

# Novel Electrophoretic Tag-Based *in vitro* Assay to Quantify Dimerization of p66 and p51 Subunits of HIV-1 Reverse Transcriptase (RT)

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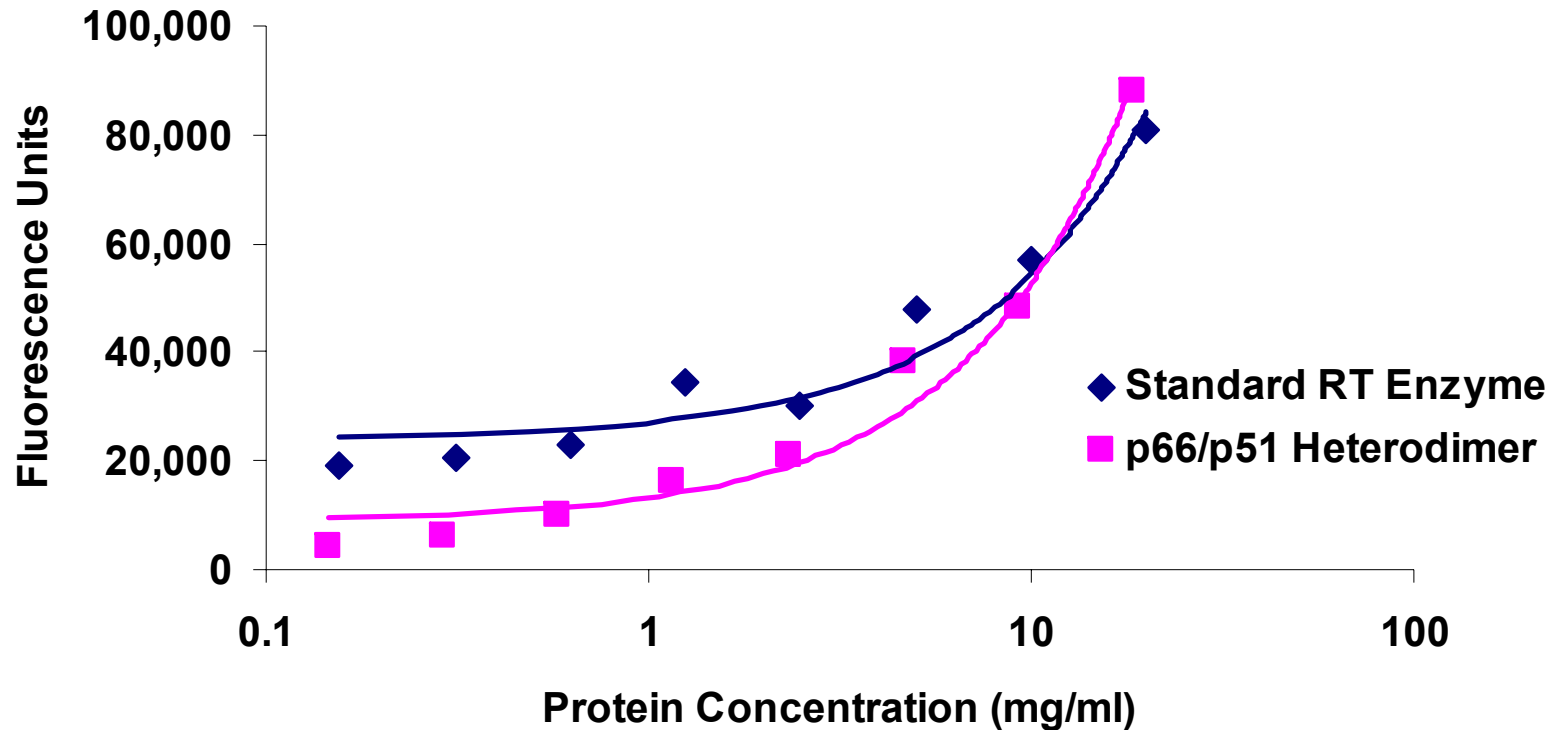
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# Background

- Dimerization is required to activate RT-polymerase function, which makes it an attractive therapeutic target.
- The mechanisms of connection domain mutations (such as N348I and T369I) are poorly understood; N348I and T369I cause reduced susceptibility to NNRTIs.
- A rapid, high-throughput, quantitative *in vitro* assay for RT dimerization should help elucidate the effect of connection domain mutants and allow the rapid screening of potential RT dimerization inhibitors.

# RT Activity of Dimers Formed *in vitro*

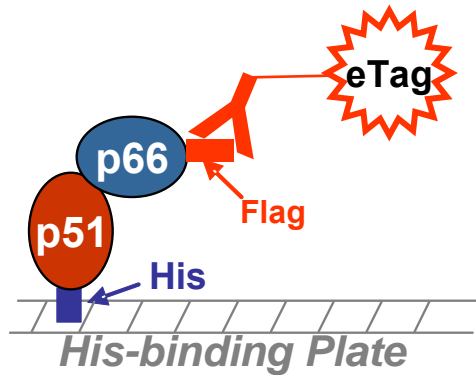
- His-tagged p51 and Flag-tagged p66 subunit proteins are expressed in bacterial expression vectors and purified using His/ Flag columns
- p66 and p51 subunits are incubated overnight at equimolar concentrations to create active heterodimers *in vitro*



Fluorescence Units (FITC) are a measure of the amount of PicoGreen ds DNA quantitation reagent (Molecular Probes) incorporated into newly made RNA-DNA heteroduplexes by the RT enzyme.

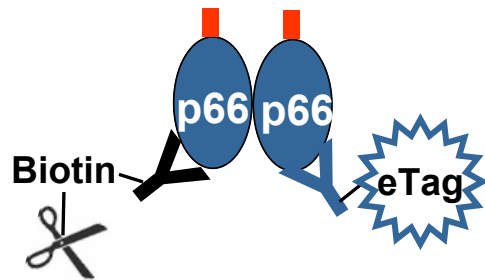
# e-Tag RT-Dimer Assays

- p66/p51 heterodimer detection



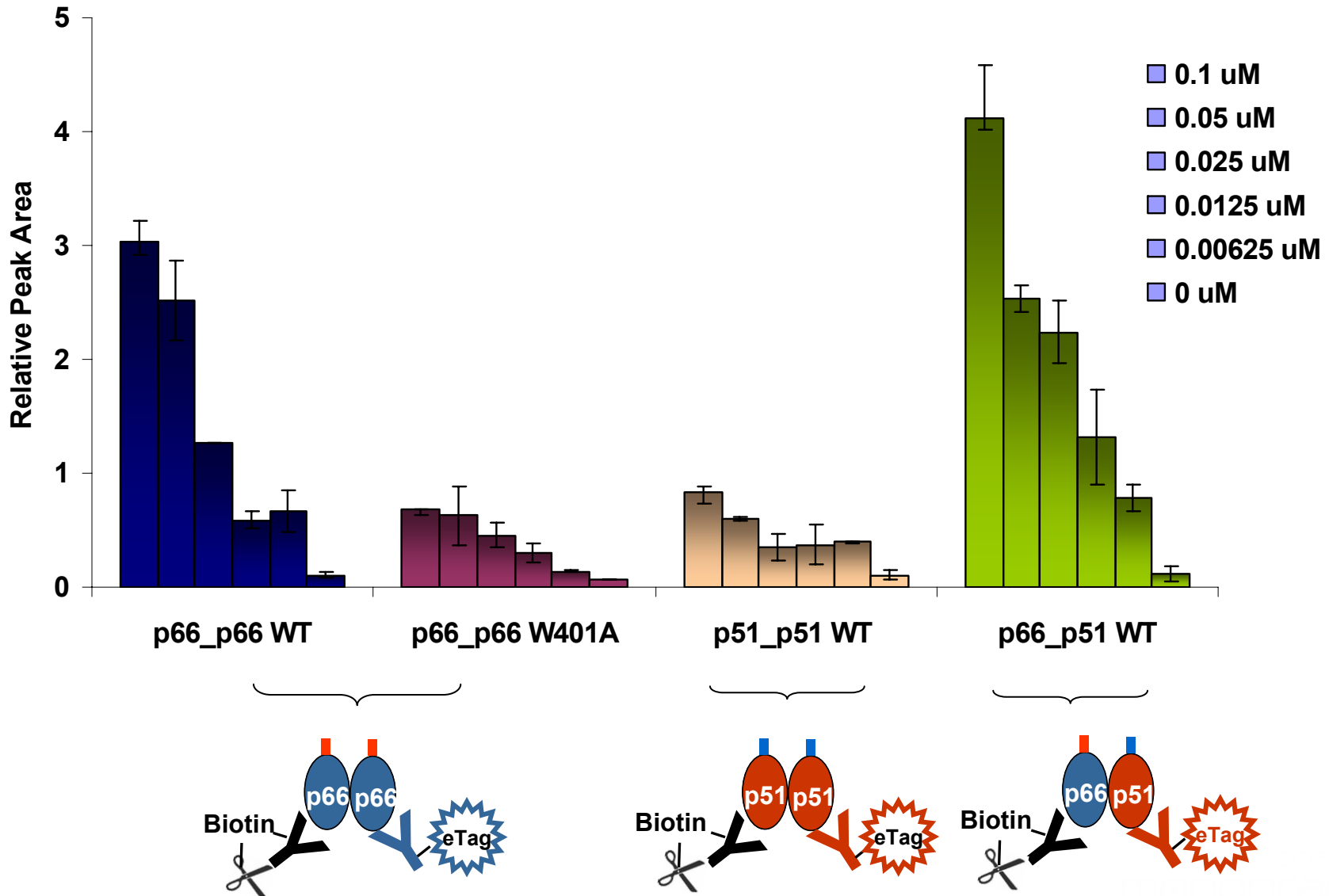
- Bind p51 subunit to His-binding plate
- Add p66 Subunit
- Add eTag-conjugated Anti-FLAG Detector Ab
- Release *eTags* with Methylene Blue
- Detect released *eTag* by CE

- p66/p66 or p51/p51 homodimer detection

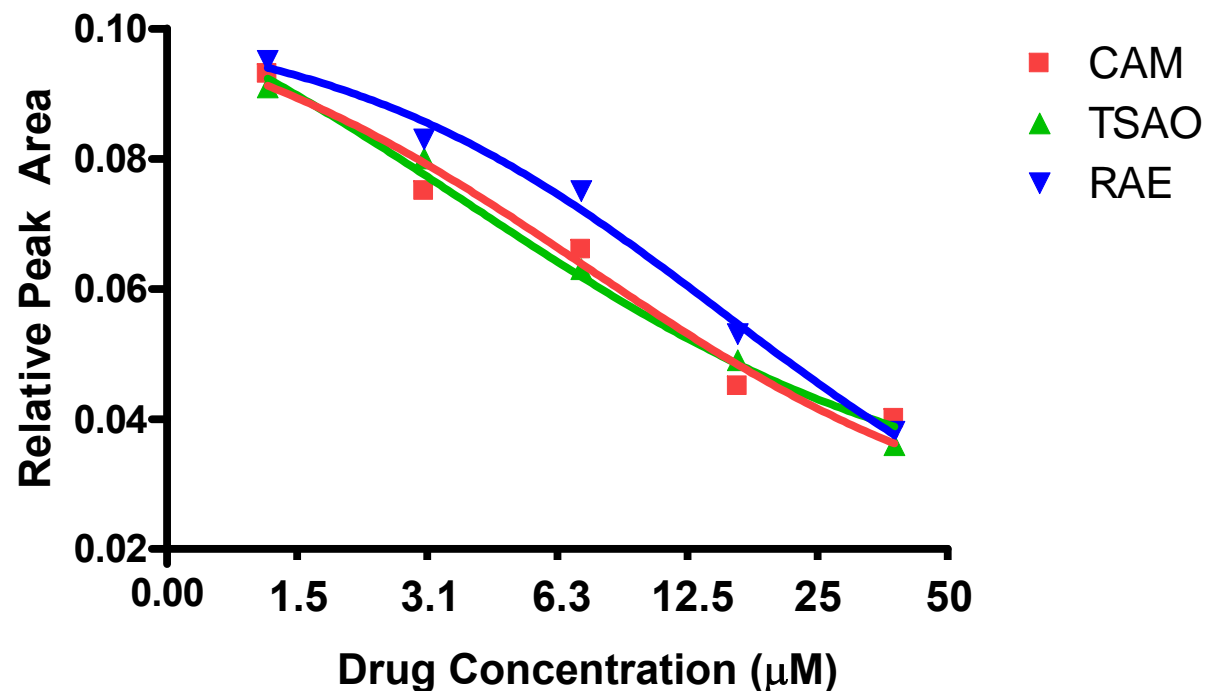
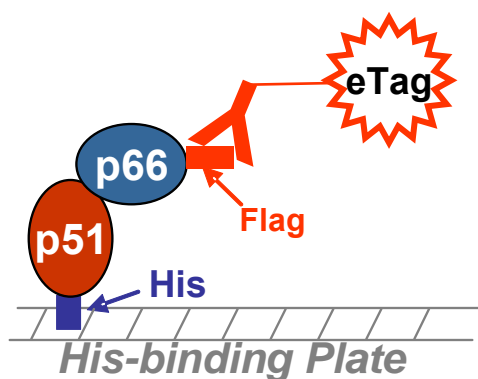


- Incubate EITHER p51 or p66 overnight
- Add scissor-conjugated biotinylated anti-RT Ab and *eTag*-conjugated anti-RT detector Ab
- Transfer reaction Streptavidin coated plates
- Release *eTags* in proximity of scissors by light activation
- Detect released *eTags* by CE

# Formation of p66 Homodimers *in vitro* is Very Efficient



# Dose-Dependent Inhibition of p66/p51 Dimerization by CAM, TSAO-3mT and RAE



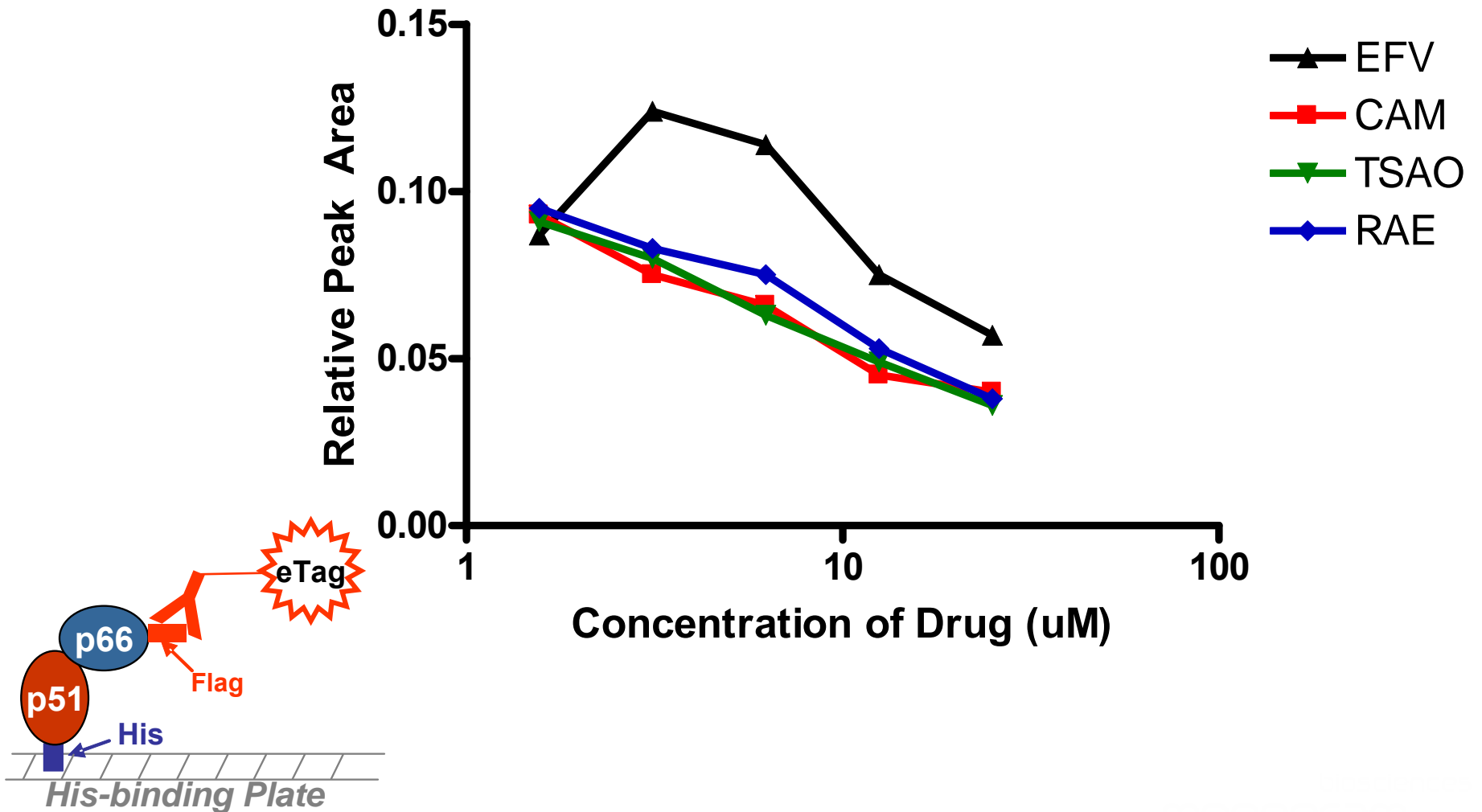
## *In vitro* Dimerization IC<sub>50</sub>

	MGRM IC <sub>50</sub> (µM)	Published IC <sub>50</sub> * (µM)
CAM	3.7	1.9
TSAO-3mT	6.4	6.7
RAE	11.6	11.7

\*Current Pharmaceutical Design, 2006, 12, 1895-1907, M.J. Camarasa *et al*

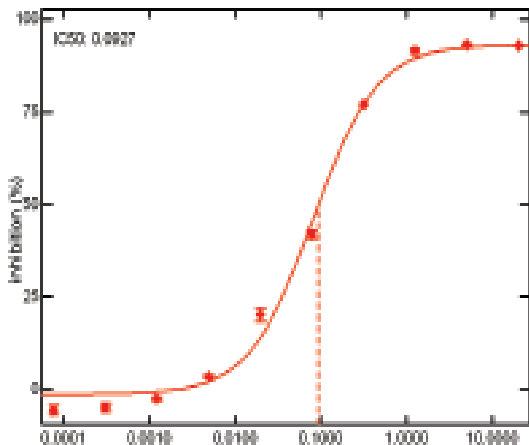
# Low concentrations of EFV Increase p66/p51 Dimerization

p66/p51 Dimers in the Presence of EFV

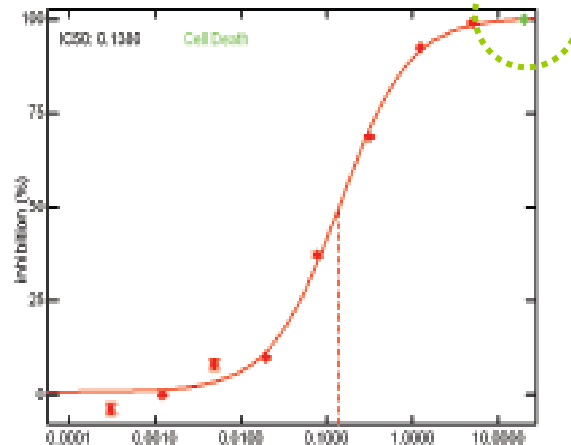


# Dimerization Inhibitors Block Replication of HIV NL4-3

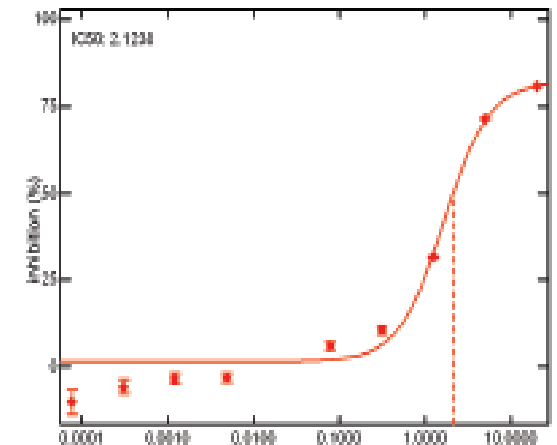
**TSAO-3mT**  
**IC<sub>50</sub> 0.09 μM**



**CAM**  
**IC<sub>50</sub> 0.14 μM**

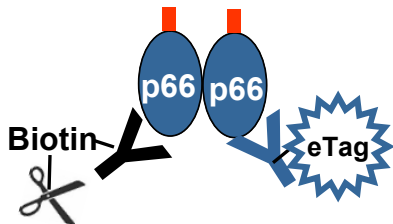
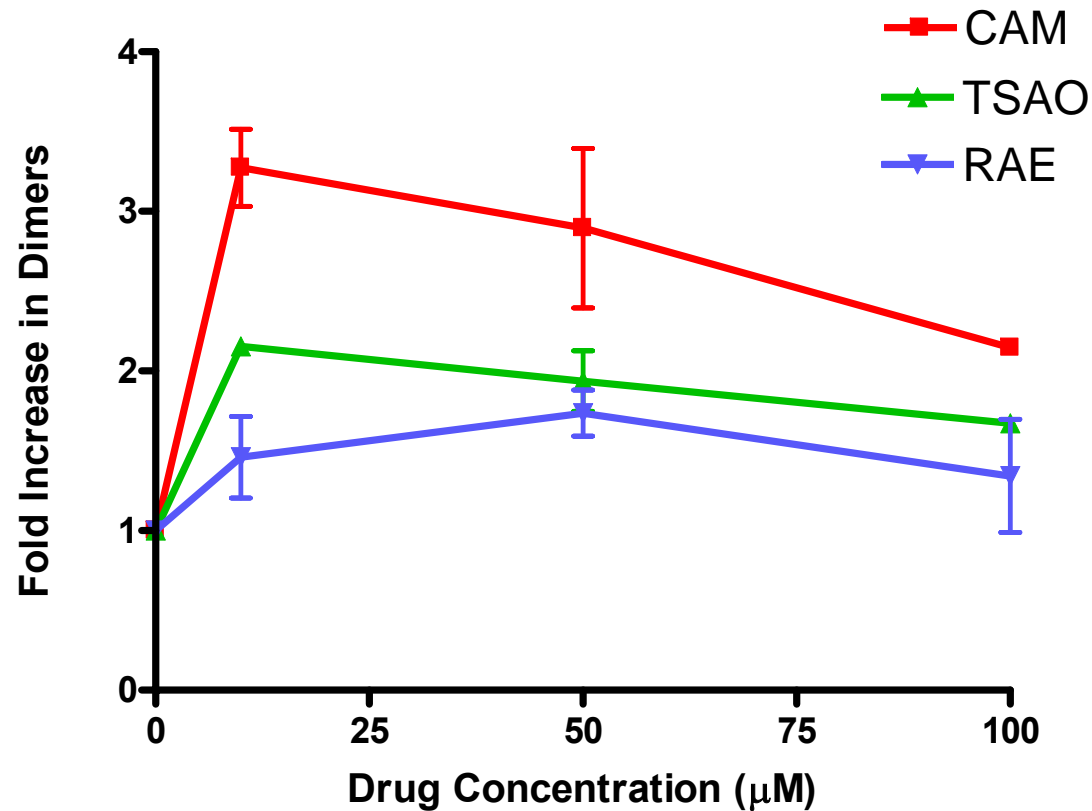


**RAE**  
**IC<sub>50</sub> 2.1 μM**



# Increase in Homodimerization Correlates with Decrease in Heterodimerization

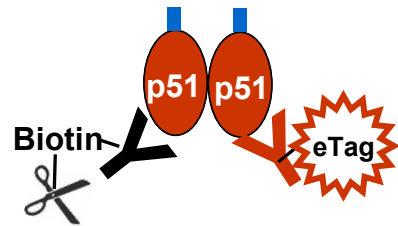
## p66 Homodimers in the Presence of CAM, TSAO or RAE



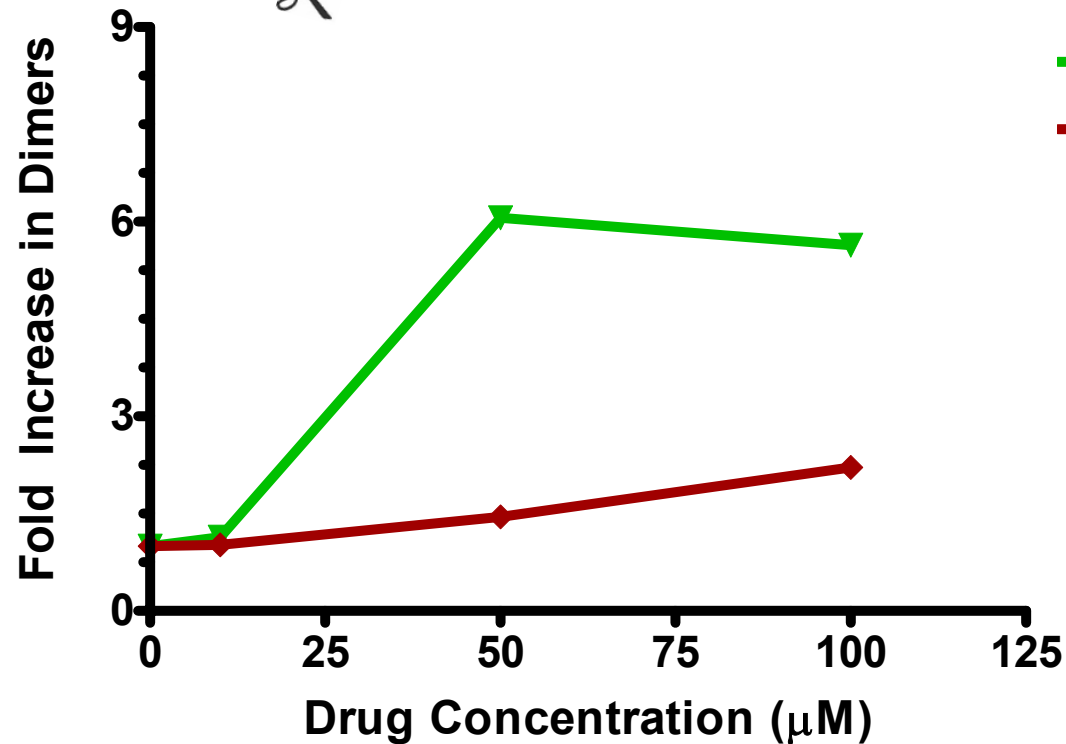
Experiment was performed two times, each in triplicate

# Mutant E138K on p51 Exhibits Reduced Sensitivity to CAM

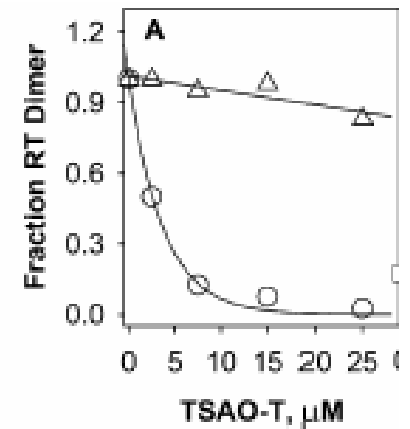
p51/p51 Homodimers



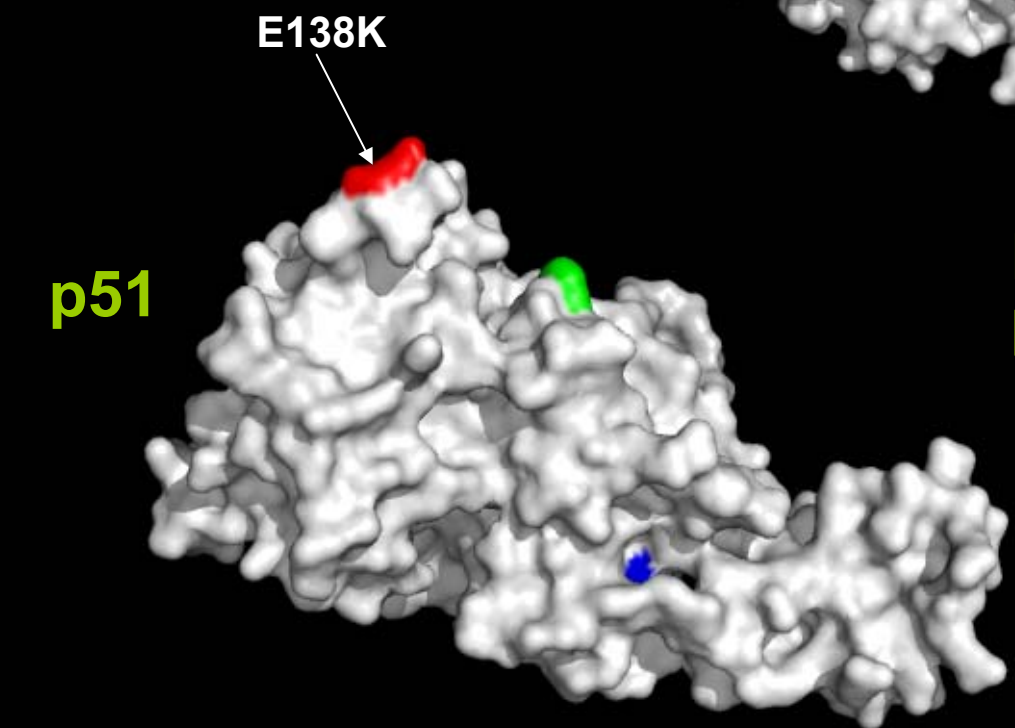
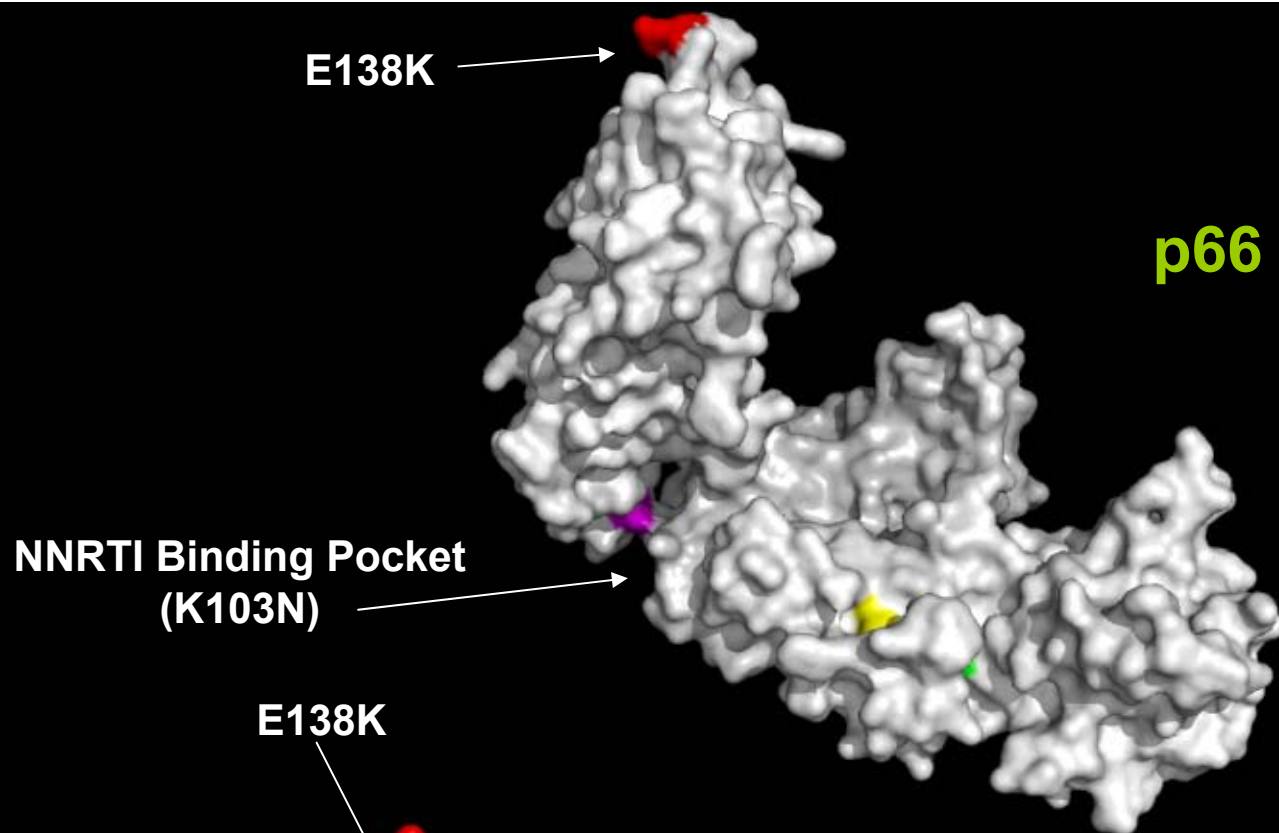
p66/p51 Heterodimer\*



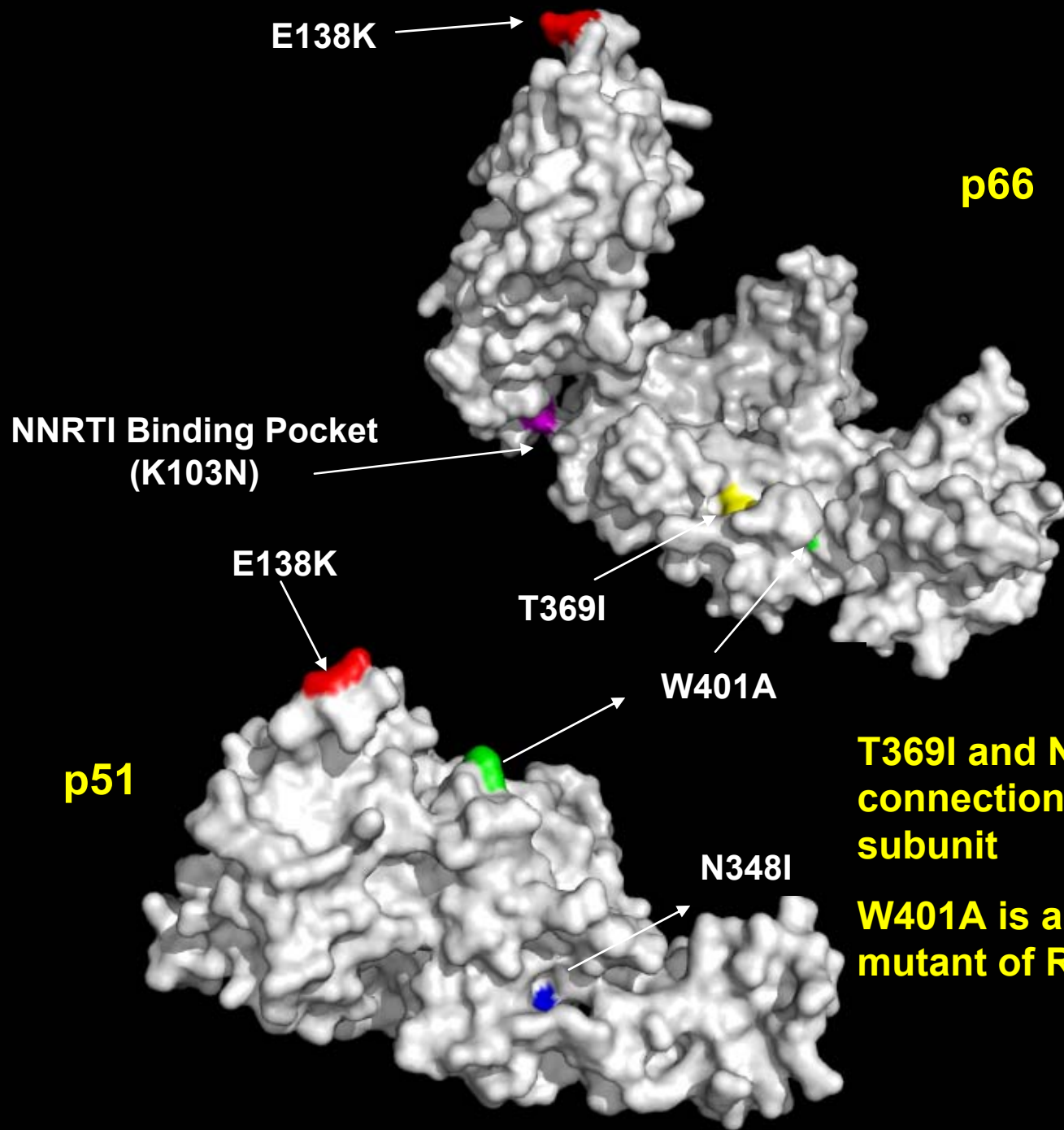
▲ p51 WT  
◆ p51 E138K



\*J. Med. Chem., 2006, 49, 4834-4841, M.J. Camarasa *et al*; using wt p51 RT (O) and E138K p51 RT (Δ)



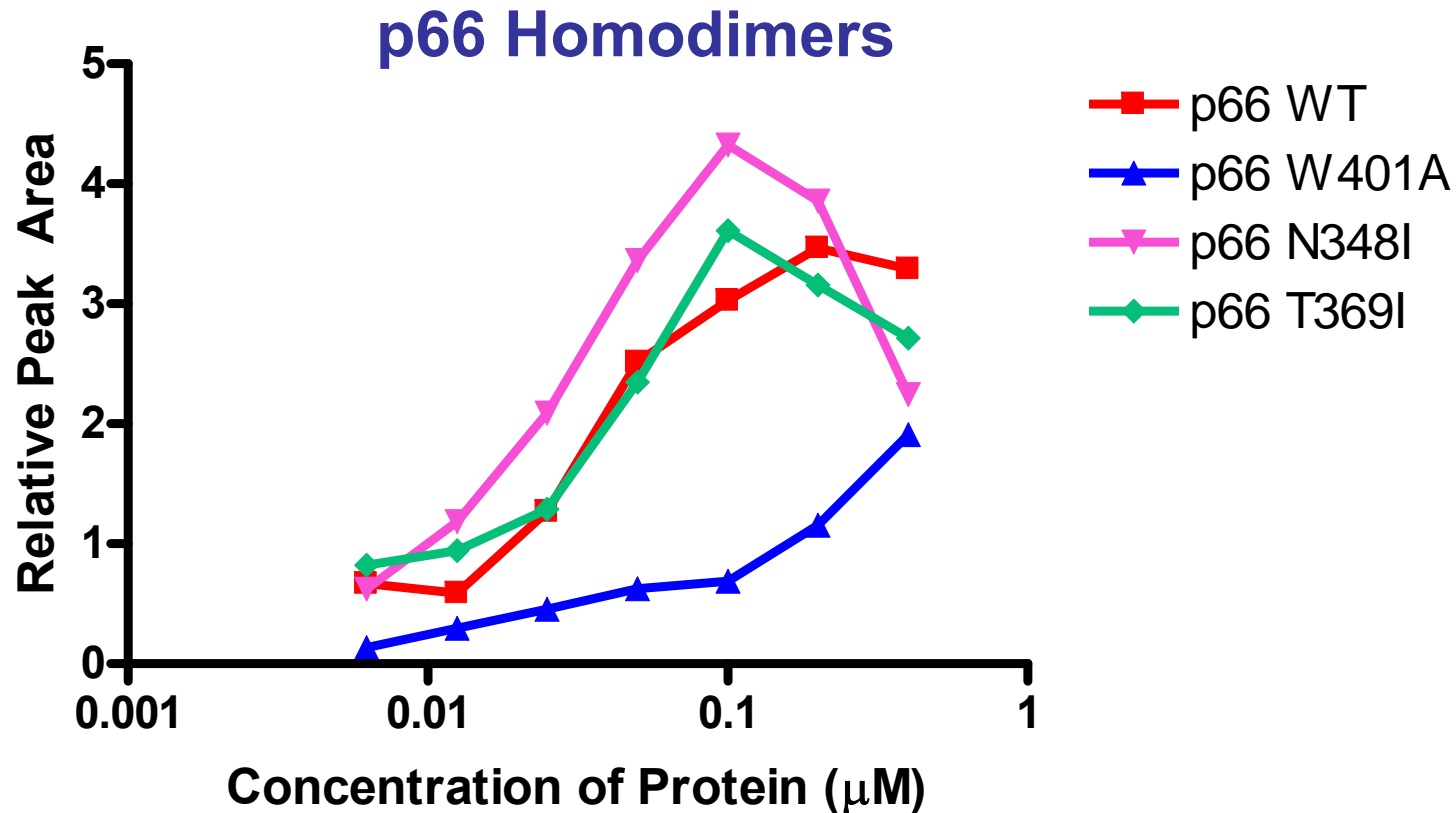
**Residue E138 on p51 is a key point of contact for CAM**



**T369I and N348I are located in the connection domain of the p66 subunit**

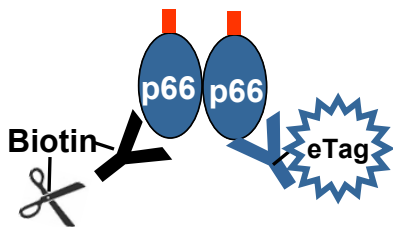
**W401A is a dimerization defective mutant of RT**

# N348I Increases p66 Homodimers



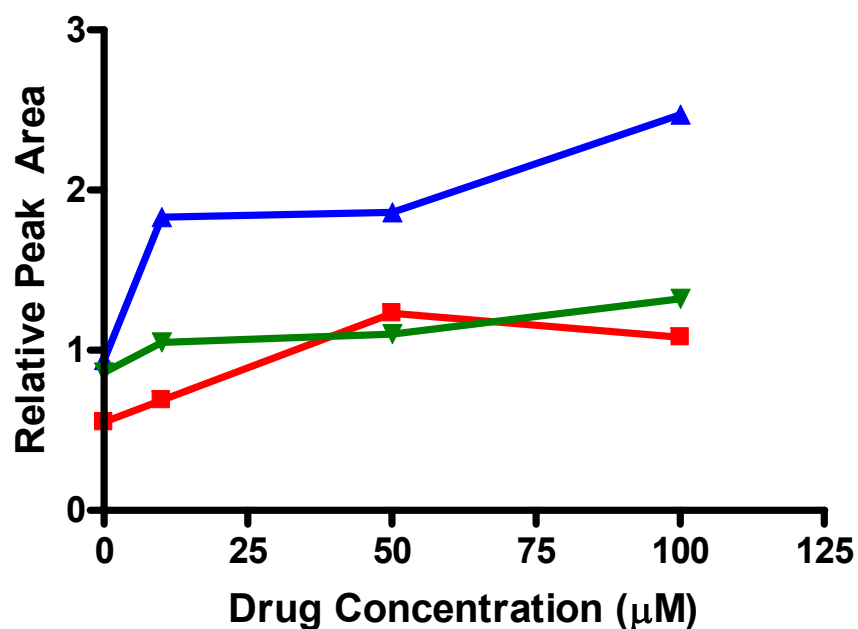
## IC<sub>50</sub> Fold Change

Sample ID	DLV	EFV	NVP
N348I	3.3	2.3	4.3
T369I	4.6	2.9	7.9
N348I/T369I	31.4	11.7	59.7

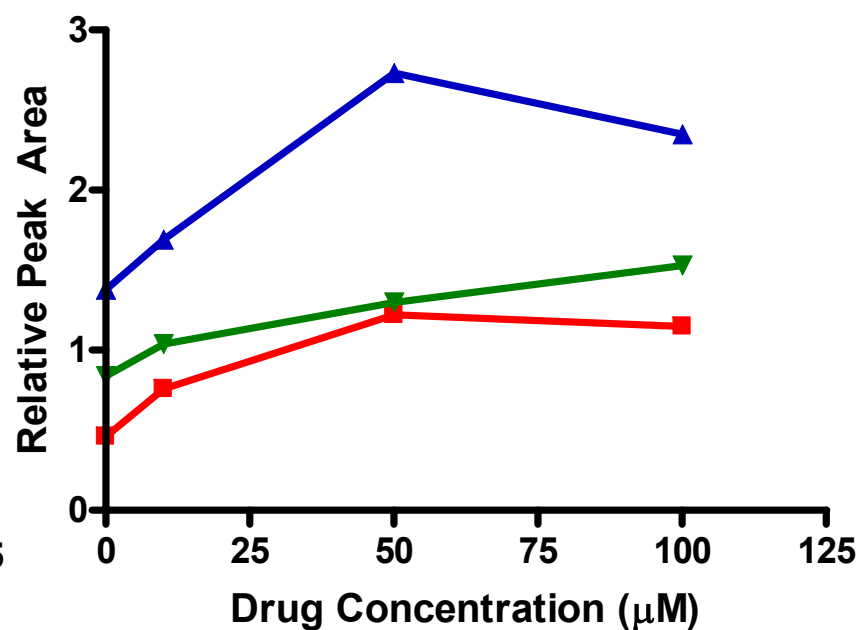


# p66 N348I Homodimer Formation is Increased by EFV and NVP

p66 Homodimers in the Presence of EFV



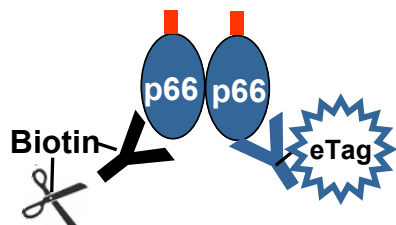
p66 Homodimers in the Presence of NVP



■ p66 WT

▲ p66 N348I

▼ p66 T369I



# Conclusions

- *In vitro* formation of p66/p66 and p51/p51 homodimers as well as p66/p51 heterodimers were quantified using *eTag* assays
- Inhibition of p66/p51 heterodimer formation by TSAO-based dimerization inhibitors - CAM, TSAO-3mT and RAE - was also demonstrated by the *eTag* assay
- An increase in p66 and p51 homodimer formation was observed in the presence of these TSAO-based inhibitors
- The connection domain mutant N348I, but not T369I, which reduces susceptibility to NNRTIs, demonstrated increased p66 homodimer formation *in vitro*
- *eTag*-based dimerization assays can be a useful tool for screening potential dimerization inhibitors of RT and for furthering our understanding of the effect of connection domain mutations on RT dimerization

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