IN VITRO AND *IN VIVO* CHARACTERIZATION OF NEUTRALIZING MONOCLONAL ANTIBODIES AGAINST *CLOSTRIDIUM DIFFICILE* TOXINS A AND B

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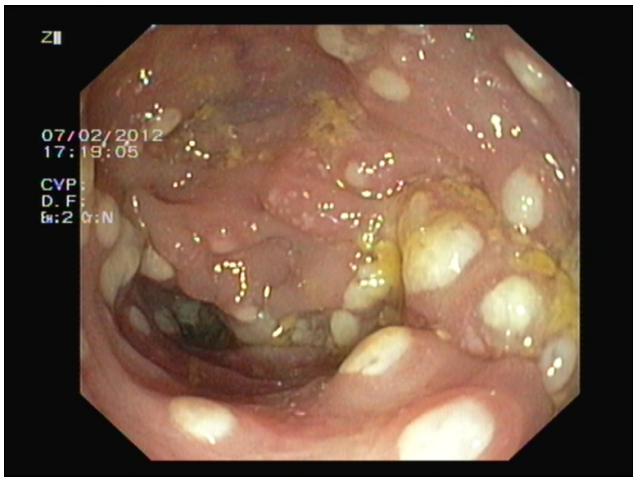
Overview

- Background
- Aims
- Strategy
- Results
- Future perspectives

Background – *C. diff* associated disease

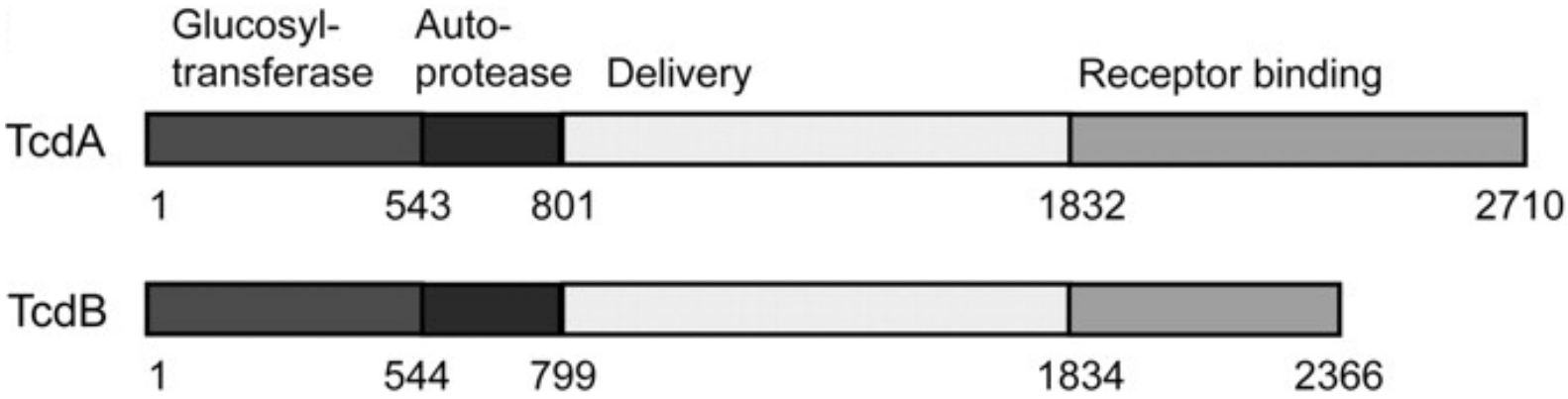


Pseudomembranous colitis, characterized by the formation of an adherent inflammatory membrane overlaying the site of injury[1]

Toxicity is determined by two toxins; TcdA and TcdB[2,3]:
→ Glucosylation of Rho GTPases → loss of cytoskeletal integrity, disruption of protein synthesis, inflammatory response and eventually cell death[4]

[1]Carroll et al., Annu Rev Microbiol. 2011.
[2]Kuehne *et al.*, Nature. 2010.
[3]Lyras *et al.*, Nature. 2009.
[4]Navaneethan *et al.*, Nat Clin Pract Gastroenterol Hepatol. 2008.

Structure of TcdA and TcdB



ABCD model of TcdA and TcdB[1]

A-domain: biologically active glucosyltransferase
B-domain: receptor binding
C-domain: cysteine protease domain (processing and cutting of the toxin)
D-domain: delivery of the catalytic domain into the cytosol

Figure adapted from Pruitt R N et al. PNAS 2010.
[1]Jank T *et al.*, Trends Microbiol. 2008.

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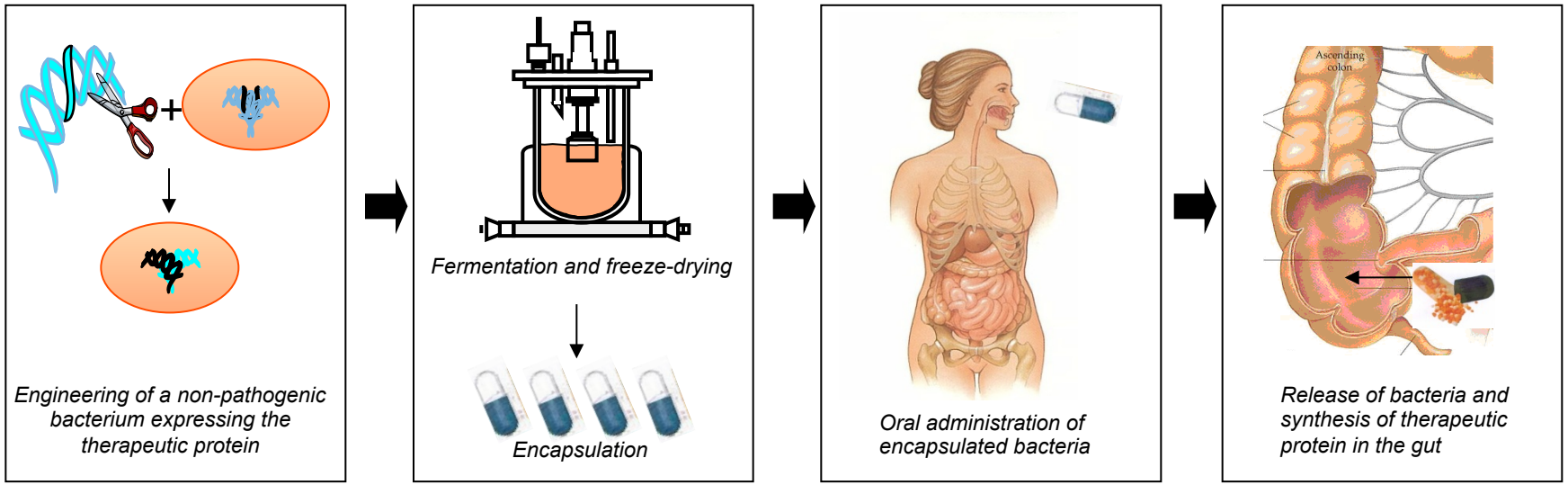
Aims

1 Obtain purified mAbs and corresponding Fabs with proven *in vitro* and *in vivo* toxin A/B neutralizing activity

2 Provide proof-of-concept for the efficacy of targeted mucosal delivery of toxin neutralizing Fabs in the hamster model of CDAD

Background – *ActoBiotics*™



Advantages:

- Oral administration of proteins and peptides
- Targeted topical release in GI tract
- Good safety profile
- Reduced systemic side-effects
- Low production costs

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Funnel-shaped strategy

Monoclonal antibody formation

↓

In vitro neutralization assays
(screening)

↓

In vitro characterization
(selection)

↓

In vivo neutralization

↓

In vivo neutralization by ActoBiotics

Generation of antibodies was performed in collaboration with PharmAbs, ActoBiotics are created by ActoGeniX

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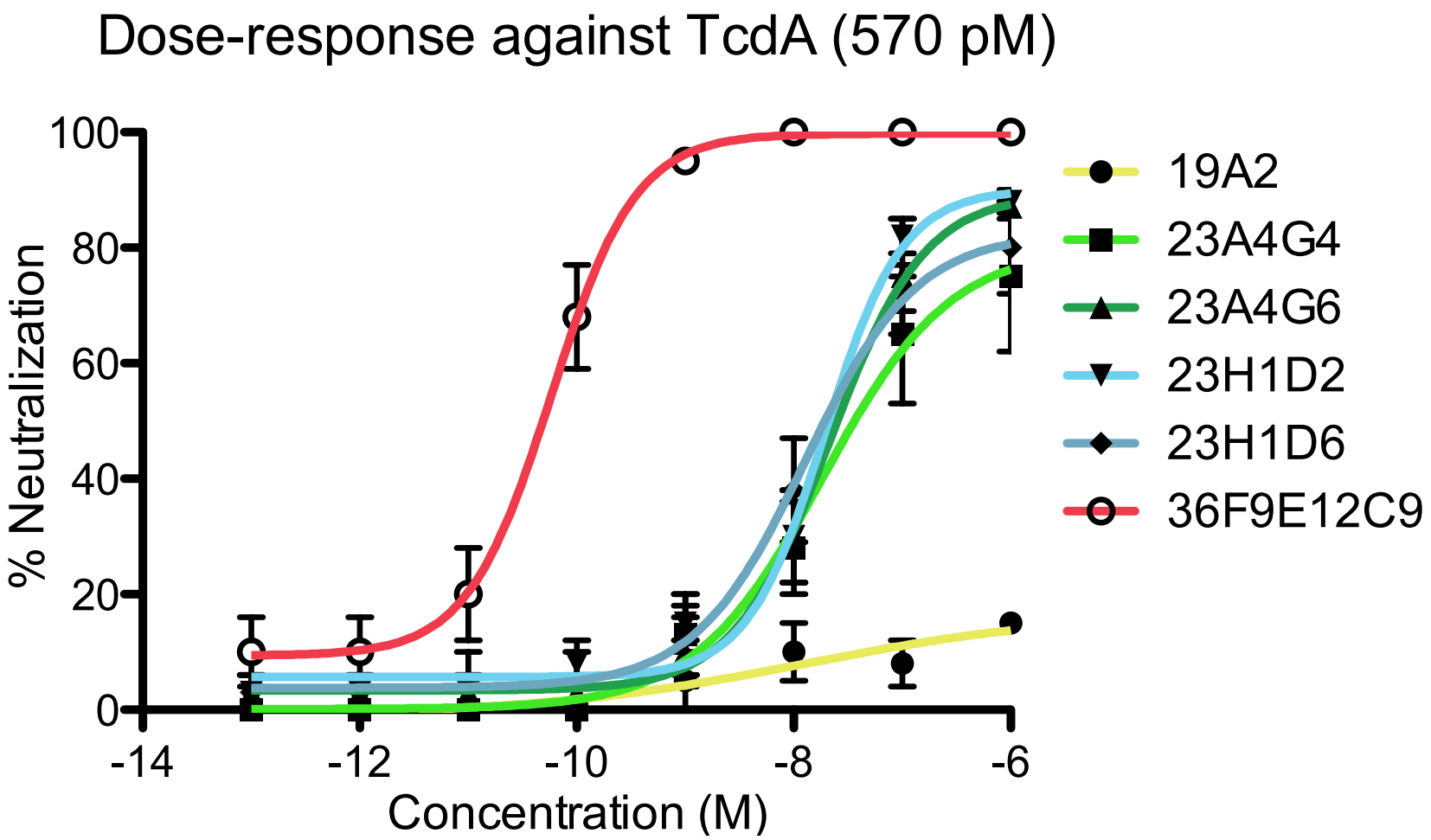
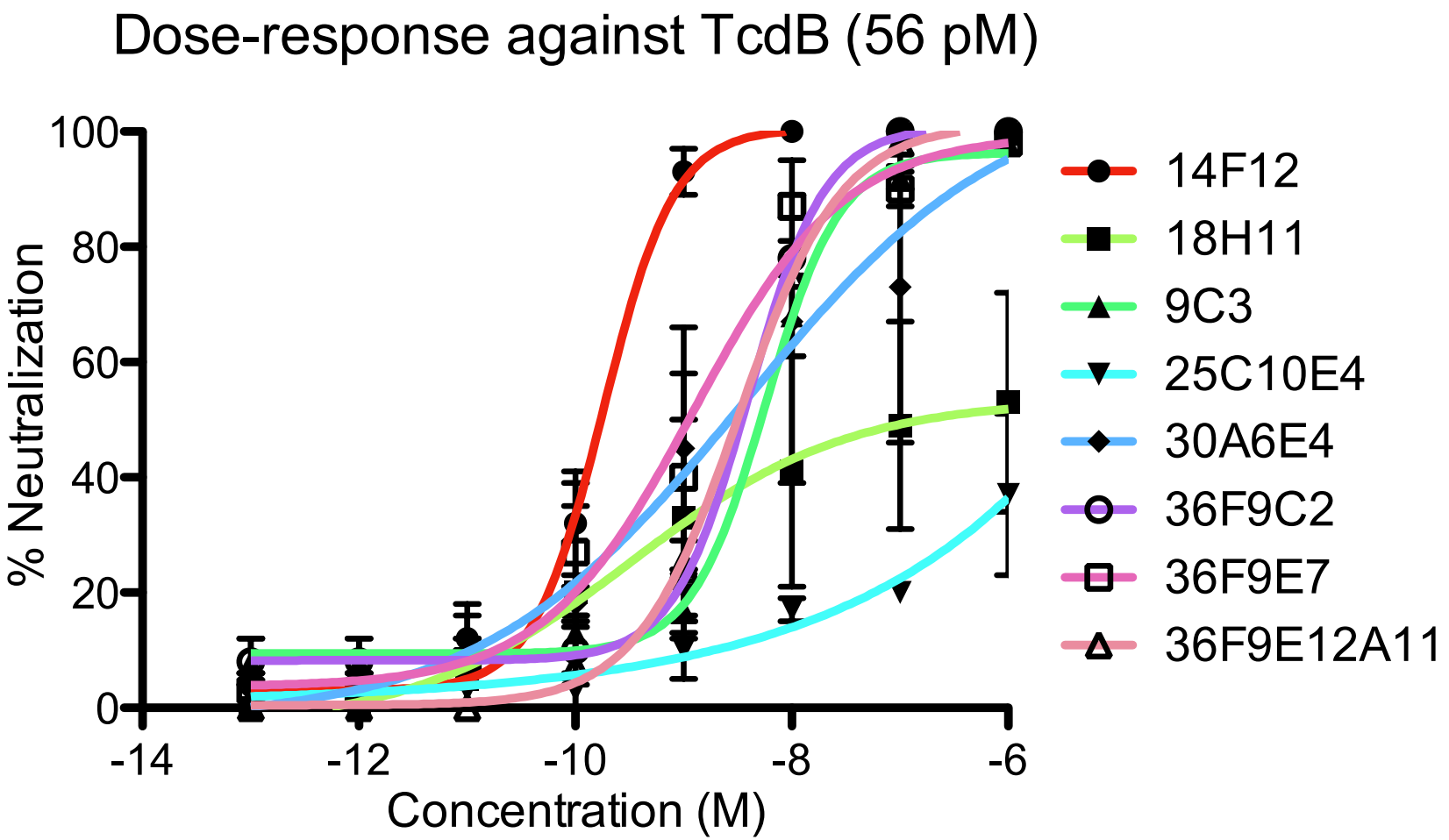
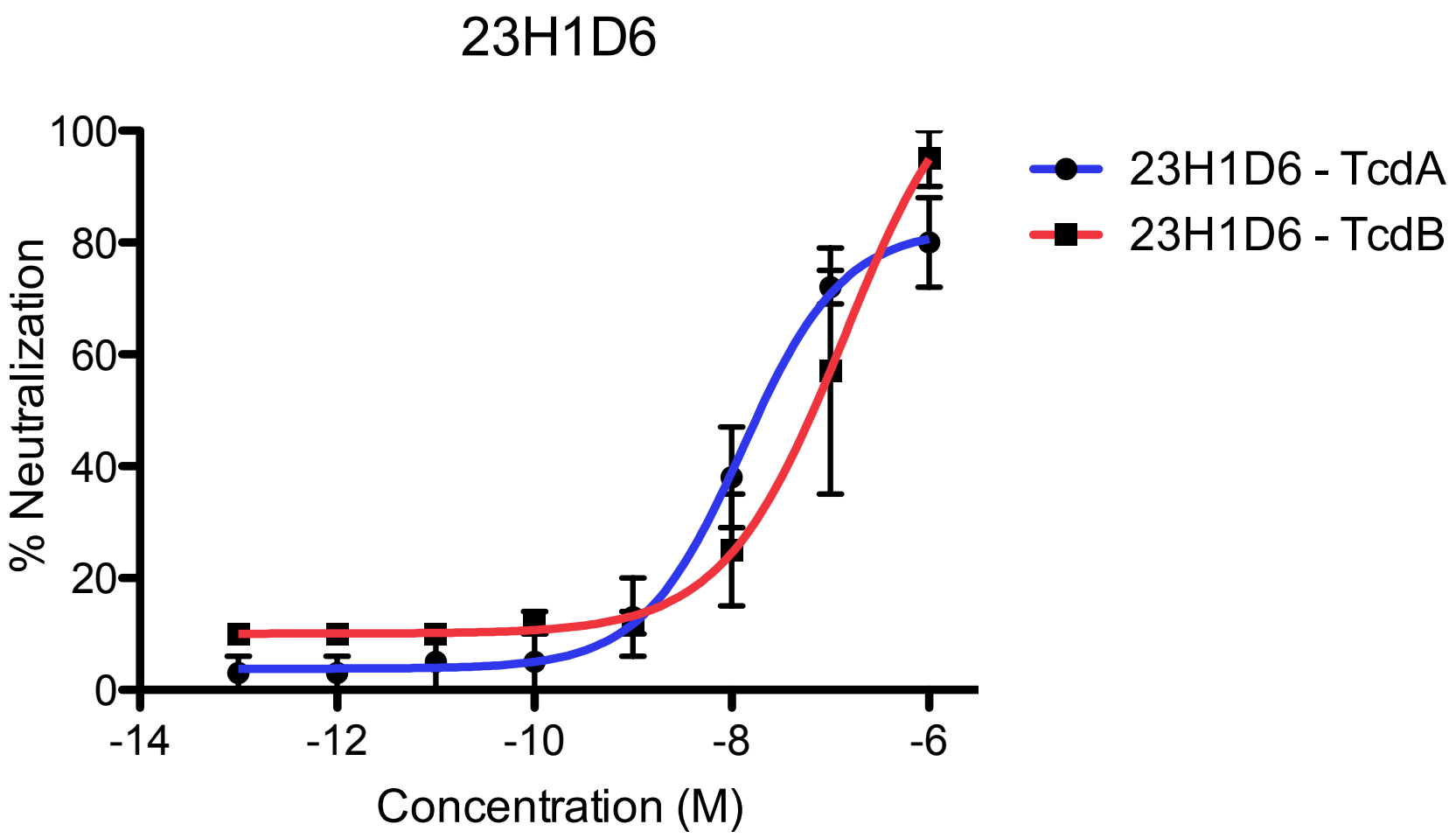
In vitro neutralization of TcdA (mAbs bind to TcdA on ELISA)



In vitro neutralization of TcdB (mAbs bind to TcdB on ELISA)



In vitro neutralization by 23H1D6



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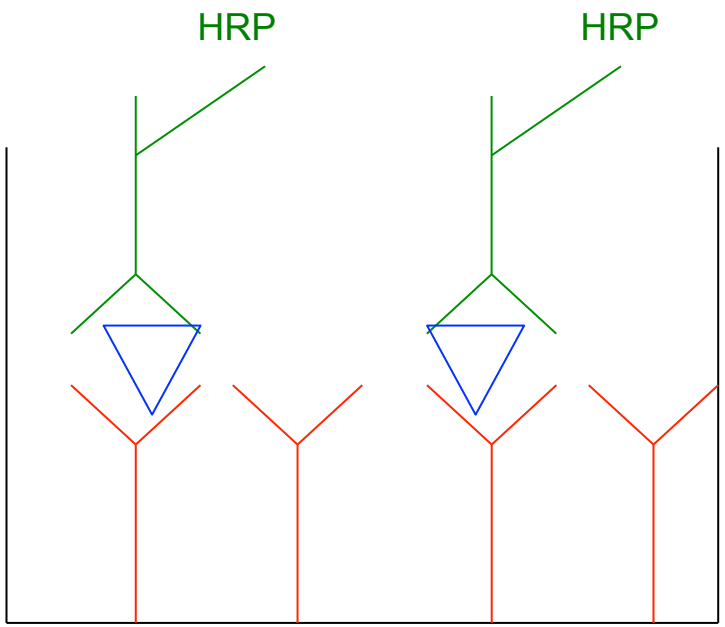
Characterization - Sandwich-ELISA

Do the different antibodies bind to the same epitope ?



Basic principle:

Coat Ab1

Incubate Antigen, i.e. TcdA or TcdB
(3-fold dilutions of the antigen)

Develop HRP-labeled-Ab2

Read-out:

- positive signal was determined as an OD which is 10-times higher than the negative control.
- negative signal was evaluated as the antibodies bind at the same epitope or in the same epitope region.

Antibodies against TcdA bind to 3 epitopes and against TcdB bind to 5 epitopes

Characterization - Biacore

	ka	kd	KA (1/M)	KD (M)
23A4G4	1,64e+04	6,34e-05	2,80e+08	3,85e-09
23A4G6	2,37e+04	9,11e-05	2,58e+08	3,92e-09
23H1D2	4,60e+04	5,45e-05	8,70e+08	1,17e-09
23H1D6	3,76e+04	2,62e-05	7,13e+10	6,26e-10

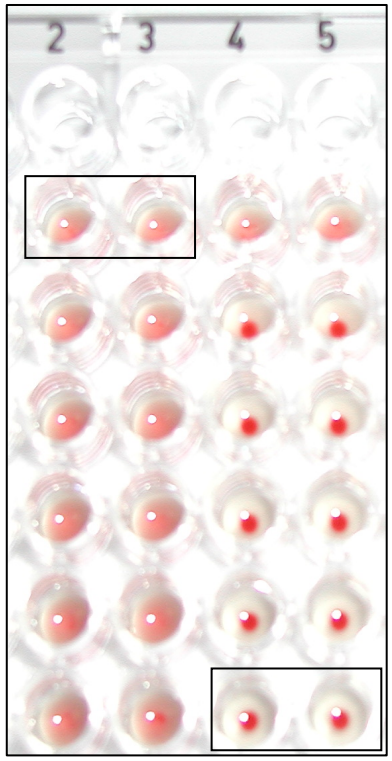
TcdA coupled at 1305 RU's, 110 µl, 5 µg/ml

In general, results indicate that although the mAbs have a slow association rate, they also have a (very) low dissociation rate.

There are no big differences between the mAbs tested.

Experiments were performed on Biacore 3000 at the Laboratory for Pharmaceutical Biology, KU Leuven

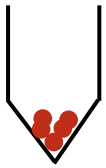
Characterization - Hemagglutination-assay



Highest conc. TcdA

Lowest conc. TcdA

Rabbit RBCs + TcdA
TcdA prevents clotting



Rabbit RBCs + TcdA +
mAb binding at receptor binding domain of TcdA

Rabbit RBCs + TcdA +
mAb **NOT** binding at receptor binding domain of TcdA

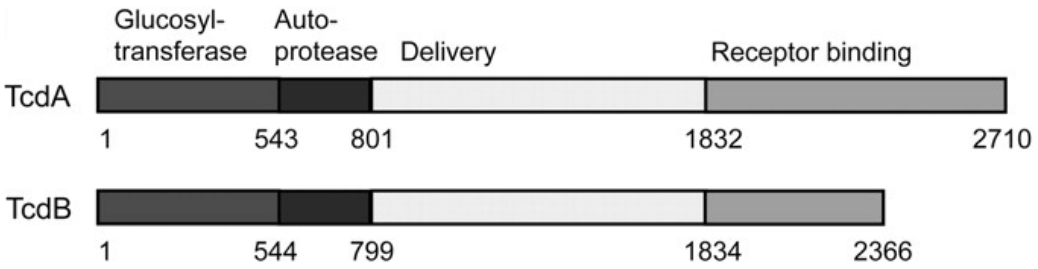


Figure adapted from Pruitt R N et al. PNAS 2010.

Characterization - Hemagglutination-assay

mAb	Concentration of the mAb					
	5,33 µM	533 nM	53,3 nM	5,33 nM	533 pM	53,3 pM
36F9E12C9	1	1	1	1,5	1	1
23A4G4	3	3	1,5	1	1	1
23A4G6	3	3	2	1	1	1
23H1D2	2,5	1,5	1	1	1	1
23H1D6	2,5	2	2	1	1	1
Conditions	TcdA (16,23 nM), Rabbit red blood cells (2%), average values of 2 experiments					

Scoring: 1 Hemagglutination, no clotting
2 Mixed phenotype
3 No hemagglutination, clotting

- 36F9E12C9 does not seem to bind at the receptor binding domain of the toxin
- 23H1D2/23H1D6 and 23A4G4/23A4G6 act very similar

Summary of *in vitro* results

- *In vitro* cell rounding assay
  - Strong neutralizers against TcdA and TcdB
  - One mAb shows neutralizing capacity against both toxins

- Sandwich-ELISA:
  - TcdA: mAbs recognize 3 different epitopes
  - TcdB: mAbs recognize 5 different epitopes

- Biacore:

  mAbs tested have similar Ka and Kd values

- Hemagglutination-assay
  - TcdA: 4 mAbs bind to the receptor binding domain, 1 does not
  - TcdB: not applicable

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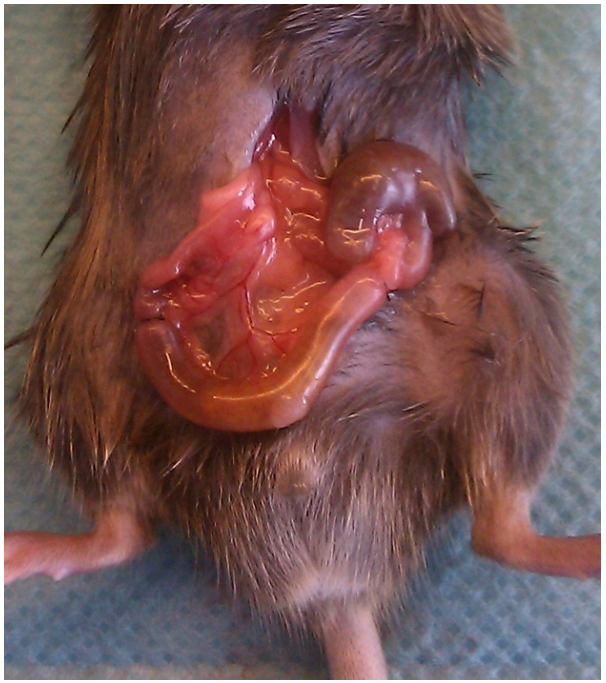
In vivo neutralization - Mouse Ileal Loop

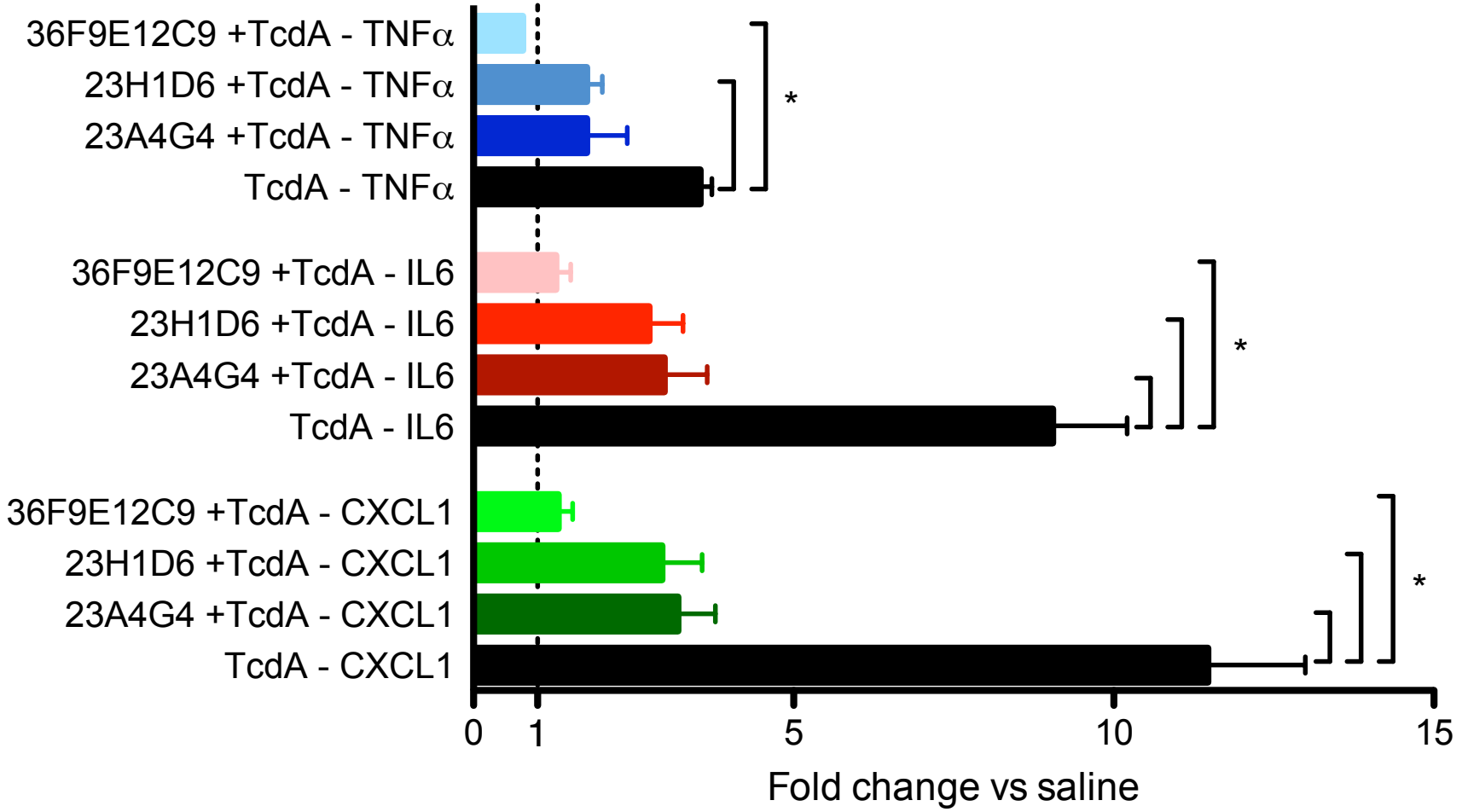
Set-up:

- C57Bl/6 mice (7-9 weeks old)
- Fasted o/n
- Loops of 2 – 3 cm long
- 2 µg TcdA/100 µl/loop
- Mice are sacrificed after 4 hours



	TcdA	23A4G4	23H1D6	36F9E12C9
Concentration per loop	64,93 nM	71,52 µM	113,31 µM	67,80 µM
Number of mice tested	9	6	6	3
Excess mAb (Molar)		1101,49	1745,11	1044,20

In vivo neutralization - Mouse Ileal Loop



Conclusions

- We were able to select in the *in vitro* cell rounding assay 5 mAbs against TcdA and 6 against TcdB.

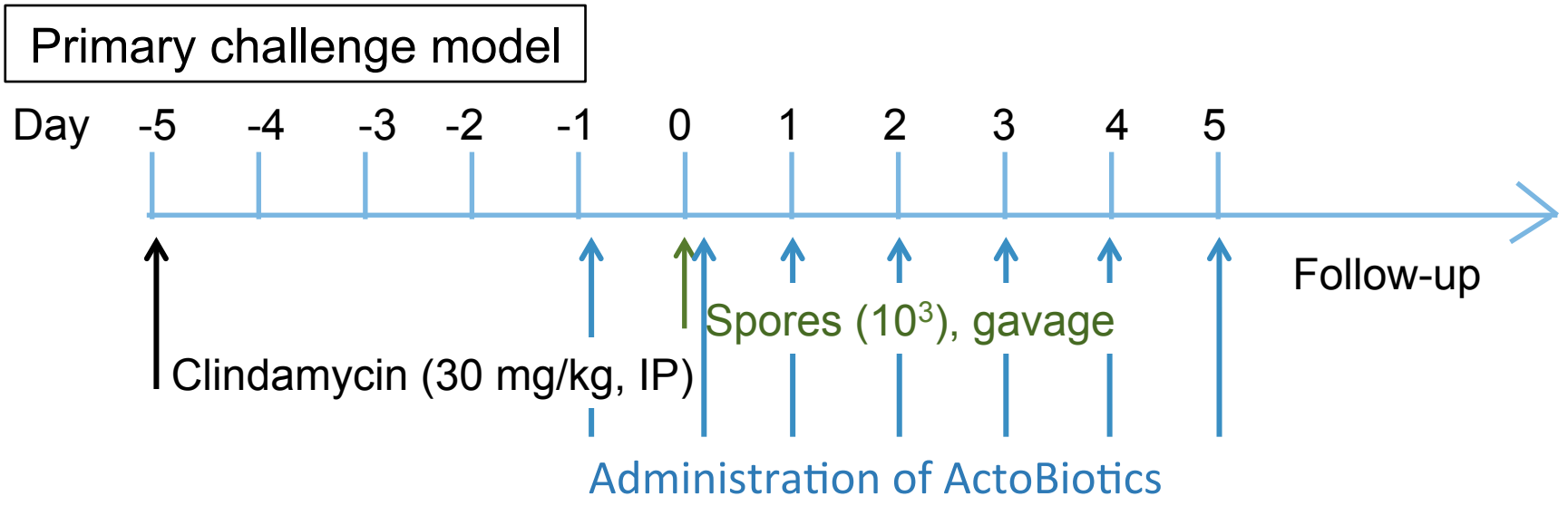
- These mAbs bind to distinct epitopes on the toxins as was seen in the sandwich-ELISA and hemagglutination assay.

- We selected 3 mAbs against each toxin and the mAbs against TcdA confirmed their neutralizing capacity *in vivo*.

Future perspectives

Fabs have been generated and will be tested for neutralizing activity in vitro in the cell rounding assay

ActoBiotics are currently generated by ActoGeniX, first tests in hamster model of CDAD will start at the beginning of 2013



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