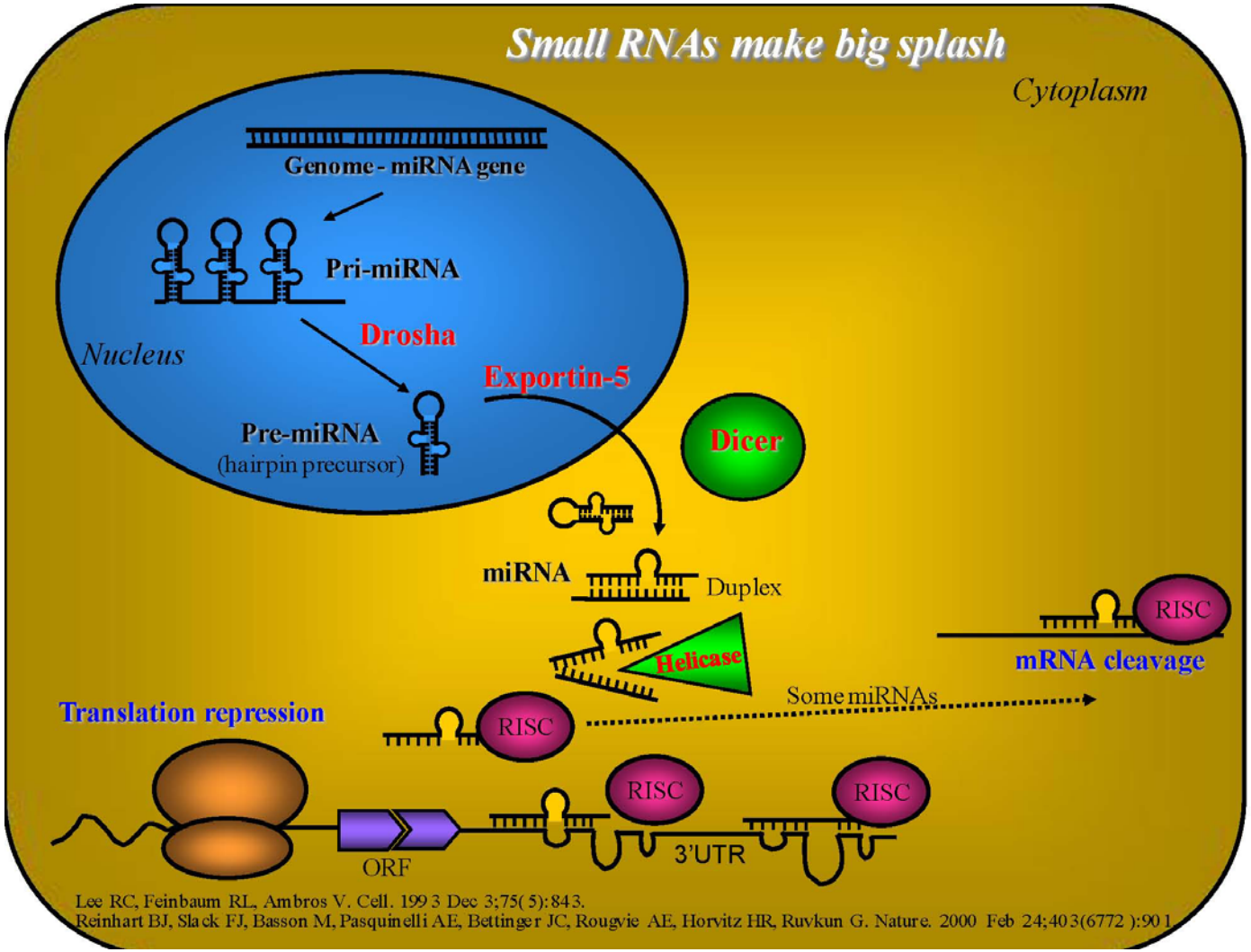
O2

Superoxide radical, e.g. O_2*-

Cascade/rapid

Reactive oxygen species, ROS e.g. H_2O_2 And Hydroxyl radicals (HO*-)

Cell death_membrane damage by lipid peroxidation



Lee RC, Feinbaum RL, Ambros V. Cell. 1993 Dec 3;75(5):843.
Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. Nature. 2000 Feb 24;403(6772):901

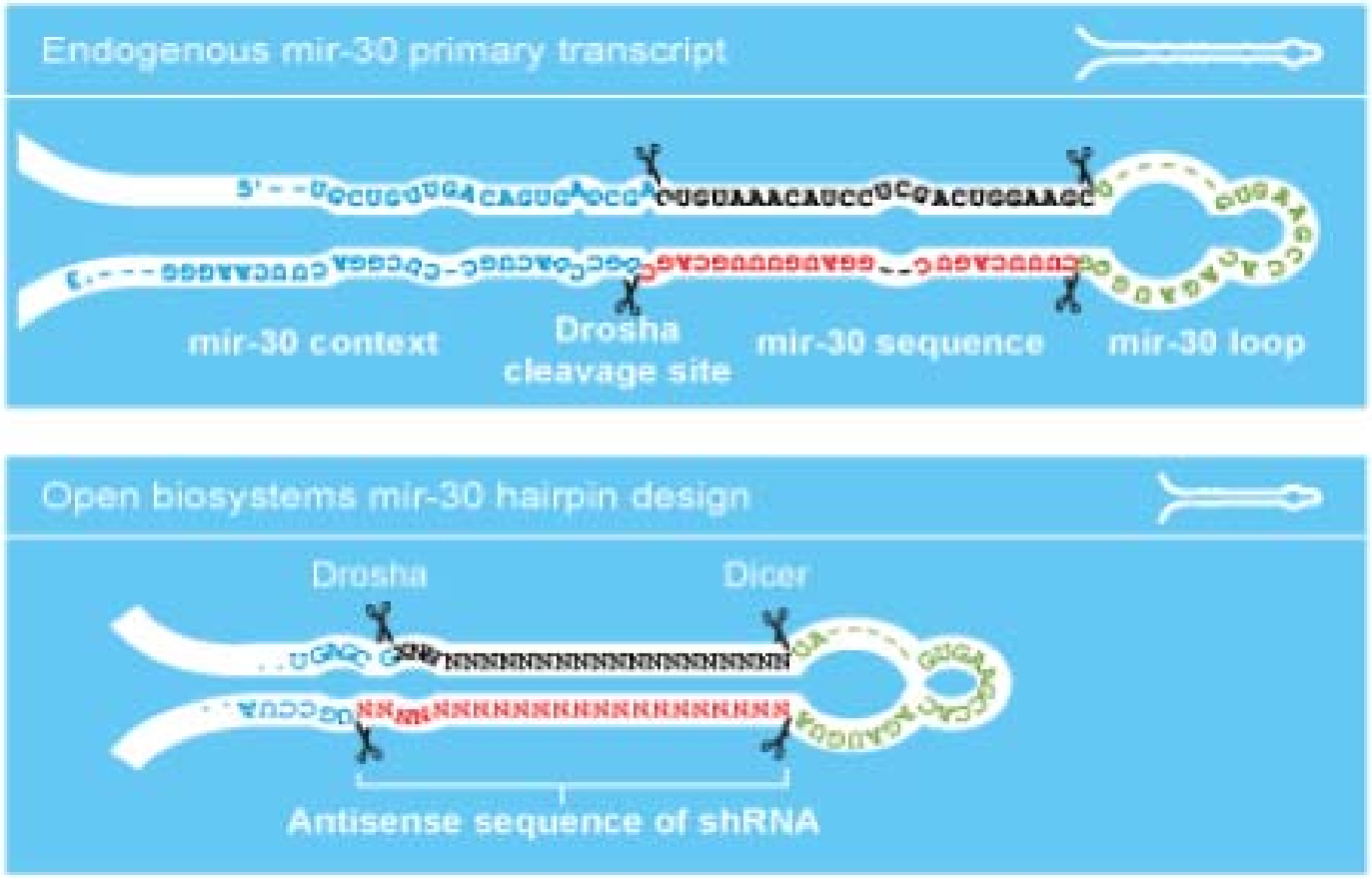
Conservation of small-RNA silencing pathways in eukaryotes

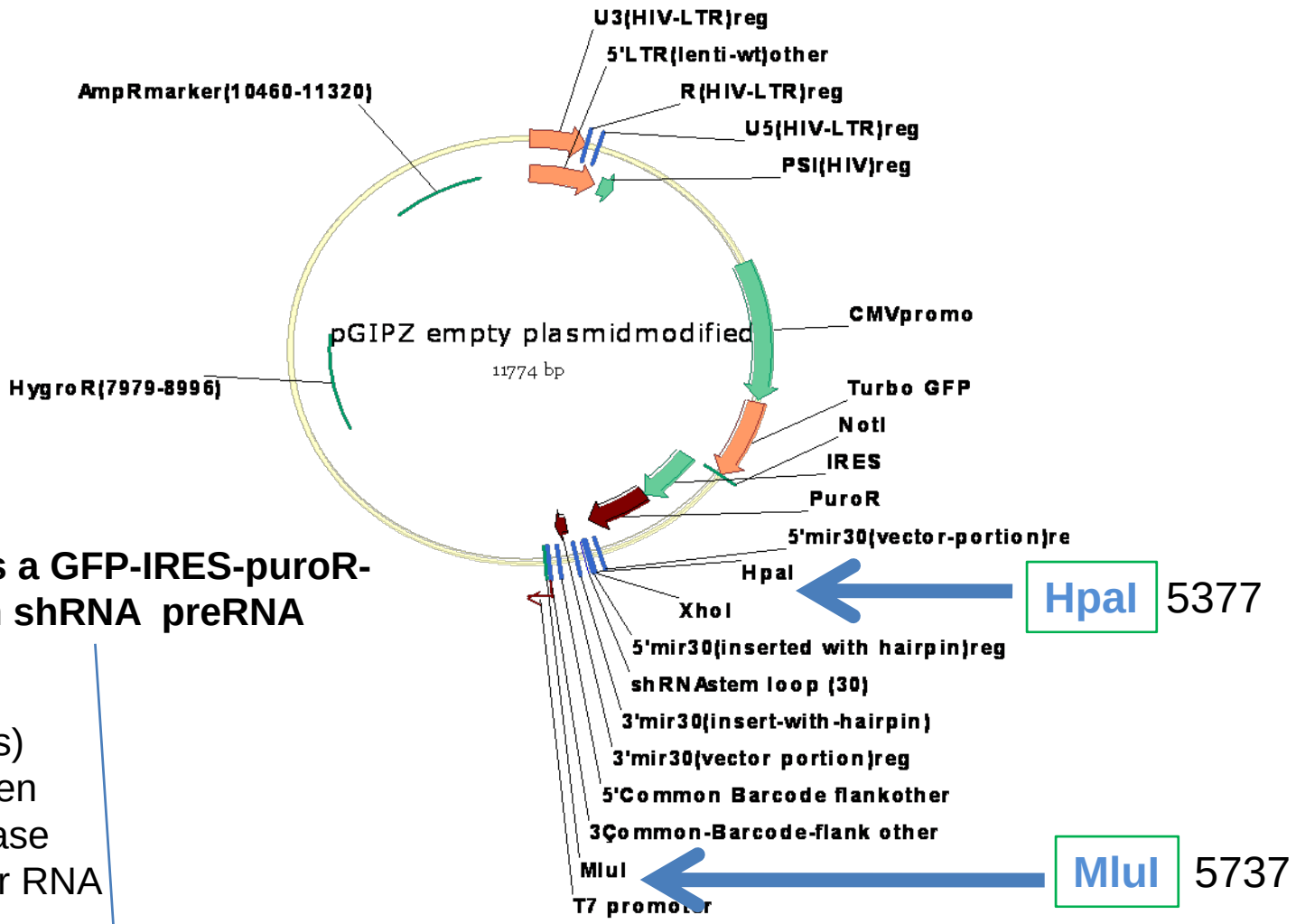
Small RNA	Size (bases)	Mechanism of action	Eukaryotes conserved in
siRNA	~21-25	PGTS (RNA degradation or translaltional arrest) CDGS	Plants, animals, fungi, ciliates
miRNA	~21-25	PTGS (RNA degradation or translational arrest). CDGS (to a lesser extent)	Plants, animals
piRNA	~24-31	PTGS (RNA degradation) CDGS (to a lesser extent)	Animals

1) PGTS-posttranslational genes silencing
2) CDGS-chormatin-dependent gene silencing pathways = assembly of small RNA complexes on nascent transcripts and includes both transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS) events.
3) CTGS_ chromatin-dependent processing and degradation of the nascent transcript.

Danesh Moazed (2009), Nature 452: 413-4

How the shRNAmir is generated by Drosha and Dicer





Generates a GFP-IRES-puroR-Parkin shRNA preRNA

Drosha (class 2 RNase III enzymes) to pri-mir RNA, then Dicer (endonuclease of RNase III) to mir RNA

GFP-IRES-puroR mRNA + parkin shRNAmir

PROMEGA NORADIOACTIVE CELL PROLIFERATION ASSAYS

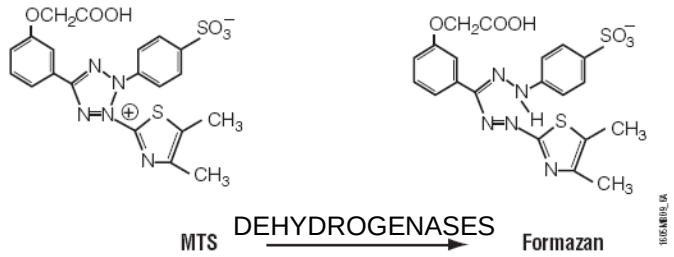


Figure 1. Structures of MTS tetrazolium salt and its formazan product.

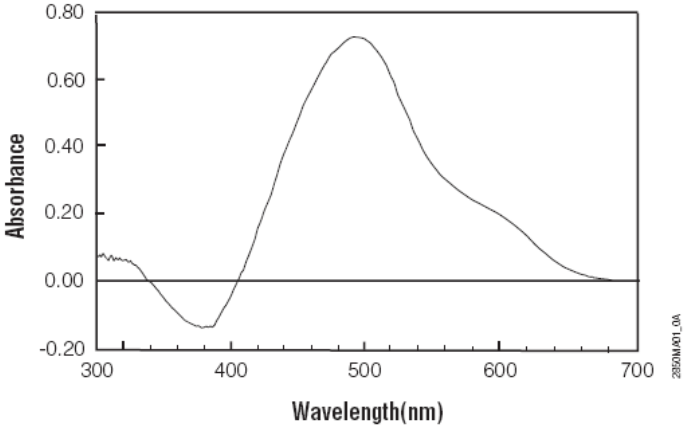


Figure 4. Absorbance spectrum of MTS/formazan after bioreduction by K562 cells. The K562 cells were cultured in RPMI 1640 supplemented with 10% FBS. The blank used to generate this absorbance spectrum was culture medium containing MTS that was not bioreduced by cells. The negative absorbance values (382nm) correspond to the disappearance of MTS as it is converted into formazan.

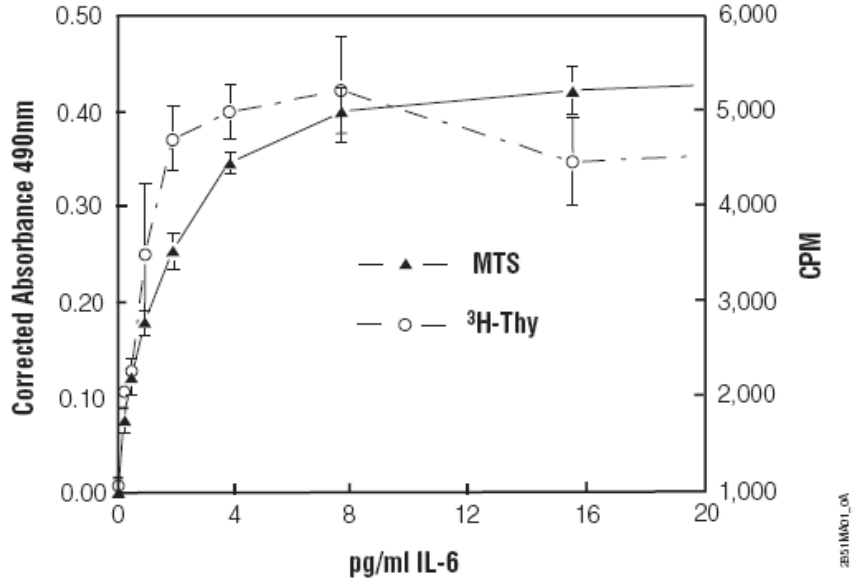
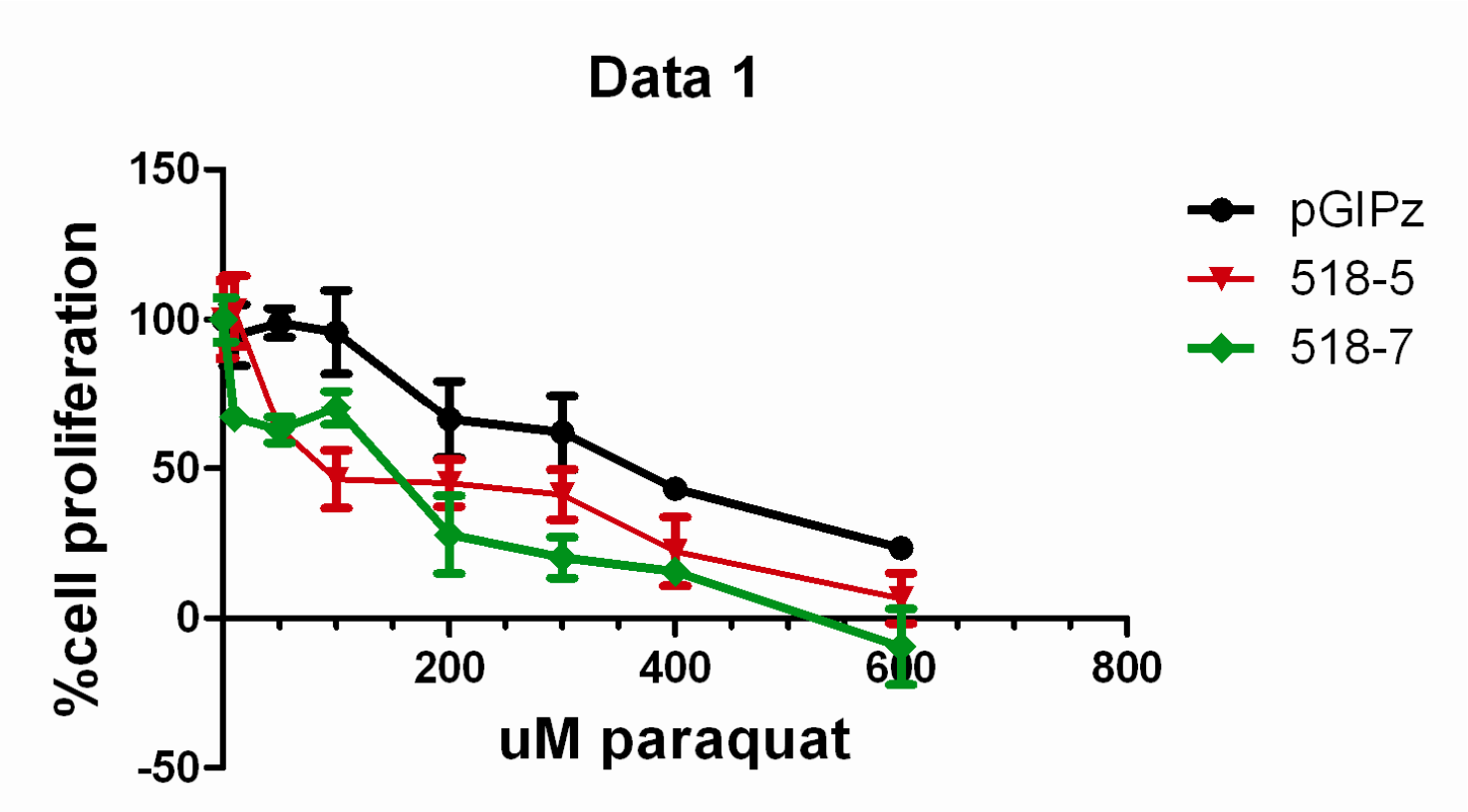


Figure 3. Proliferation of B9 cells in response to various concentrations of IL-6 measured using the CellTiter 96® AQ_{ueous} Assay and [^{3}H]-thymidine incorporation assays.

SOLUBLE, ABORBANCE AT 450-490 nm.

Abs is DIRECTLY PROPORTIONAL TO THE NUMBER OF LIVING CELLS.


Data 1
%cell proliferation
150
100
50
0
-50
200
400
600
800
uM paraquat
pGIPz
518-5
518-7

CELL LINE

- PARKIN shRNAmir: started out 27 got 15 different cell lines for 3 different shRNAmirs (pGIPz)
- DJ-1 shRNAmir: started out 9 got 8 cell lines for one pGIPz shRNAmir, started 36 cell colonies, got 38 for 5 different pLKO shRNAs.
- PINK1 shRNAmir: started 18, got 15 different cell lines for pGIPZ shRNAmir
- Alpha synuclein: started at 9, got 9 for one pGIPZ
- ATP13A2: started 9, got 9 for one pGIPz
- sytI: started out 18, got 17 for 2 pGIPz
- sytII: started out 18, got 14 for 2 pGIPz
- **TOTAL TIMES: 4 MONTHS**