Assessing putative mammalian adenylyl cyclase inhibitors

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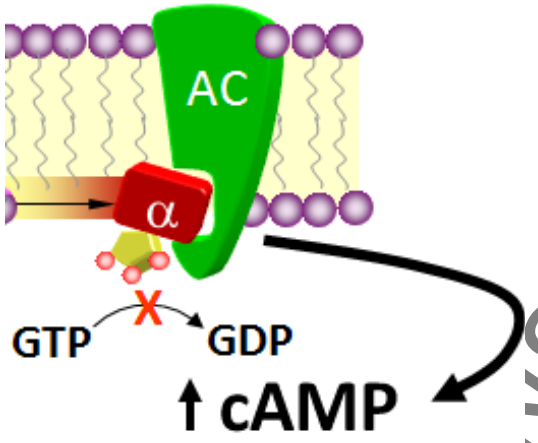
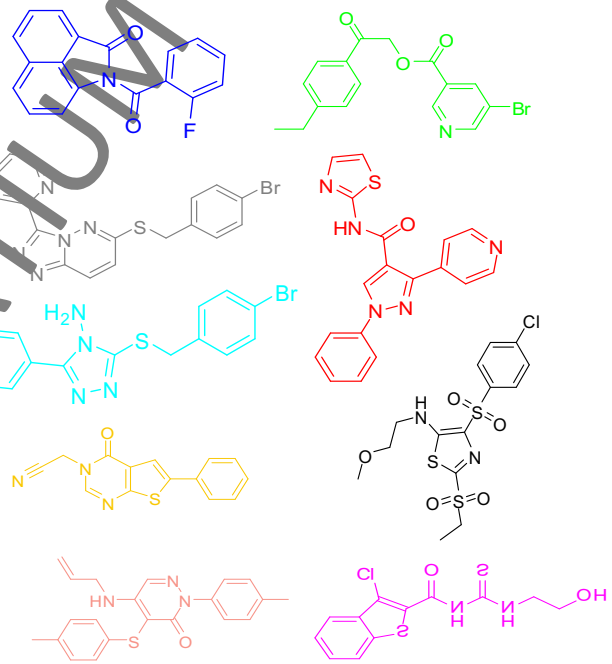
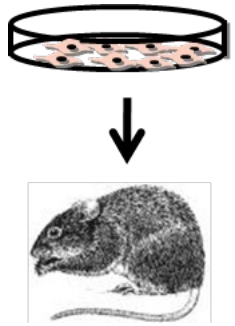
A general approach to discover molecules to be turned into drugs

Identify target → Create assay(s) → Test 1,000s -1,000,000s chemicals for activity → Modify chemicals to make suitable for human use → Preclinical testing for safety/efficacy → Clinical trials in humans







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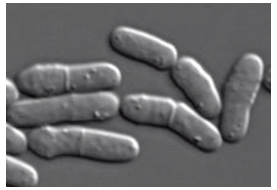
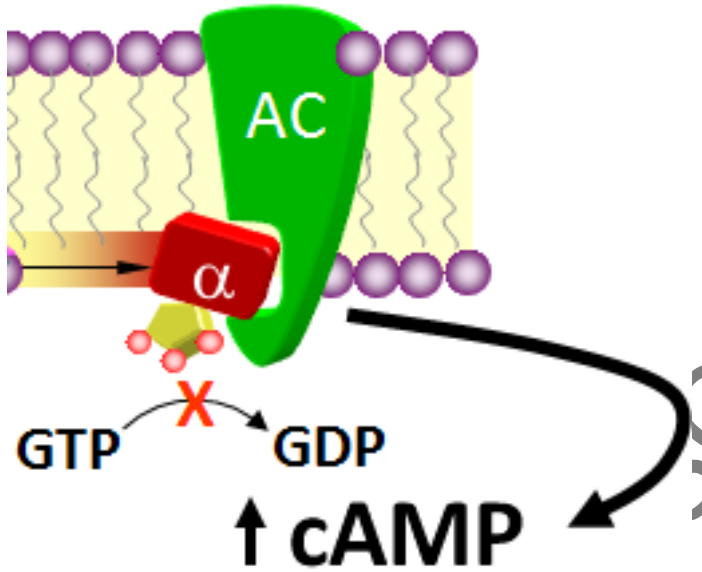


What's the problem in MAS?

- The GNAS Gsα protein is broken – stuck in the "on" position
- It acts through adenylyl cyclases (ACs; there are 9 of them)
- Gsα has been very difficult to directly target
- Our screens targeted either GNAS or the ACs expressed in bone progenitor cells



Because the ACs are embedded in a membrane, they are difficult to work with by a traditional screening assay that uses purified protein

We express ACs in a fission yeast strain that lacks its own AC so that we can assess their activity in live cells while they are in the intact membrane similar to how they are found in human cells. These strains also express $GNAS^{R201C}$ to stimulate the AC

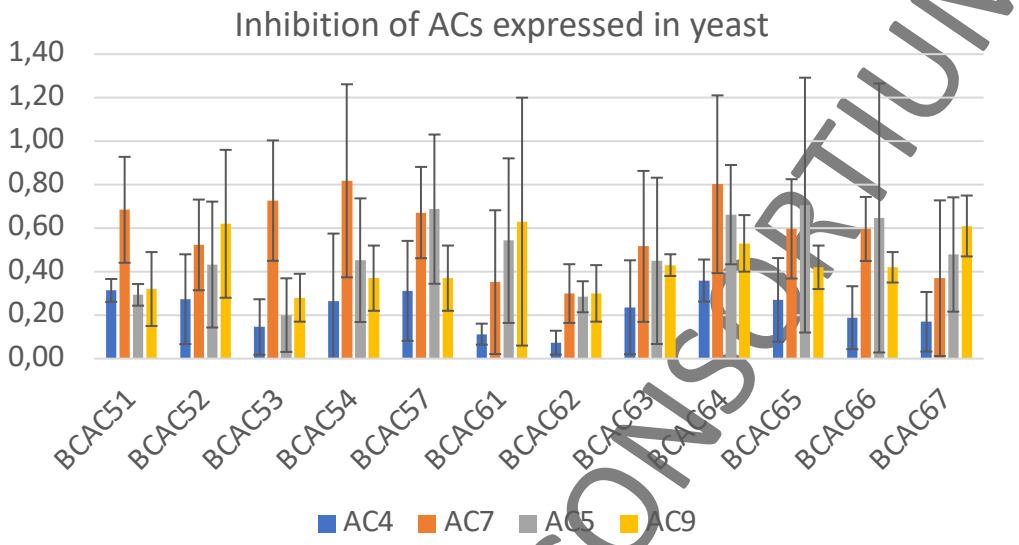
Our yeast strains are engineered to "tell" us when a compound inhibits the AC to lower cAMP levels by causing the expression of the jellyfish green fluorescent protein (GFP)

AC inhibitor screening via expression in fission yeast

High throughput screens using fission yeast strains that express individual mammalian ACs together with GNAS^{R201C}

~125,000 compounds screened against strains expressing AC4 and AC7 (the most highly expressed ACs in bone progenitor cells)

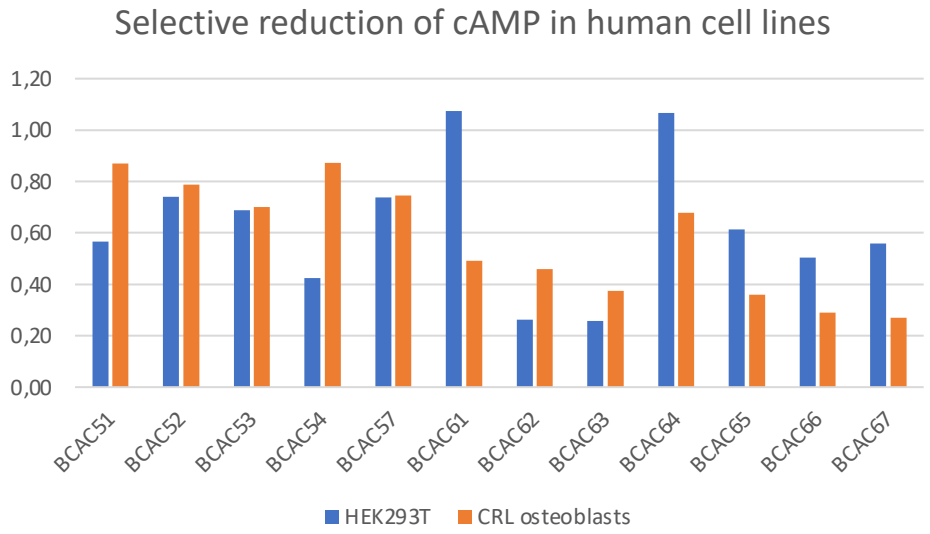
Compounds were identified that could elevate expression of a reporter by lowering cAMP levels



MDBR Funding Results

Direct assessment of 47 compounds on cAMP production validated two groups of compounds

One group shows the potential for drug development as many related compounds can lower cAMP levels in both yeast that express mammalian ACs and in human cell culture



The lower the bar, the more a compound reduces cAMP levels

Another Research Team Discovered BCAC51 Scaffold as Potential mAC1 Inhibitors

AC10042

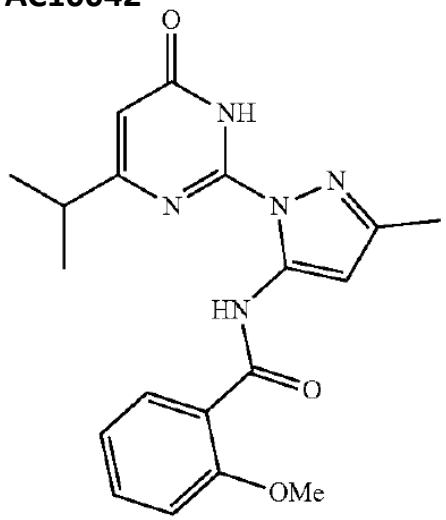


TABLE 2

Activity Table of Compounds Disclosed

Compd. ID	HEK-AC1 IC50 (μM)	HEK-AC1 % Inhibition (30 μM)	HEK-AC8 IC50 (μM)	HEK-AC8 % Inhibition (30 μM)	HEK-AC2 % Inhibition (10 μM)	HEK-AC2 % Inhibition (30 μM)
AC10048	0.58 ± 0.2	99 ± 2	N/D*	19 ± 11	(–)186 ± 20	(–)59 ± 12
AC10049	1.3 ± 0.2	98 ± 1	68 ± 10	26 ± 4	(–)177 ± 31	(–) 128 ± 7
AC10058	1.2 ± 0.2	99 ± 1	N/D	19 ± 4	(–)226 ± 14	(–) 190 ± 21
AC10042	1.5 ± 0.3	101 ± 0.7	N/D	9 ± 4	(–)179 ± 50	(–)94 ± 35
AC10057	3.7 ± 0.2	94 ± 1	N/D	9 ± 2	(–)195 ± 26	(–)199 ± 30
AC10043	10.3 ± 2	92 ± 2	N/D	16 ± 9	(–) 109 ± 36	(–)196 ± 8
AC10050	16.7 ± 1	79 ± 4	N/D	(–) 1 ± 10	(–)21 ± 10	(–) 126 ± 43
AC10051	N/D	4 ± 7	N/D	(–) 3 ± 11	(–)13 ± 12	(–) 12 ± 11

*N/D: not determined

IC50: Amount of drug needed to inhibit biological process by half. Lower IC50 values indicate highly potent drug.

- Val Watts' lab identified the same scaffold in a HEK cell-based screen expressing AC1 to identify compounds which reduce cAMP.
- **However, *in vitro* enzyme assay using the fusion AC 5C1:2C2 and compounds from the HEK screen, revealed that the compounds did not affect cAMP levels.**
- Watts team concluded that the compounds interfere with the calmodulin signaling pathway to reduce cAMP, as that pathway is required for AC1 stimulation.

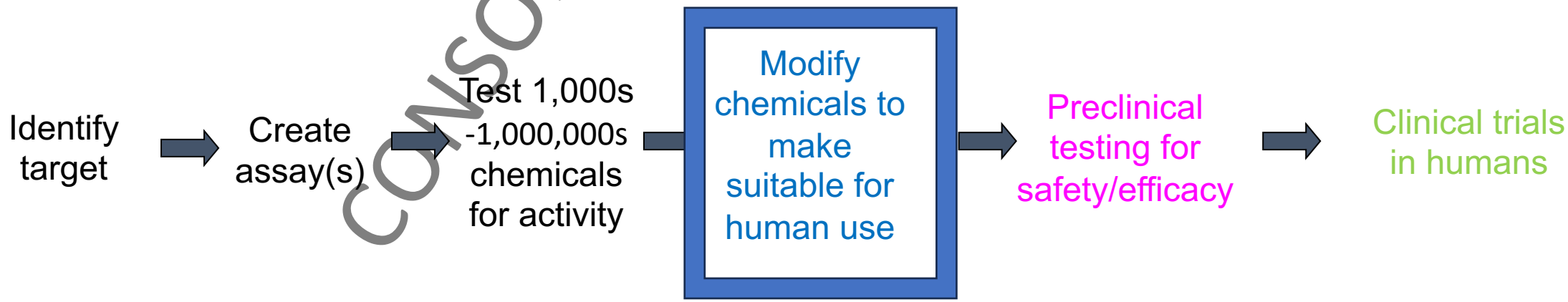
AC4, AC9, AC7 are not regulated by calmodulin signaling

Dilemma: Do these compounds act directly on mACs or indirectly through another target?

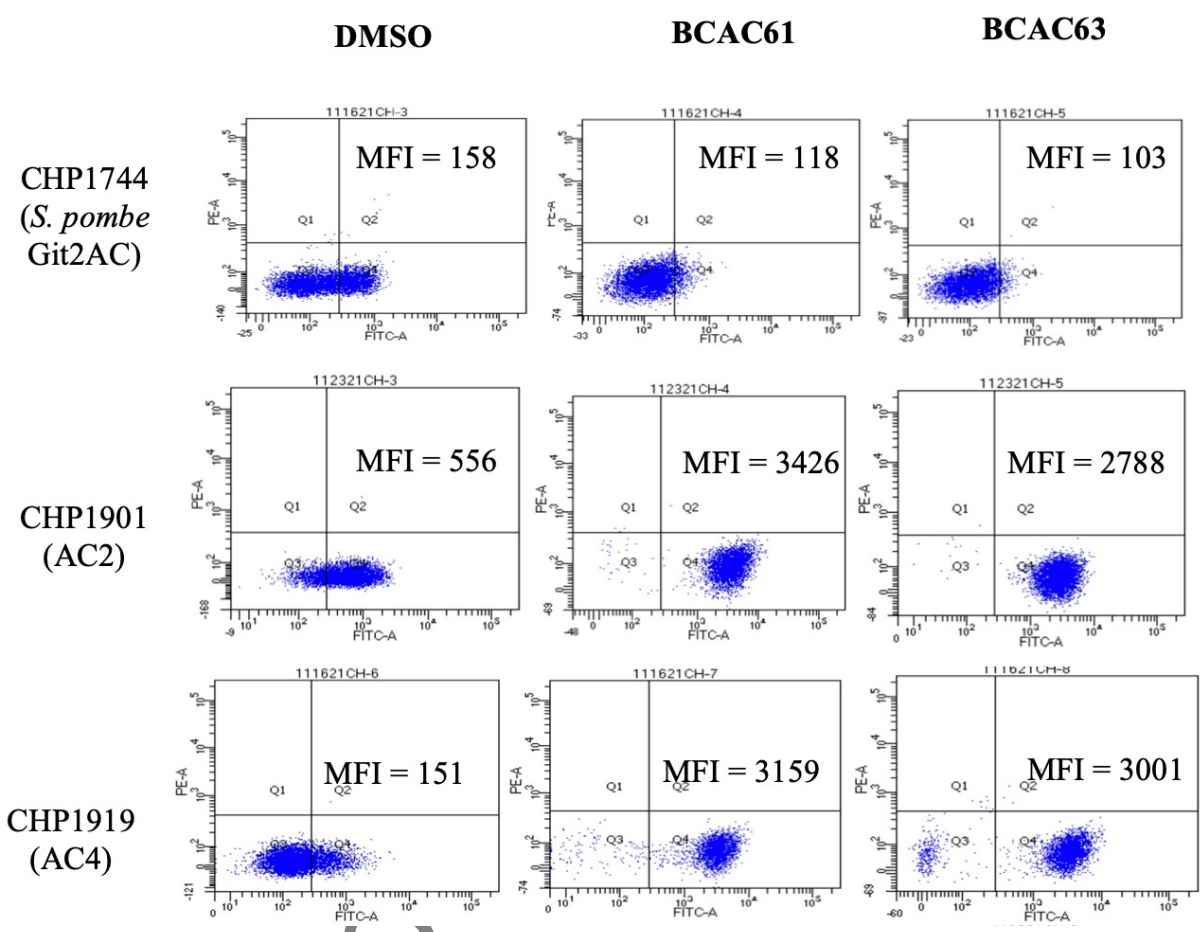
This matters because a medicinal chemist will want guidance by *in vitro* enzyme assays and study sections will be comprised of chemists who expect *in vitro* data.

Possible mode of action

- Prevent GNAS from activating
- Inhibit AC independent of GNAS function
- Act on a process that is required for AC activity



Compounds inhibit tmAC activity in the absence of GNAS

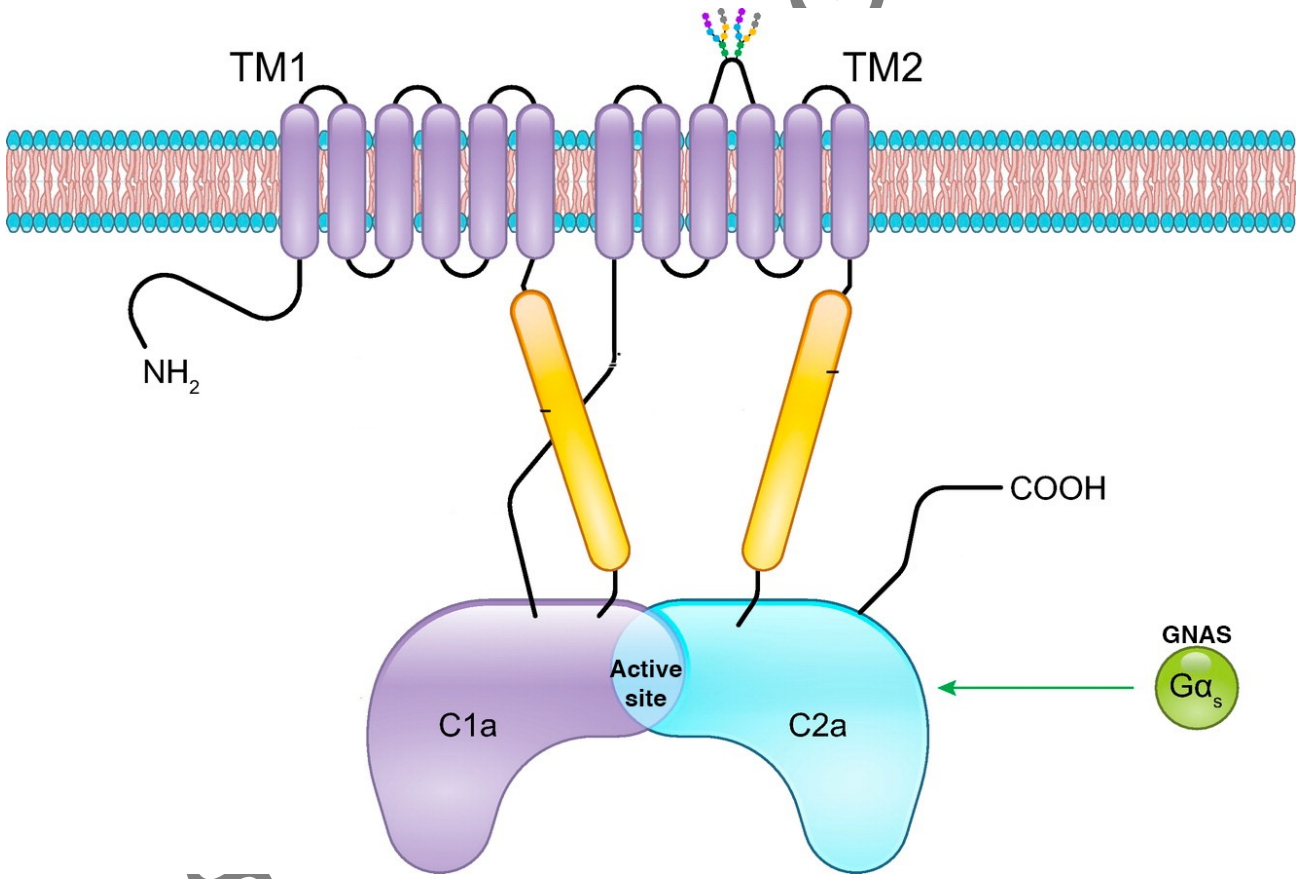


No shift means no effect

Rightward shift means inhibition

Shift also seen for AC7 and AC9, although this does not rule out indirect effect

Structure of transmembrane ACs



C1 and C2 cytoplasmic domains come together to make the portion that produces cAMP.

Modified from Ostrom *et al., Physiol Rev*. 2022 Apr 1;102(2):815-857.

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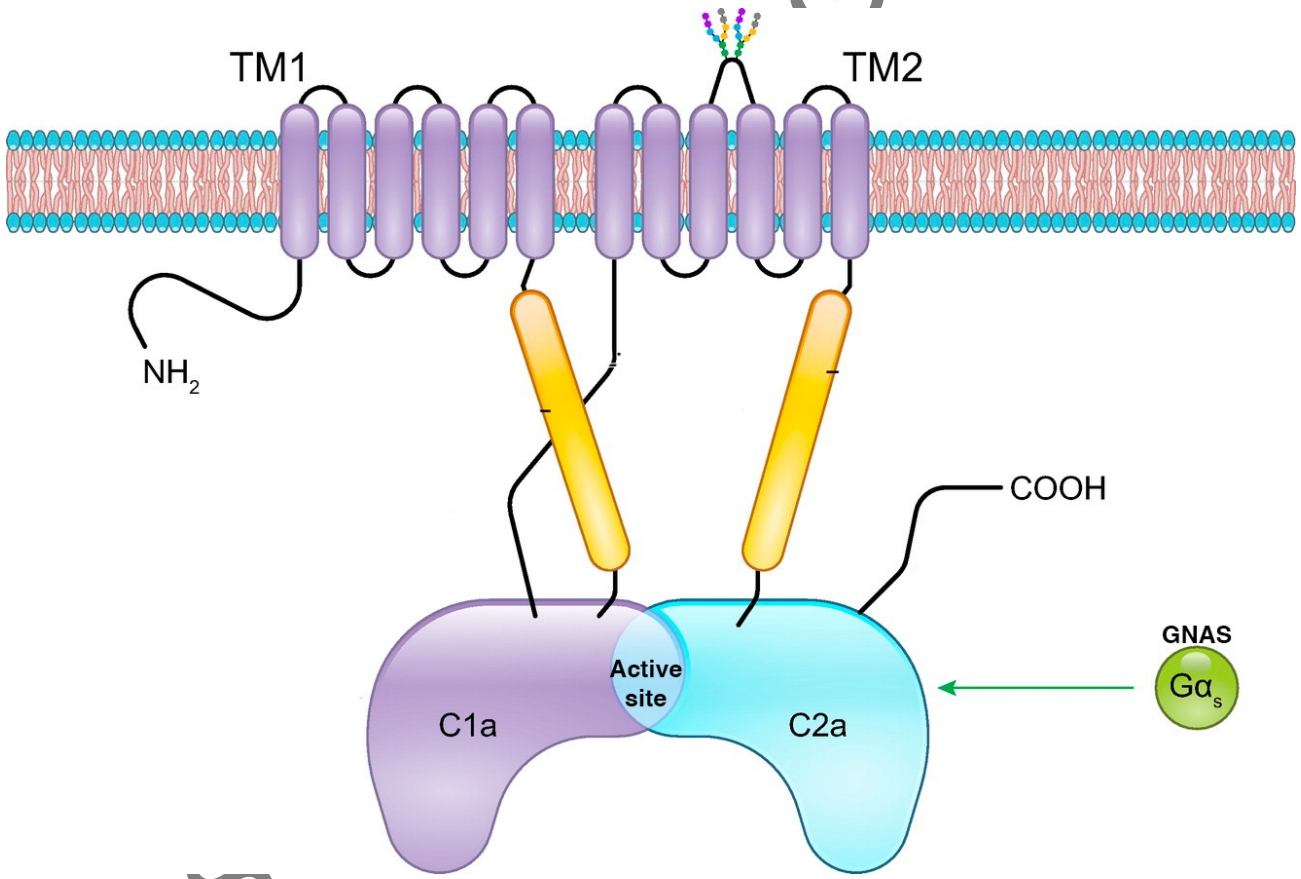

C1 and C2 cytoplasmic domains come together to make the portion that produces cAMP.

Q: Do the inhibition profiles suggest a direct or indirect mode of inhibition?

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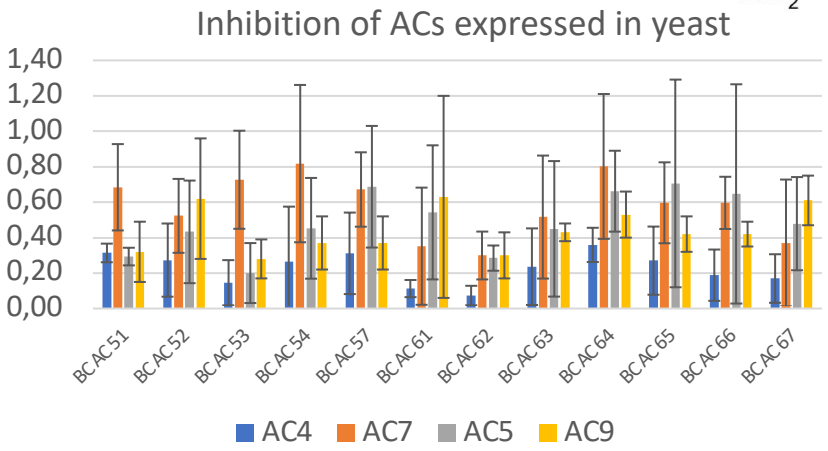
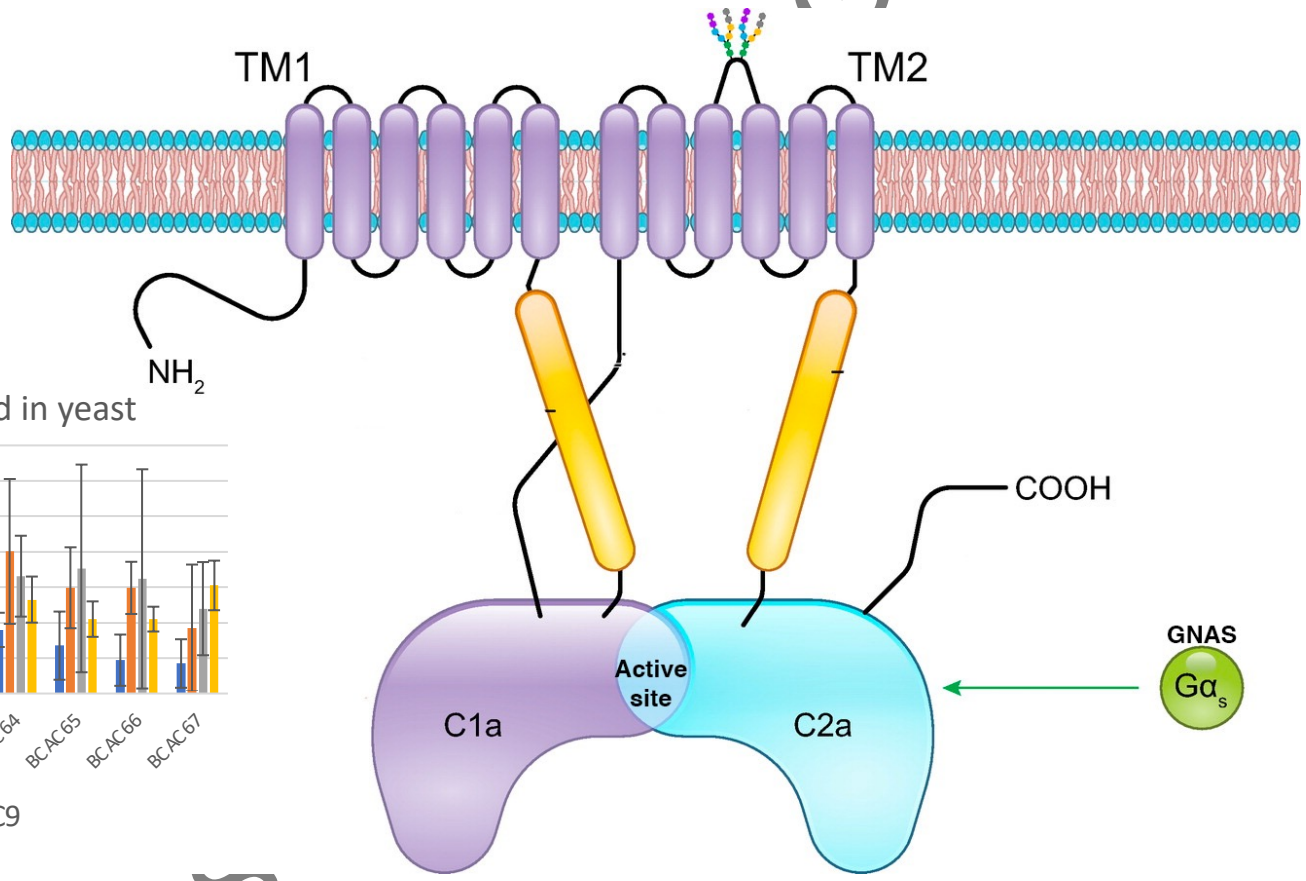
Compounds appear to display distinct patterns of inhibition consistent with direct inhibition





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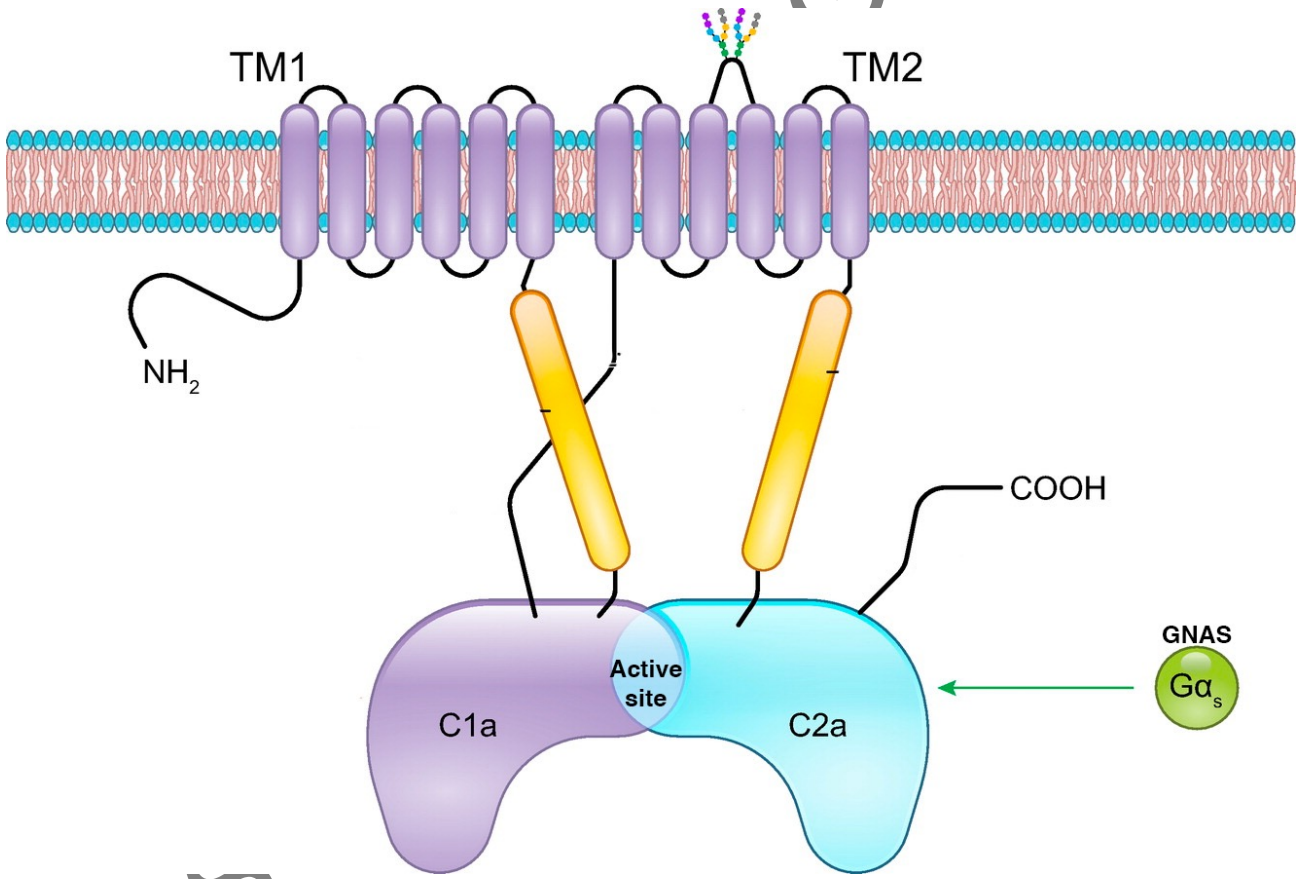
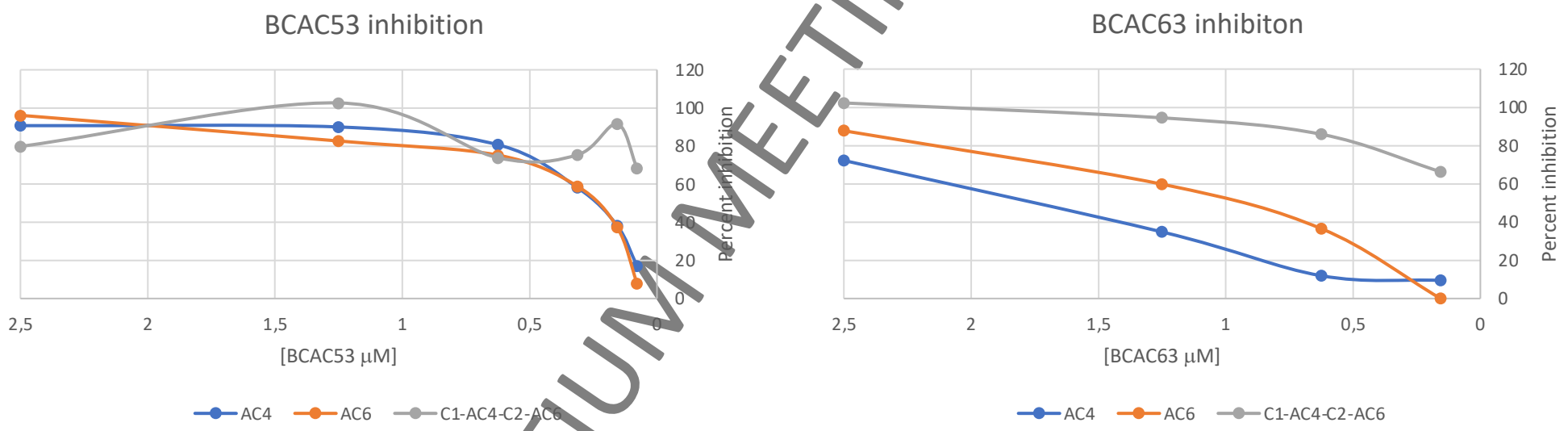


C1 and C2 cytoplasmic domains come together to make the portion that produces cAMP.
Q: Does a tmAC containing C1 and C2 domains from two different tmACs (chimeric) show a pattern of inhibition suggesting one or the other domain as the site of compound binding?

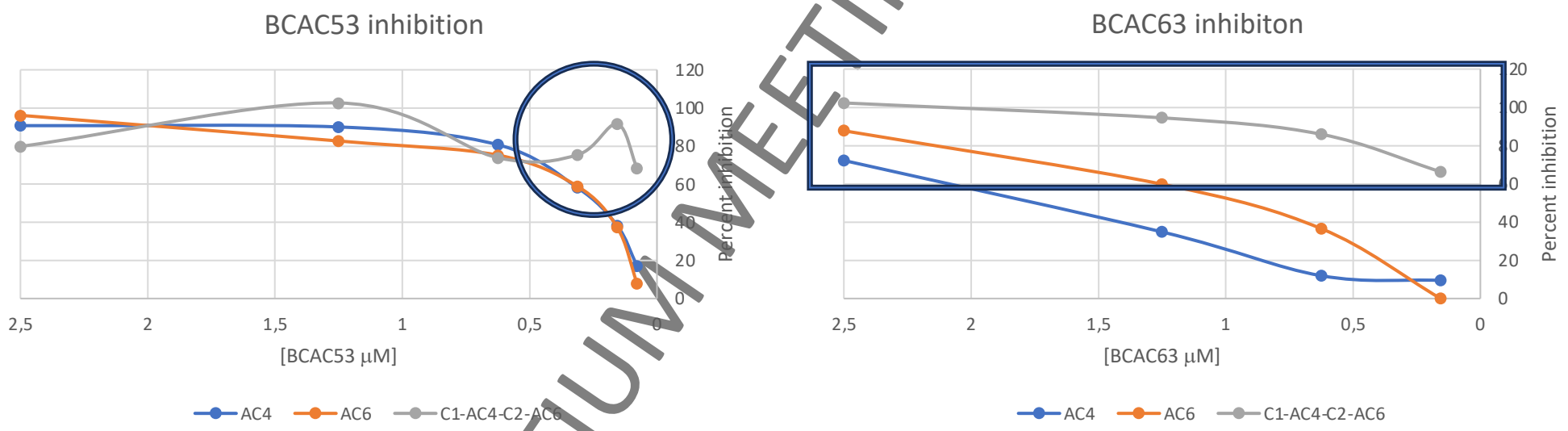
Modified from Ostrom *et al., Physiol Rev*. 2022 Apr 1;102(2):815-857.

A chimeric AC is hypersensitive to inhibition



Strains expressing either AC4, AC6 or a chimeric protein in which the AC4 C2 domain was replaced with the AC6 C2 domain were tested for compound sensitivity.

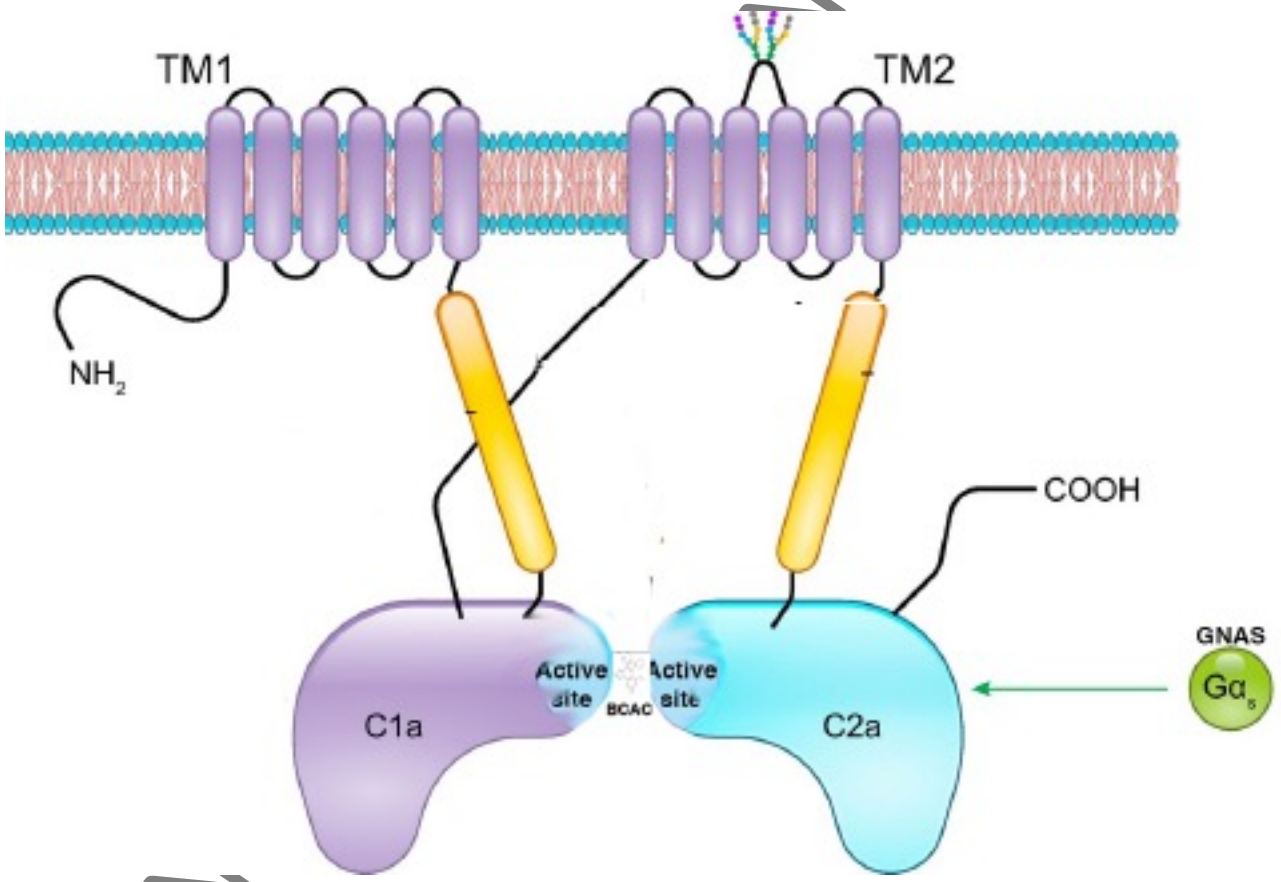
A chimeric AC is hypersensitive to inhibition



The chimeric protein is more sensitive to inhibition, consistent with a model in which the compound disrupts the interaction between the two cytoplasmic domains.

Future studies will examine whether single amino acid changes in a putative binding site can confer resistance to the compounds.

Possible mechanism of inhibition-preventing C1-C2 dimerization



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Summary

- Mutational activation of GNAS increases cAMP production by transmembrane adenylyl cyclases (tmACs)
- BCAC51 and related compounds reduce cAMP production in human cells and in yeast cells that express mammalian tmACs stimulate by $GNAS^{R201C}$
- These compounds do not show this effect in biochemical reactions
- Our data suggest that the biochemical reactions may not correctly reflect the interaction between the two cytoplasmic domains of tmACs
- Our compounds may act by disrupting the interaction between the C1 and C2 domains
- Further experiments will examine the effect of single amino acid changes in AC4 that a docking model suggests may affect compound binding by conferring compound resistance

- Thus, in the absence of a working *in vitro* enzyme assay, we hope to convince chemists that medicinal chemistry is warranted

Acknowledgments

Hoffman lab

Dr. Jeremy Eberhard
Maira Ahmed
Dan Pacella
Nick Rhodes
SeoBin Yoo
Tim Huang

Collaborators and Clones

Dr. Michael Collins	*NIH/NIDCR*
Dr. Fernando Fierro	*UCDavis*
Dr. James Inglese	*NIH/NCATS*
Trish Dranchak	*NIH/NCATS*
Dr. Val Watts	*Perdue U.*
Dr. Carmen Dessauer	*UT Houston*
Dr. Dermot Cooper	*U. Cambridge*

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Thank you!