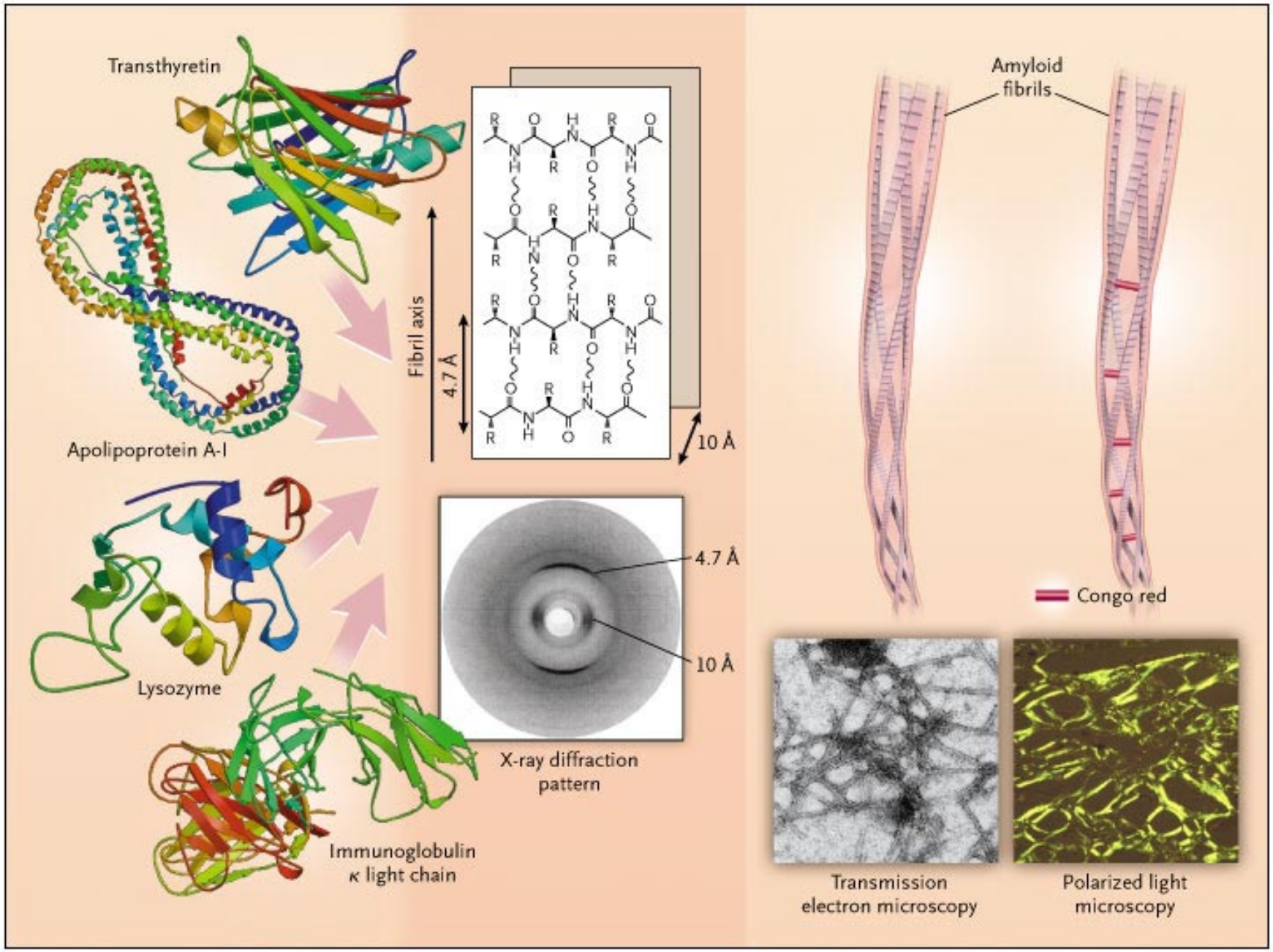
Small molecules and macromolecules for the inhibition of β 2 microglobulin amyloidogenesis.



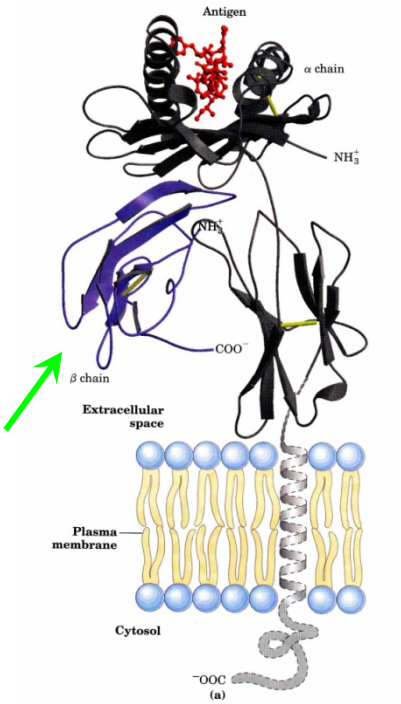
N Engl J Med 2003;349:583-96

Table 1. Amyloid Proteins and Their Precursors.*

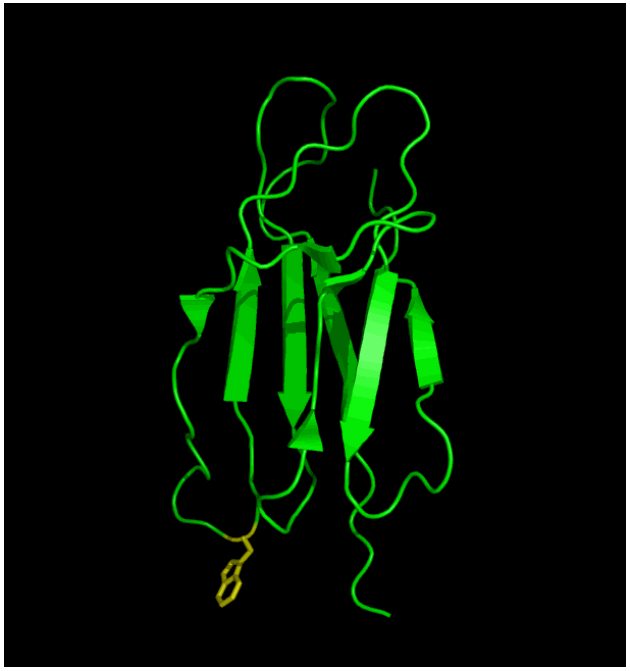
Amyloid Protein	Precursor	Distribution	Type	Syndrome or Involved Tissues
Aβ	Aβ protein precursor	Localized Localized	Acquired Hereditary	Sporadic Alzheimer's disease, aging Prototypical hereditary cerebral amyloid angiopathy, Dutch type
APrP	Prion protein	Localized Localized	Acquired Hereditary	Sporadic (iatrogenic) CJD, new variant CJD (alimentary?) Familial CJD, GSSD, FFI
ABri	ABri protein precursor	Localized or systemic?	Hereditary	British familial dementia
ACys	Cystatin C	Systemic	Hereditary	Icelandic hereditary cerebral amyloid angiopathy
Aβ2M	Beta$_2$-microglobulin	Systemic	Acquired	Chronic hemodialysis
AL	Immunoglobulin light chain	Systemic or localized	Acquired	Primary amyloidosis, myeloma-associated
AA	Serum amyloid A	Systemic	Acquired	Secondary amyloidosis, reactive to chronic infection or inflammation including hereditary periodic fever (FMF, TRAPS, HIDS, FCU, and MWS)
ATTR	Transthyretin	Systemic Systemic	Hereditary Acquired	Prototypical FAP Senile heart, vessels
AApoAI	Apolipoprotein A-I	Systemic	Hereditary	Liver, kidney, heart
AApoAII	Apolipoprotein A-II	Systemic	Hereditary	Kidney, heart
AGel	Gelsolin	Systemic	Hereditary	Finnish hereditary amyloidosis
ALys	Lysozyme	Systemic	Hereditary	Kidney, liver, spleen
AFib	Fibrinogen Aα chain	Systemic	Hereditary	Kidney

β2-m and dialysis related amyloidosis

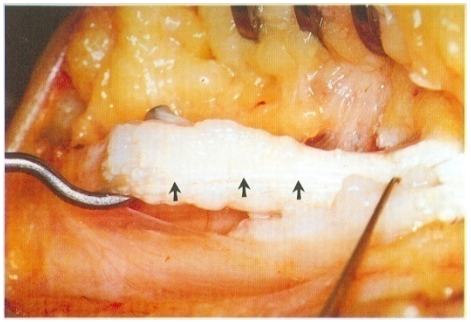
Cell membranes



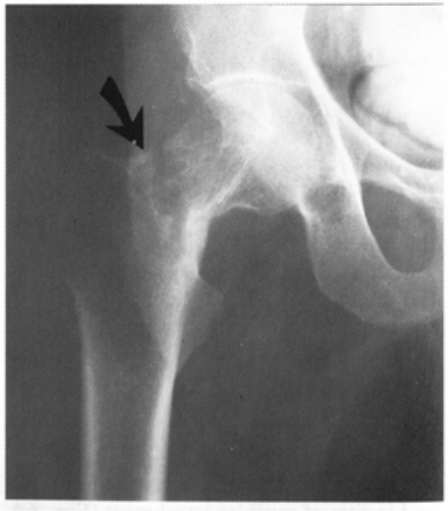
Plasma



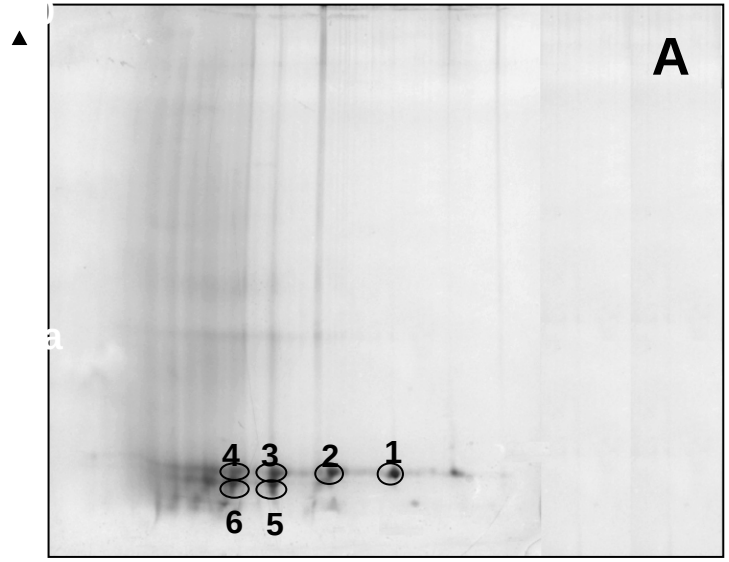
Amyloid deposits

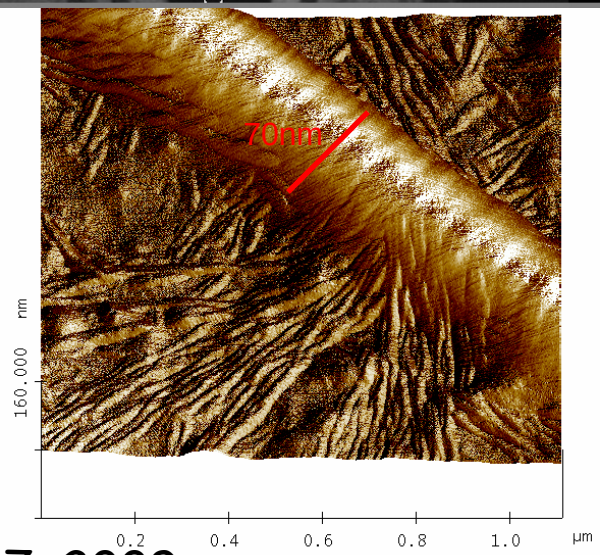


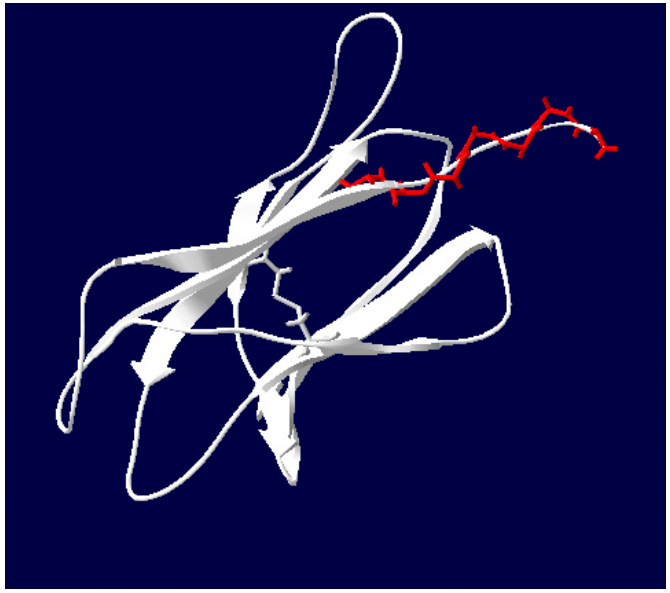
6B



Imaging, Microscopy and Proteomics analysis in molecular diagnosis





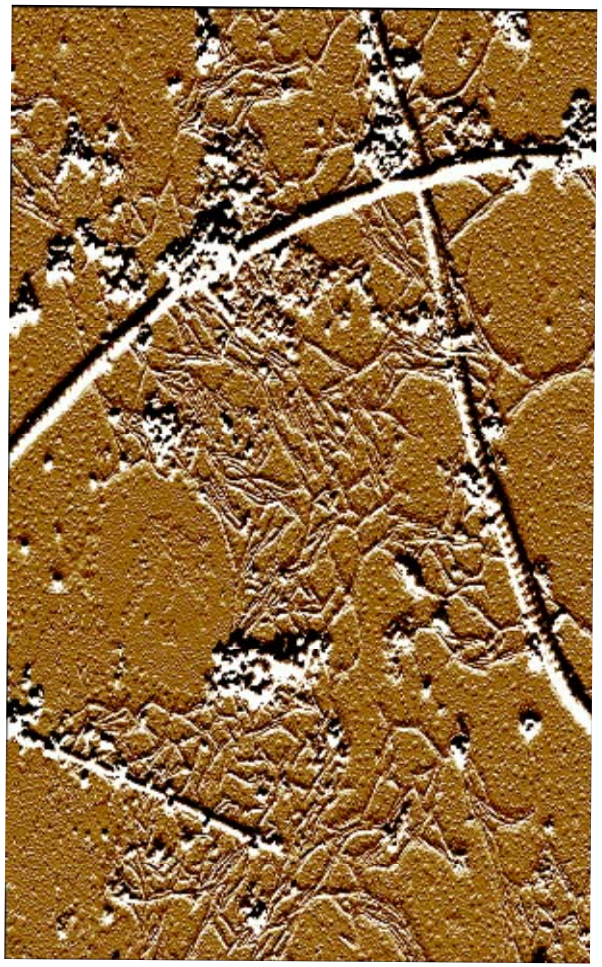


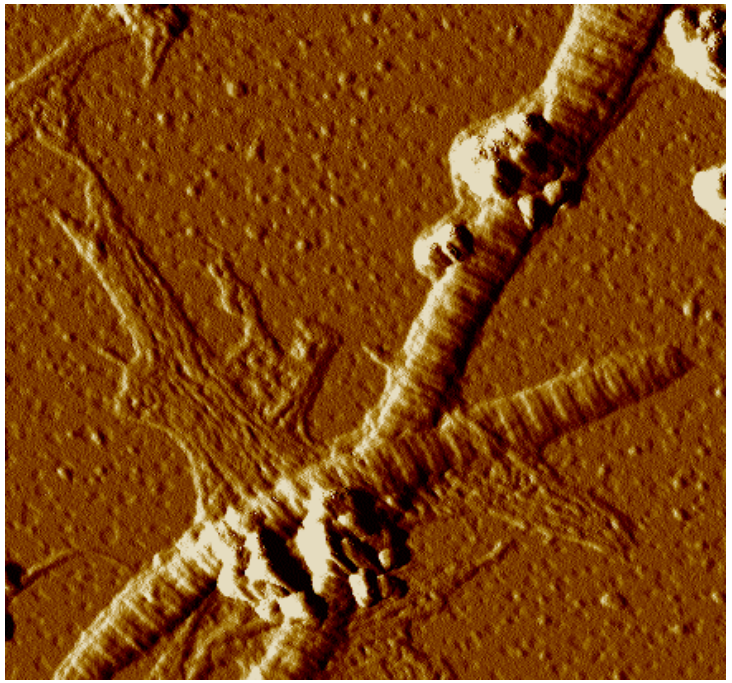
Amyloid **15**:1-7, 2009

Naiki, et al 1997.*Amyloid* **4:** 223–232	Na Citrate 50mM pH 2.5 - 4	β2-m 100 uM + seeds
McParland et al 2000. *Biochemistry* **39:** 8735–8746	Na citrate 50 mM pH 2.5 100 mM NaCl	β2-m 100 uM No seeds
Esposito et al *Protein Science* 2000, *9*:831–845.	Na Citrate 50 mM pH 6.5	β2-m N-terminal truncated 100 uM +seeds
Chiti et al *J Biol Chem.2001* 14; 276(50): 4714-21	Na Citrate 50 mM pH 7.3	Refolding intermediate 100 uM + seeds
Yamamoto al, 2004, J *Am Soc Nephrol,* 15 :126-133	Na Phosphate 50 mM 100 mM NaCl pH 7.4 20%TFE	β2-m 100 uM +seeds
Yamamoto al, *Biochemistry* 2004 43, 11075-11082 Kihara et al,2005,JBC,280:120 2-8	Na Phosphate 50 mM 100 mM NaCl pH 7.4 0.5% SDS	β2-m 25 uM +seeds
Relini A et al. J Biol Chem. 2006 ; 1:16521-9. J Biol Chem. 2008;283:4912-20	Ammonium Acetate 50mM pH 6.4, 20 uM heparin, fibrillar collagen type	β2-m 30uM
Borysik AJ, et al *Kidney Int*. 2007 2:174-81	PBS pH 7,4, GAGs	β2-m N-terminal truncated 200 υM

A potent promoter of fibrillogenesis on collagen is also heparin

β_2-m 0.1 mg/ml, heparin 3 μg/ml, t of amyloid fibrils observation= 24 h-37° c
physiologic model of β2-m amyloidogenesis



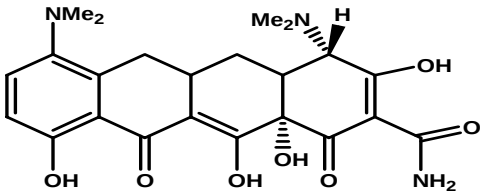


J Biol Chem. 2008;283:4912-20.

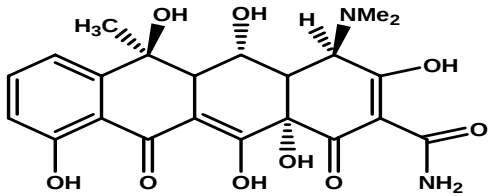
Identification of drugs targeting the amyloidogenic protein

1. Generic inhibitors of fibrillogenesis (tetracyclines)

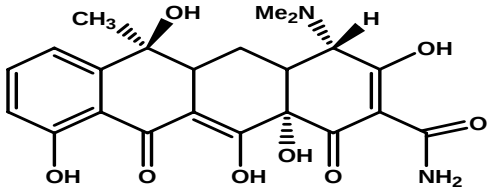
2. Specific interactors (antibodies)



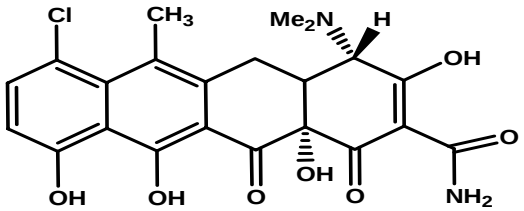
10-MINOCYCLINE



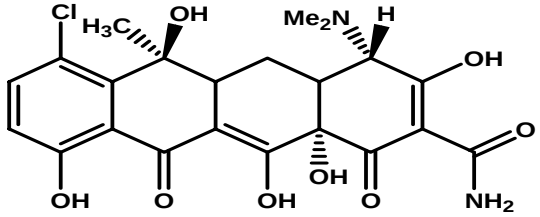
7- 4- EPIOXYTETRACYCLINE



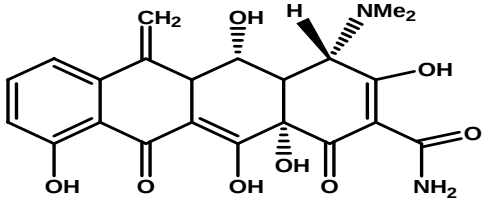
13-TETRACYCLINE



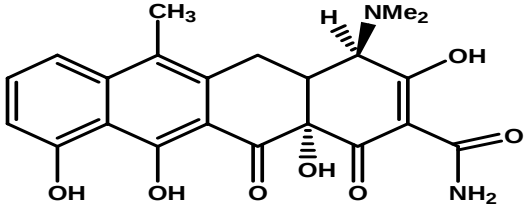
1-ANHYDROCHLORTETRACYCLINE



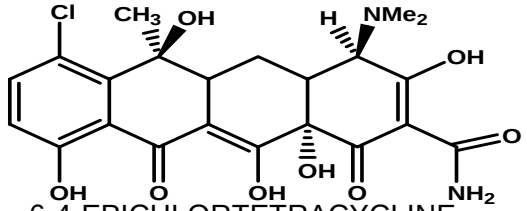
2-CHLORTETRACYCLINE



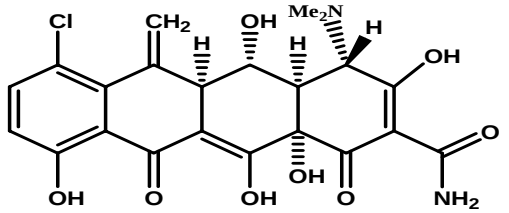
9- METHACYCLINE



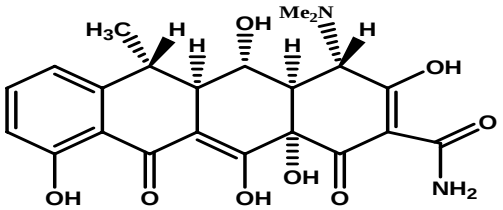
5-4-EPIANHYDROTETRACYCLINE



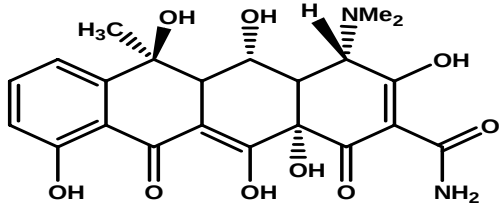
6-4-EPICHLORTETRACYCLINE



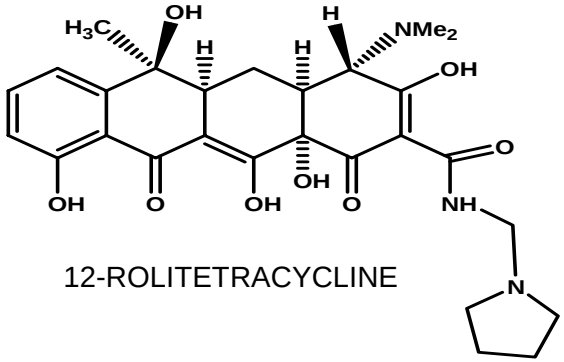
8-MECLOCYCLINE



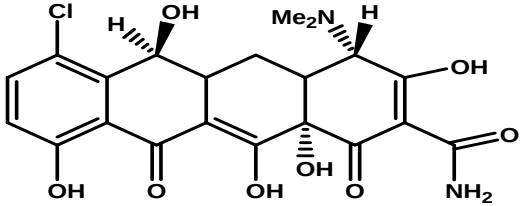
4-DOXYCYCLINE



11-OXYTETRACYCLINE



12-ROLITETRACYCLINE

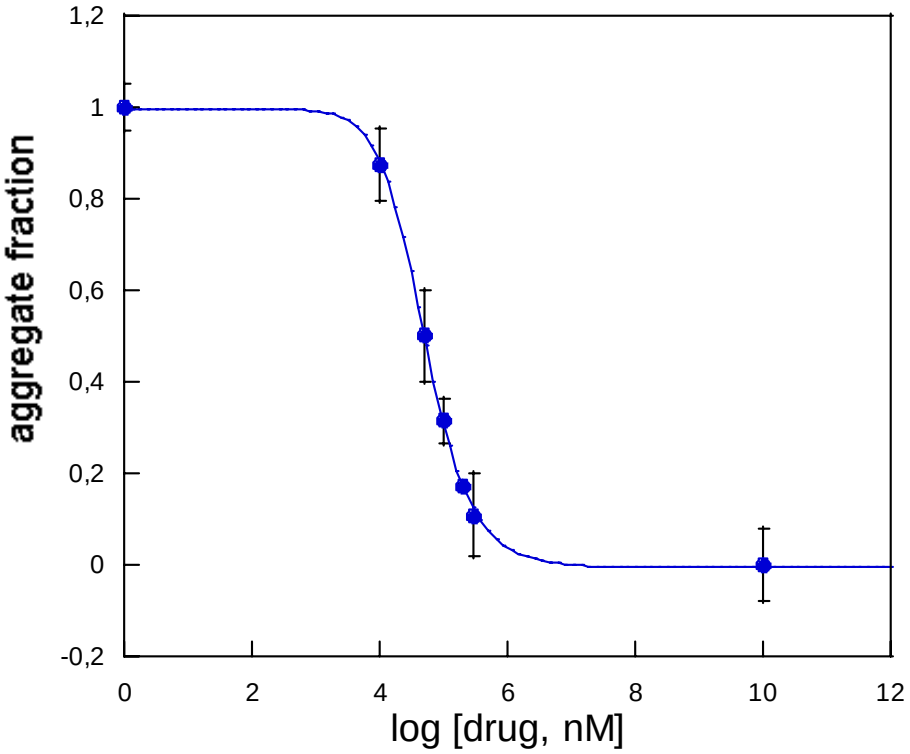


3-DEMECLOCYCLINE

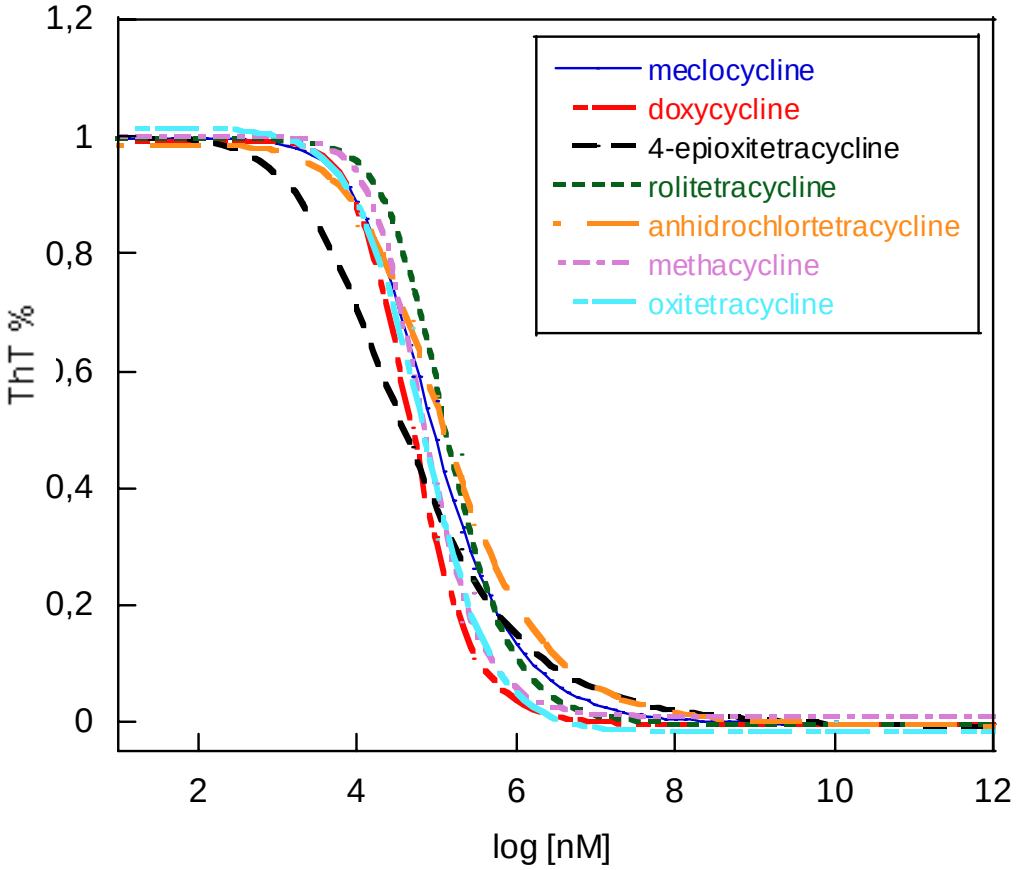
In collaboration with Mario Salmona Istituto Mario Negri

β2-m fibrillogenesis in the presence of TFE

Yamamoto S, et al. J Am Soc Nephrol. 15:126-33, 2004

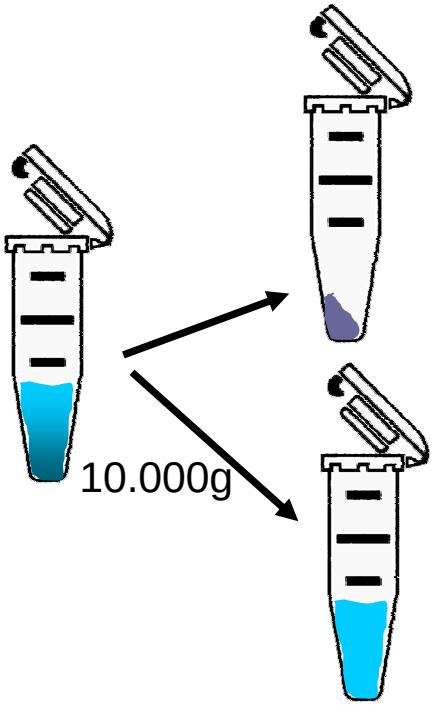


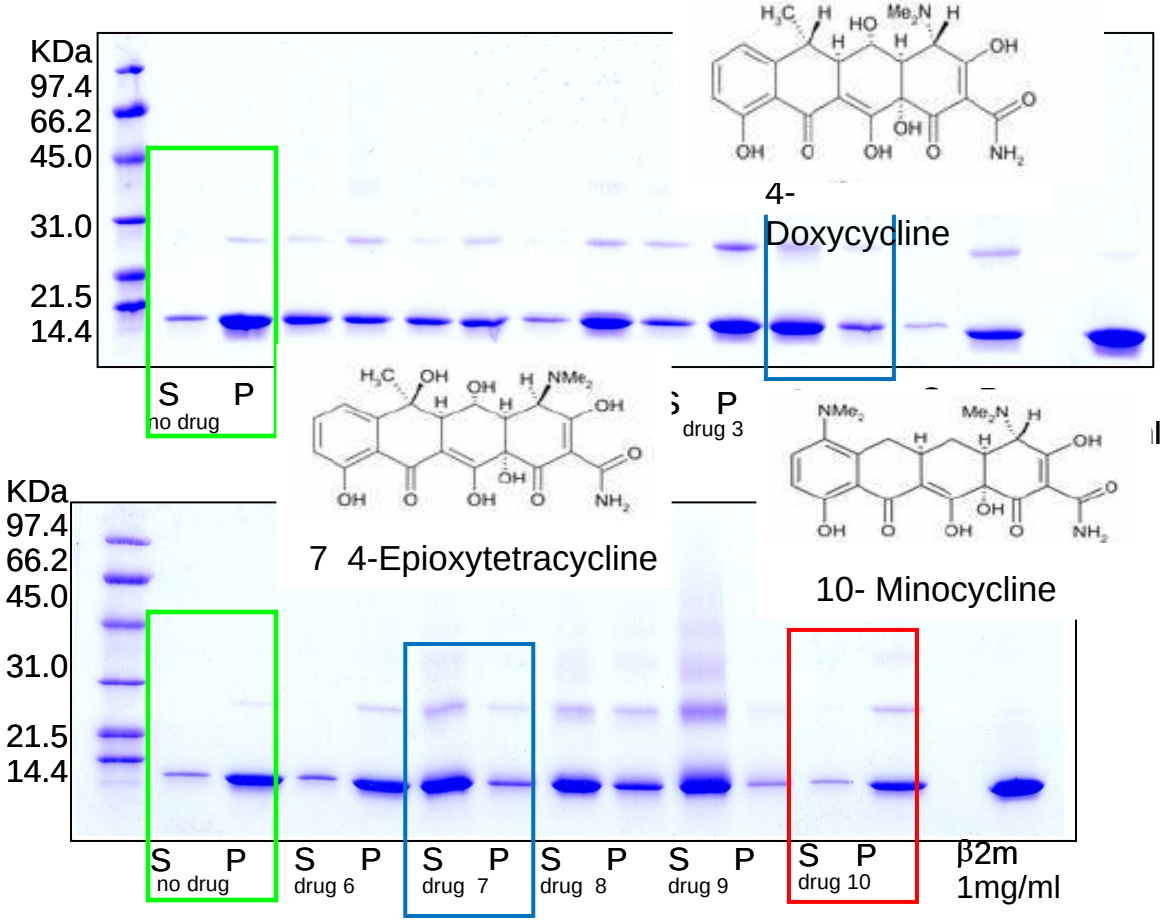
IC 50 : 50 ± 10μM

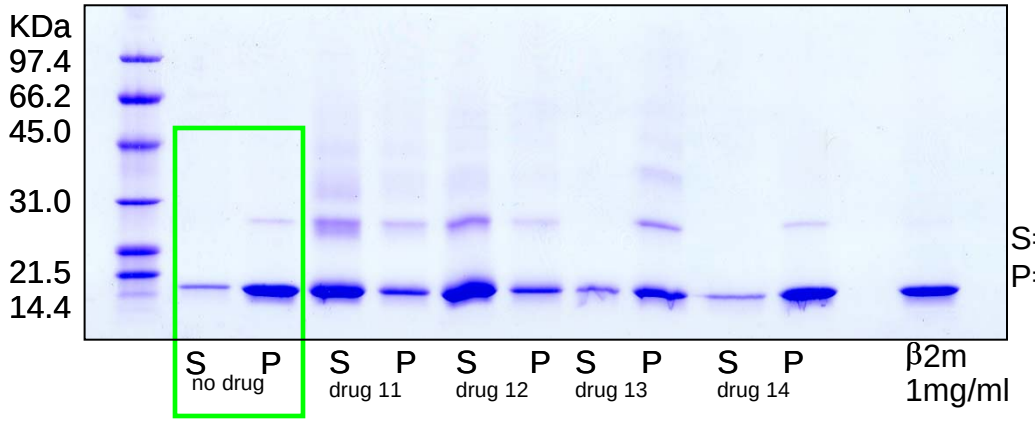


Drug	IC_{50}(μM)
Anhidrochlortetracycline	135±9
Methacycline	71±9
Oxitetracycline	69±5
Doxycycline	50±5
4-epioxitetracycline	40±9
Meclocycline	94±12
Rolitetracycline	135±10

Analysis of soluble fraction

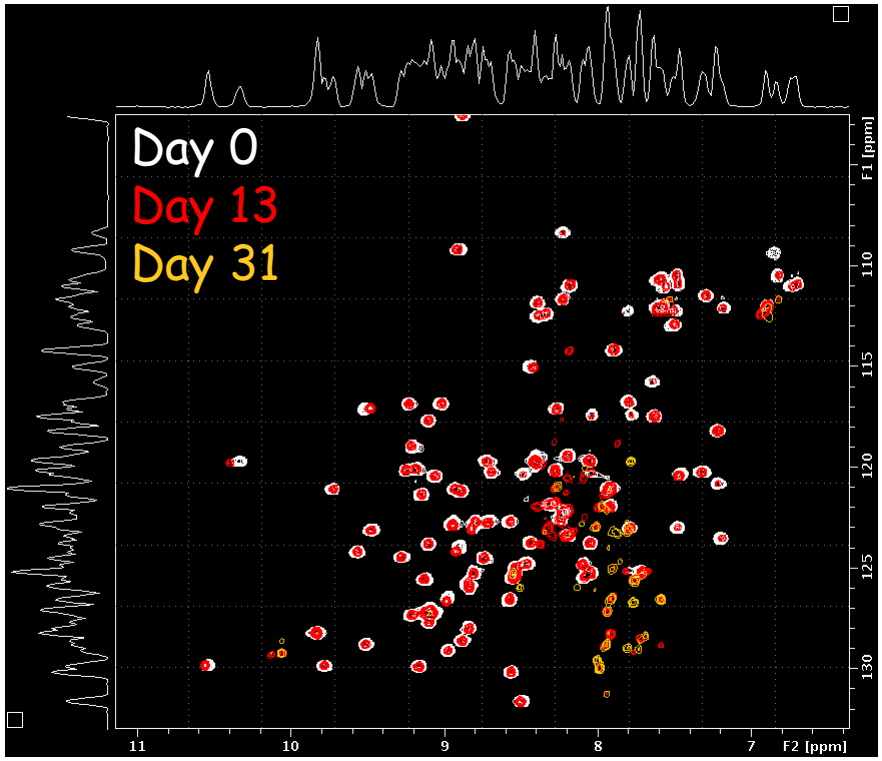




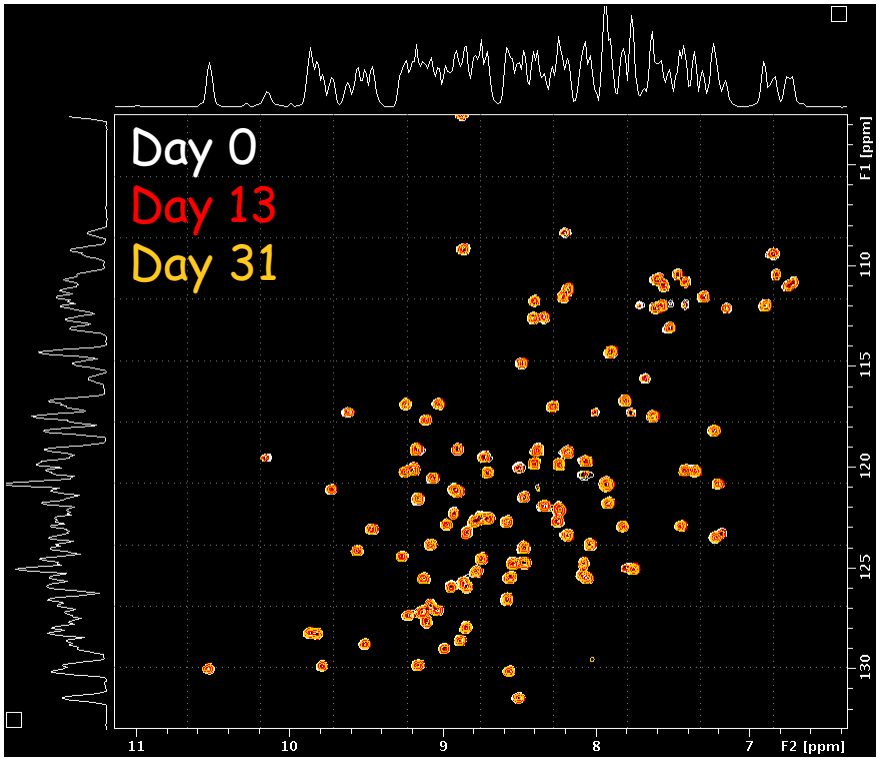


S= supernatante
P= pellet

Doxycycline stabilyzes the native form of β2-m



- doxycicline



+ doxycicline

3:1

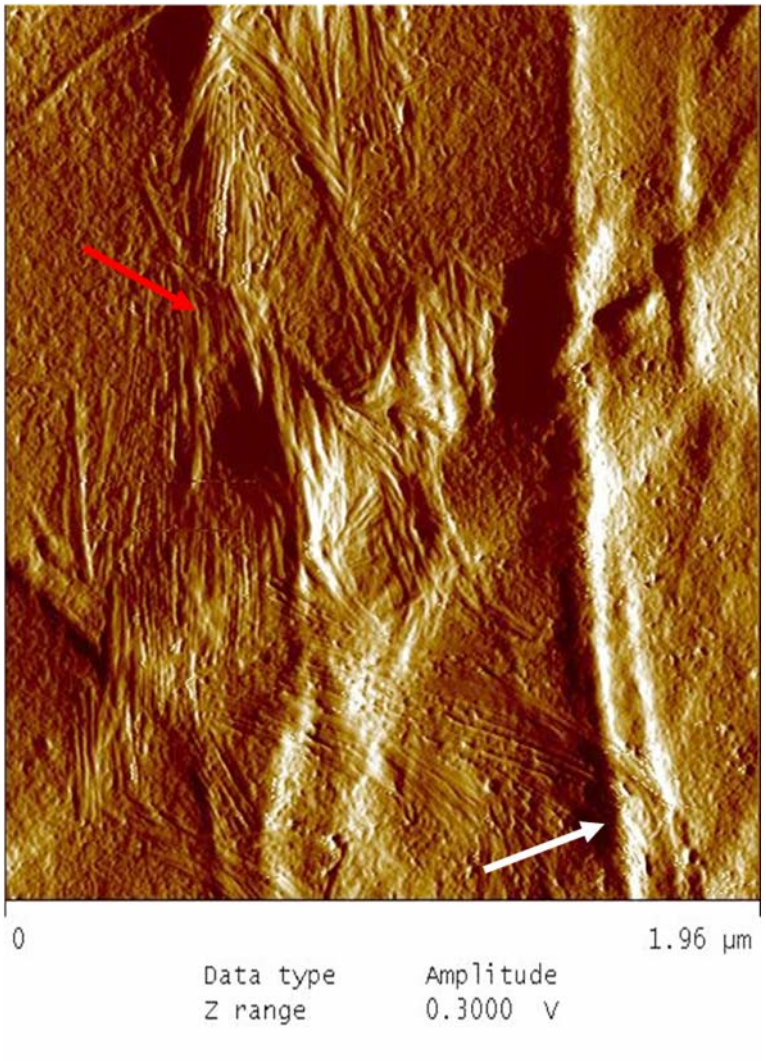
strand A-B
Loop AB

N-terminal
loop BC,DE,FG

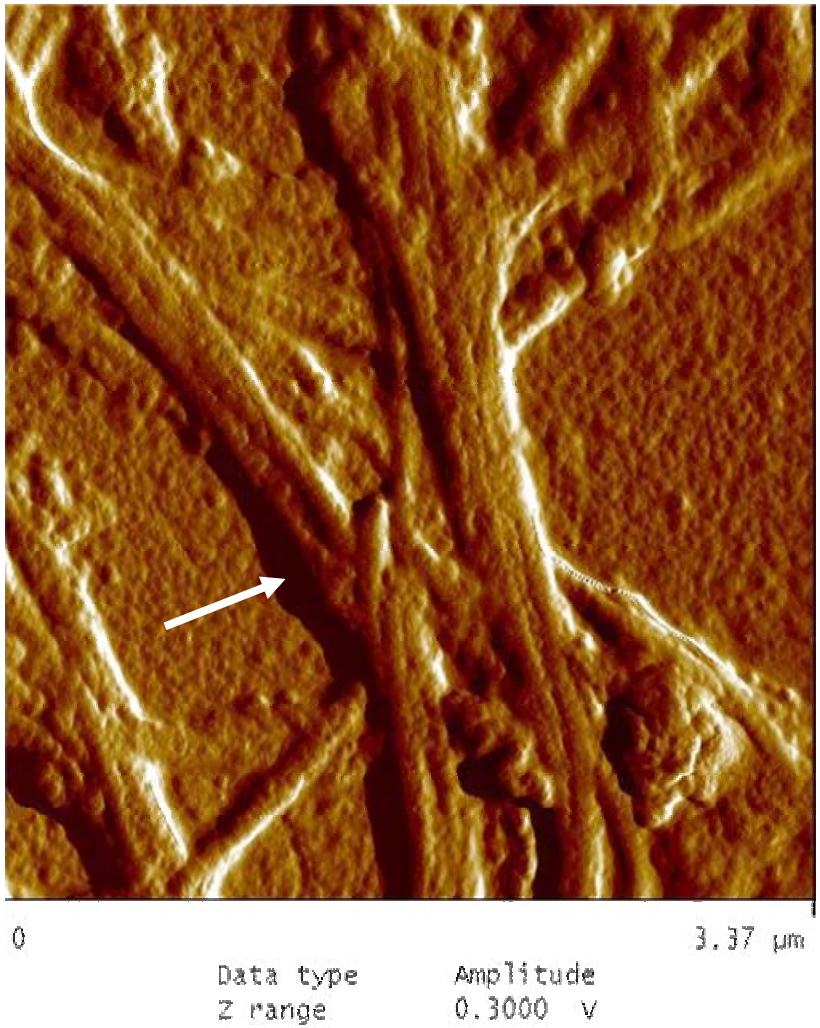
$\overline{\Delta\delta} + \sigma < \Delta\delta \leq \overline{\Delta\delta} + 2\sigma$

$\Delta\delta > \overline{\Delta\delta} + 2\sigma$

β2-m fibrillogenesis in the presence of fibrillar collagen

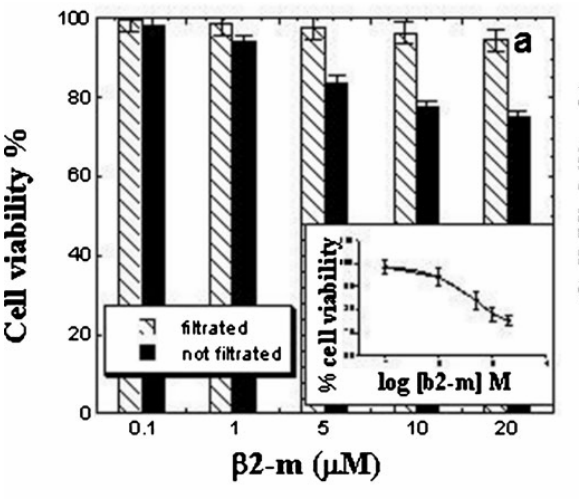


- doxycycline

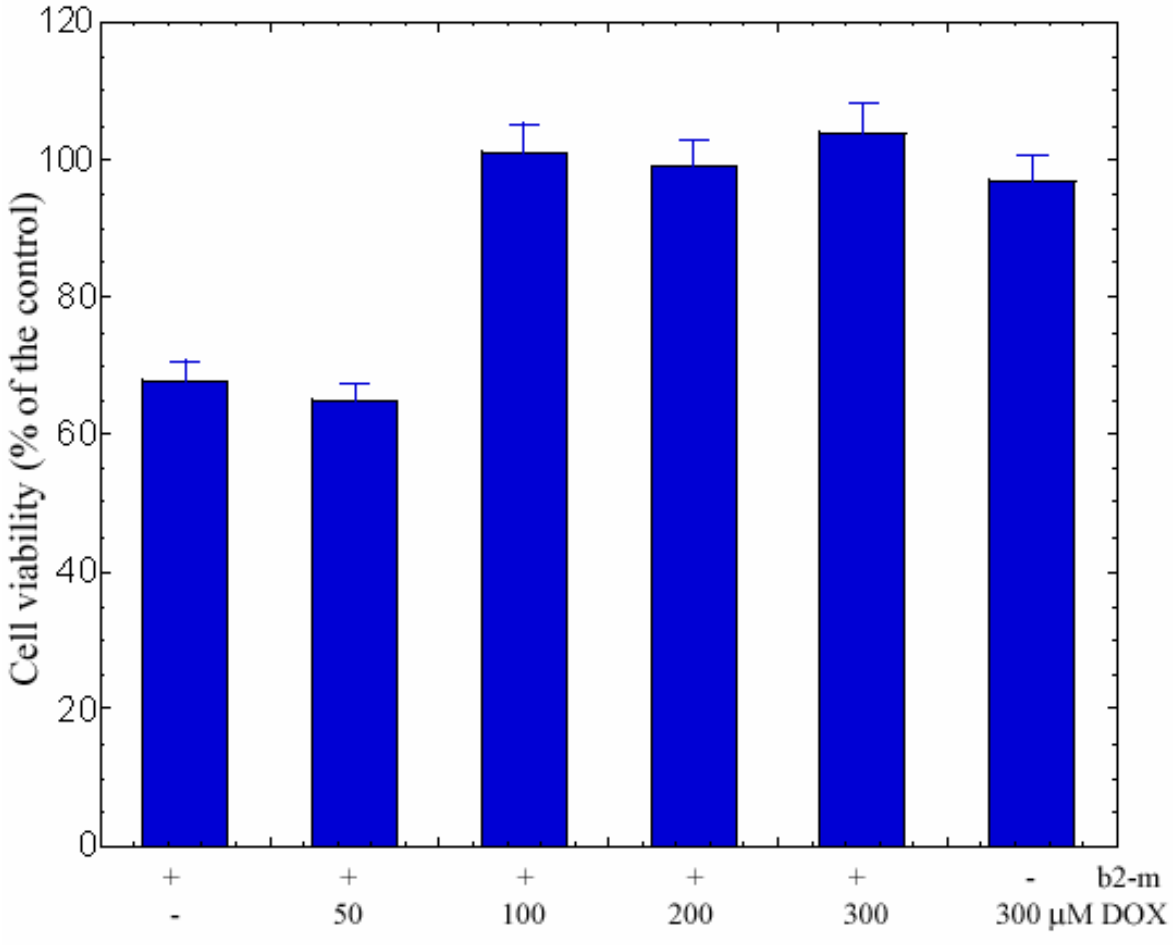


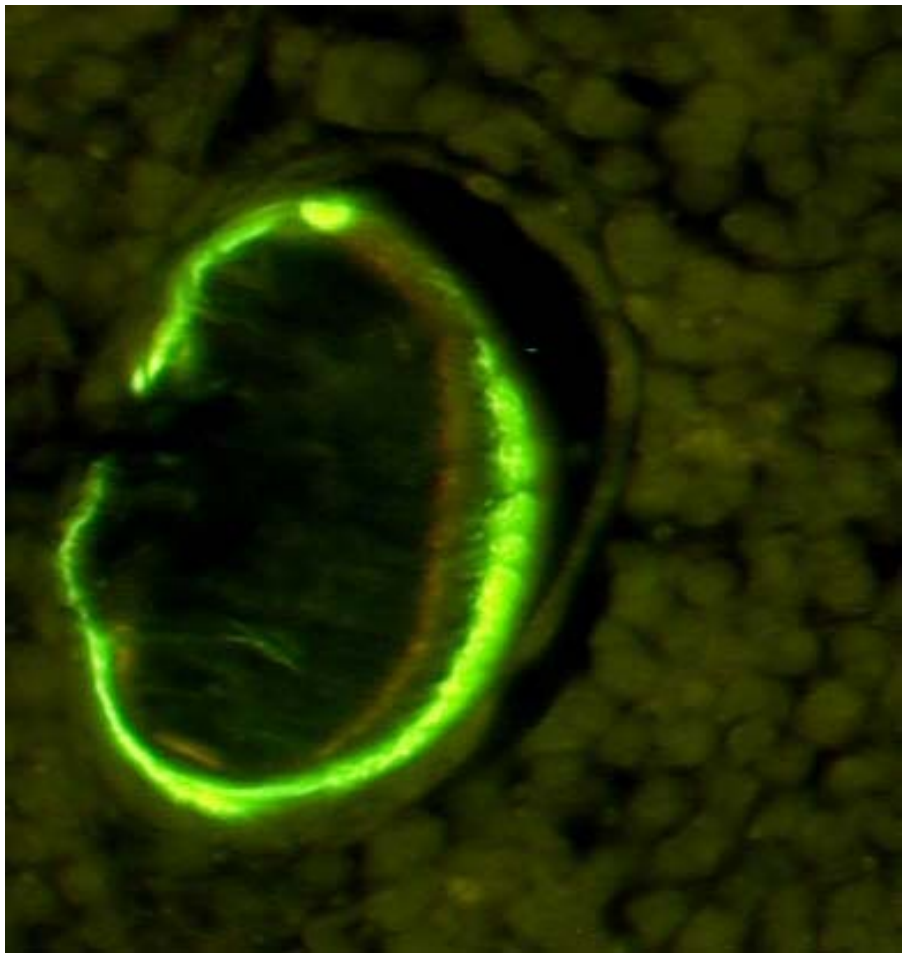
+ doxycycline

The cytotoxic effect of β2-m soluble in the presence of different amount of Doxycycline on SHSY-5Y cell viability valued by MTT reduction test.



Girgetti et al. Nephrol Dial Transplant. 2009;24:1176-81





(rate of concentration tygecycline bone/plasma > 2000)

Agwuh et al. J Antimicrobial Chemotherapy 58, 256-65, (2006)

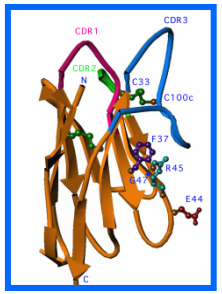
Conclusion:

In-vitro tuning of bio-mimicking models of amyloidogenesis provide a strong support for translating into clinical trial the evidences of the potent anti-amyloidogenic properties of the tetracyclines selected analogues

Lode Wyns

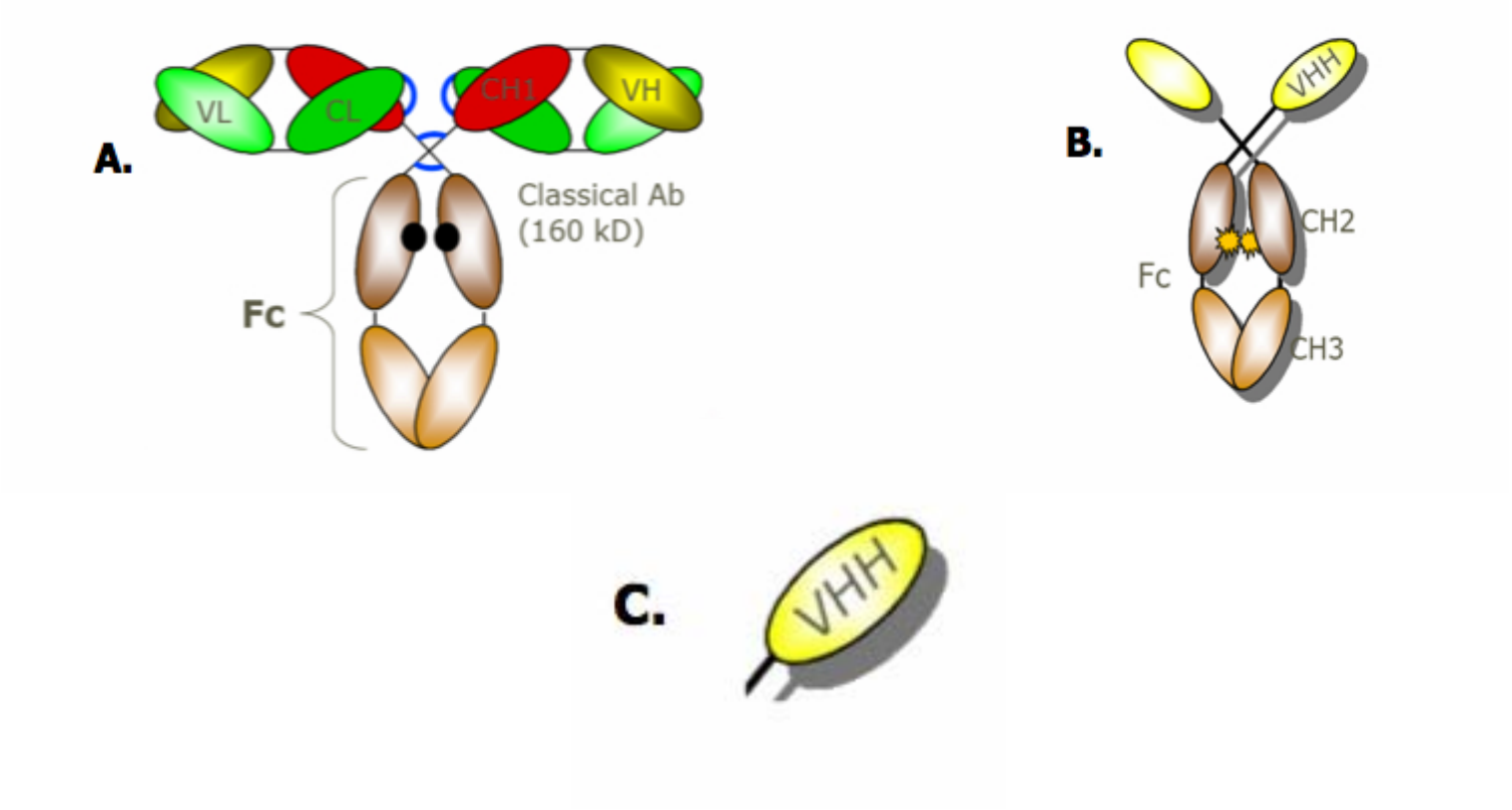
Mireille Dumoulin

http://www.vib.be/VIB/EN/



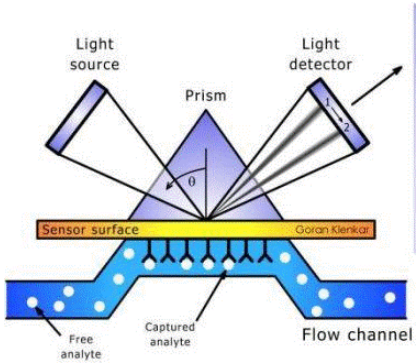
Nanobodies

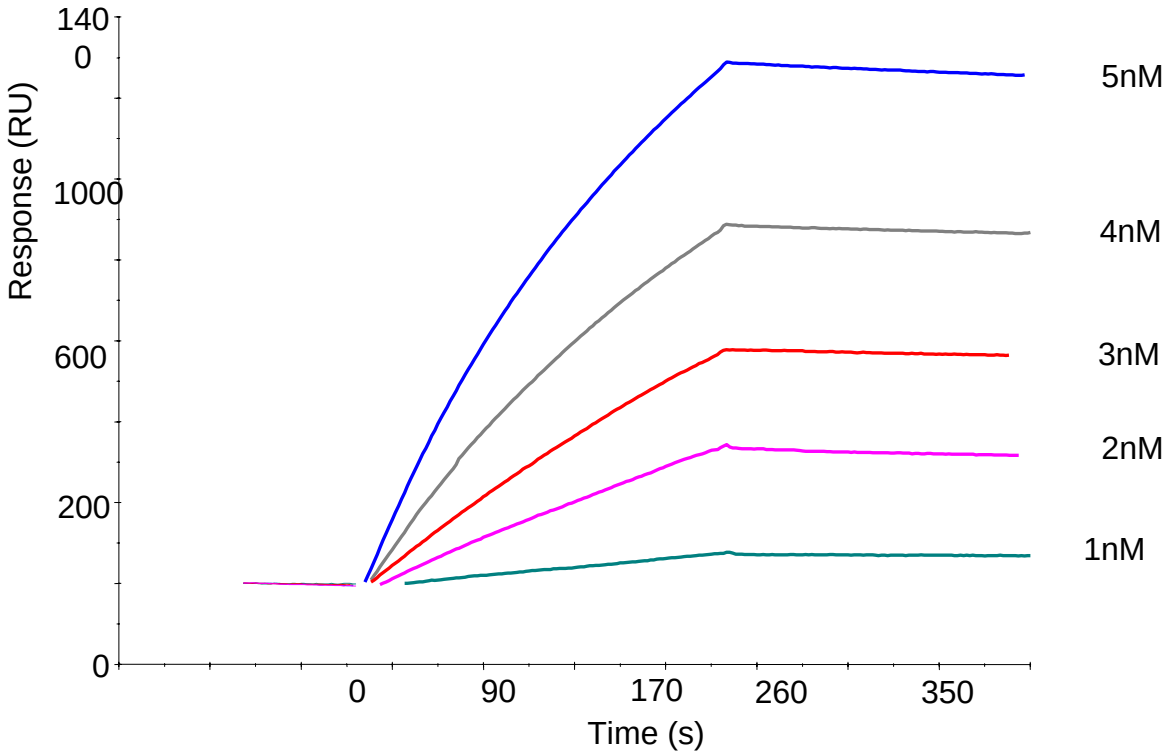
	ml tot	Mg tot
Nb_20a (=Nb_b2m1a) EP502 1,333mg/ml VUB-ULTR 07/03/07	5ml	6,665mg
Nb_20b (=Nb_b2m1b) EP503 1,017mg/ml VUB-ULTR 07/03/07	5ml	5,085mg
Nb_21(=Nb_b2m4) EP539 0,752mg/ml VUB-ULTR 07/03/07	6,5ml	4,888mg
Nb_22a (=Nb_b2m2a) EP505 1,153mg/ml VUB-ULTR 07/03/07	4ml	4,612mg
Nb_24 (=Nb_b2m3) EP506 1,989mg/ml VUB-ULTR 07/03/07	5ml	9,945mg
Nb_25 (=Nb_b2m5) EP668 1,916mg/ml VUB-ULTR 07/03/07	4ml	7,664mg
Nb_29a (Δb2m) CA94 0,441mg/ml VUB-ULTR 07/03/07	10ml	4,41mg
Nb_29c (Δb2m) CA69 0,437mg/ml VUB-ULTR 07/03/07	13,5ml	5,8995mg
Nb_31 (Δb2m) CA7069 0,417mg/ml VUB-ULTR 07/03/07	9,5ml	3,9615mg



- High affinity
- High stability
- High diffusibility
- Easy humanization
- Easy engineering

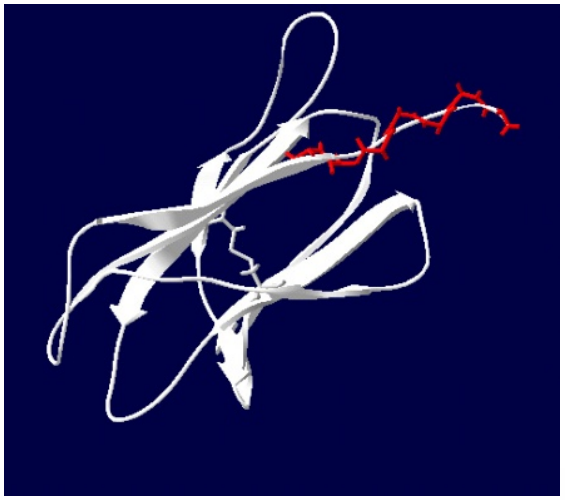
Affinity characterization by surface plasmone resonance

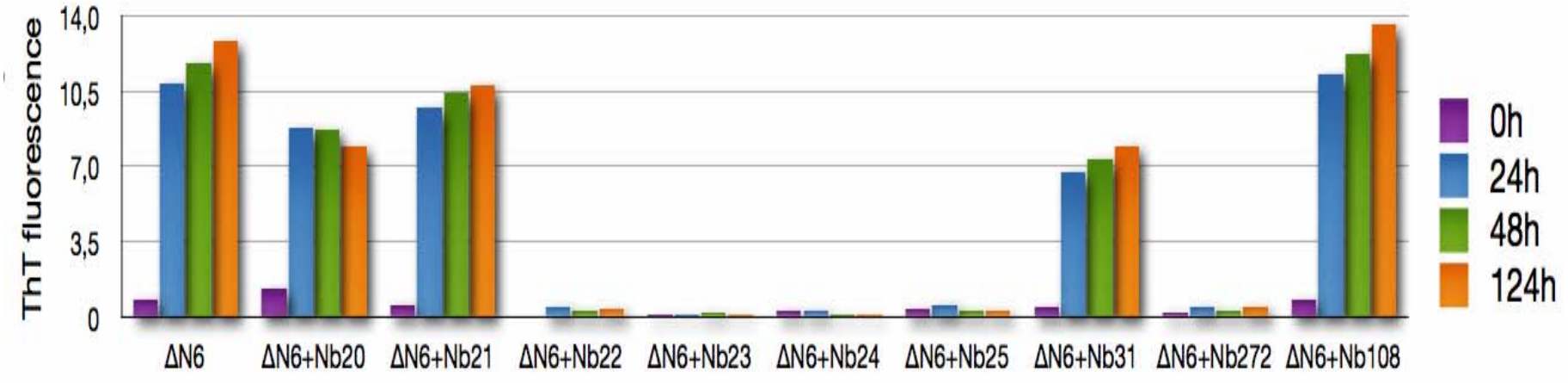




Dissociation constants in nM:

Nb	**20a**	**22a**	**23a**	**24**	**30a**	**30b**	**31**	**272b**	**273**
K_D [nM] b2m	24	269	50	58	2,6	1,6	6,8	129	52
K_D [nM] ΔN6 b2m	35	330	54	44	11,0	6,7	8,4	72	50

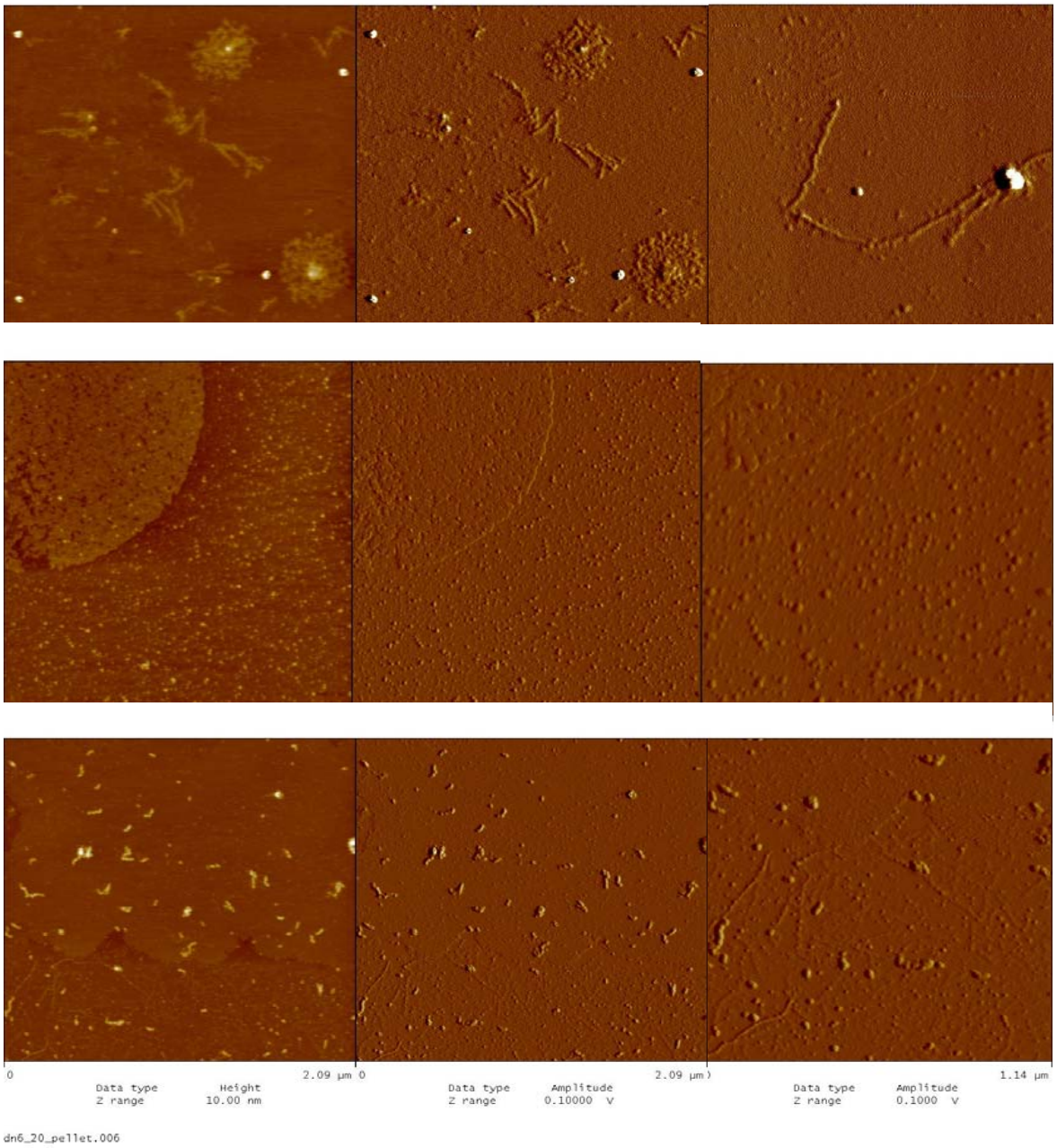



ThT fluorescence
14,0
10,5
7,0
3,5
0
ΔN6
ΔN6+Nb20
ΔN6+Nb21
ΔN6+Nb22
ΔN6+Nb23
ΔN6+Nb24
ΔN6+Nb25
ΔN6+Nb31
ΔN6+Nb272
ΔN6+Nb108
0h
24h
48h
124h

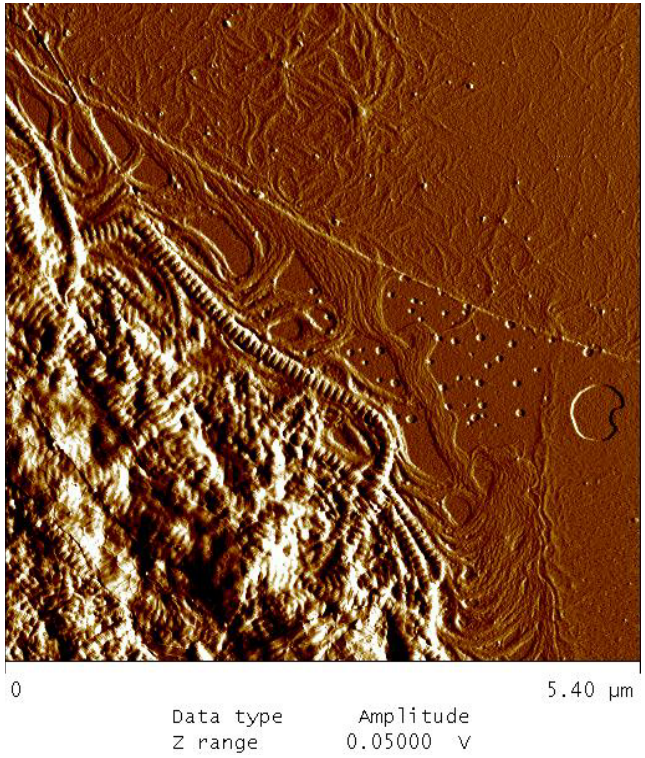
ΔN6b2m

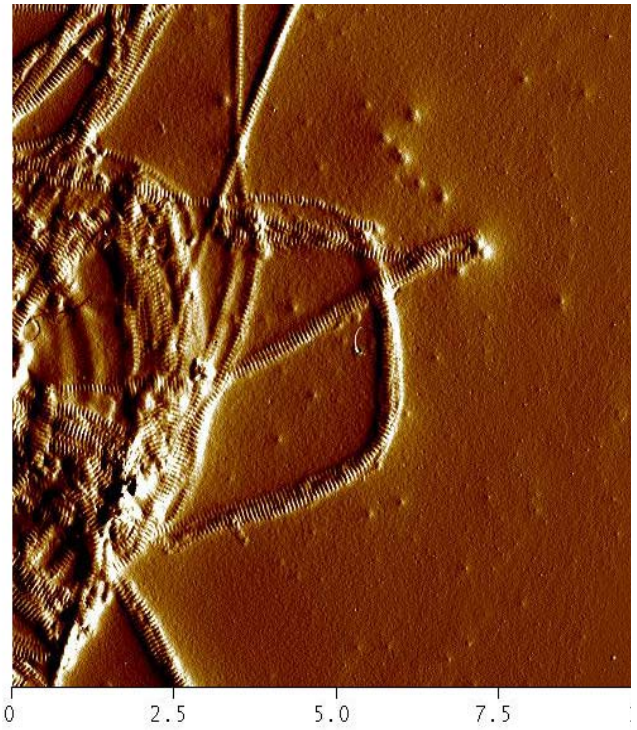
ΔN6b2m+Nb23a

ΔN6b2m+Nb20a



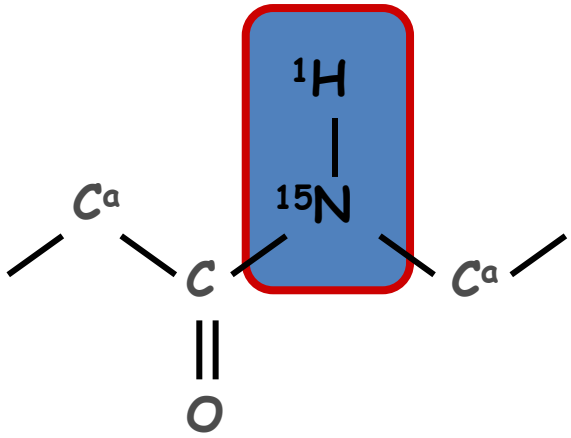
β2-m fibrillogenesis in presence of fibrillar collagen





Epitopes characterization by **NMR studies**

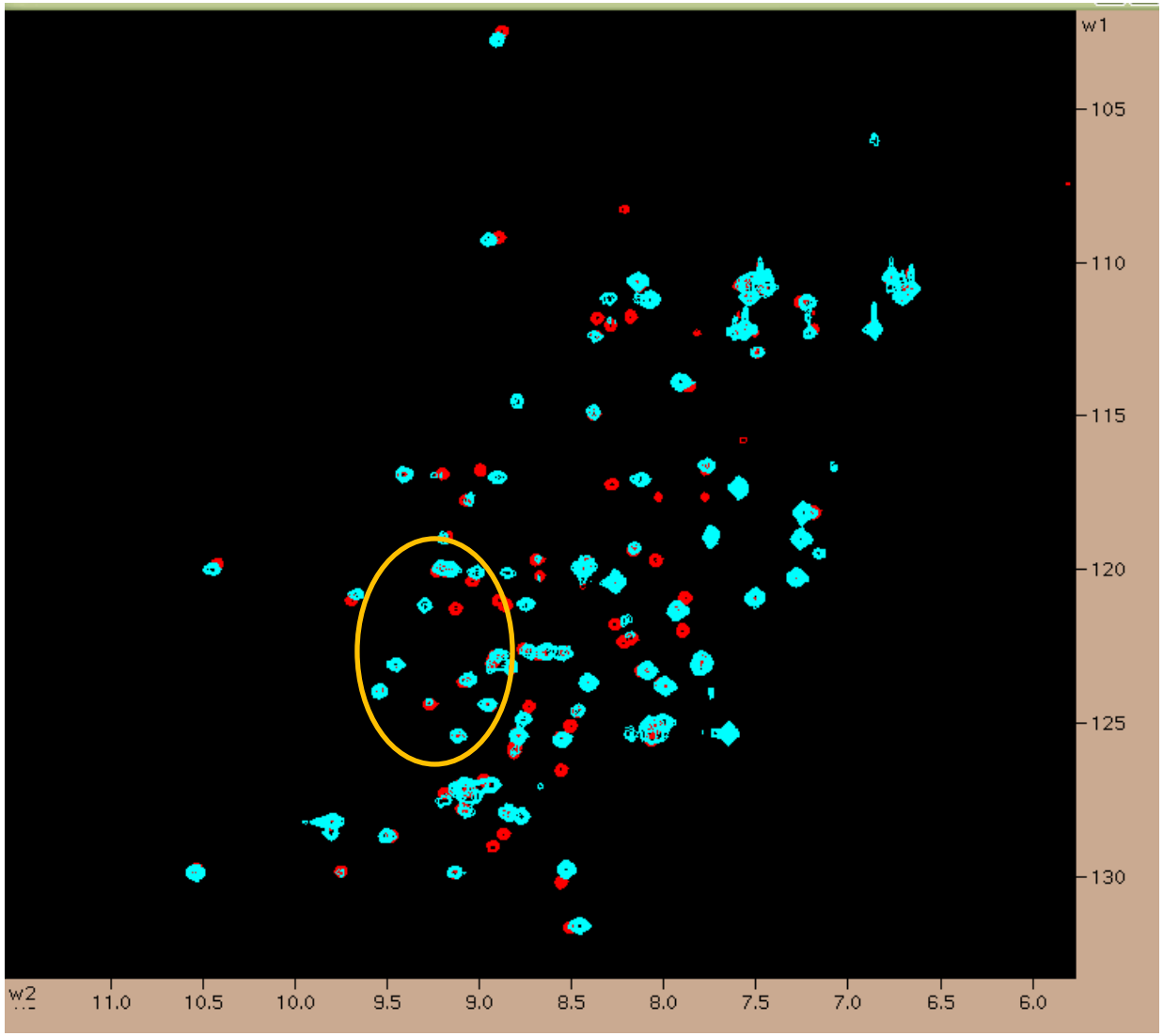
^{15}N-HSQC: Titration of Unlabelled nb-23a into Labelled β2m

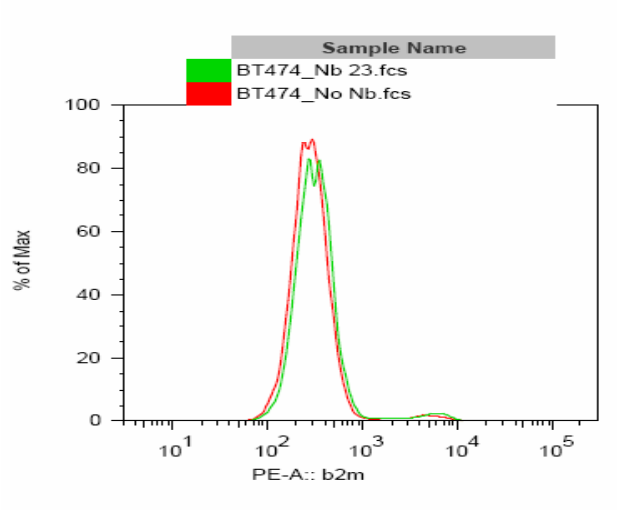


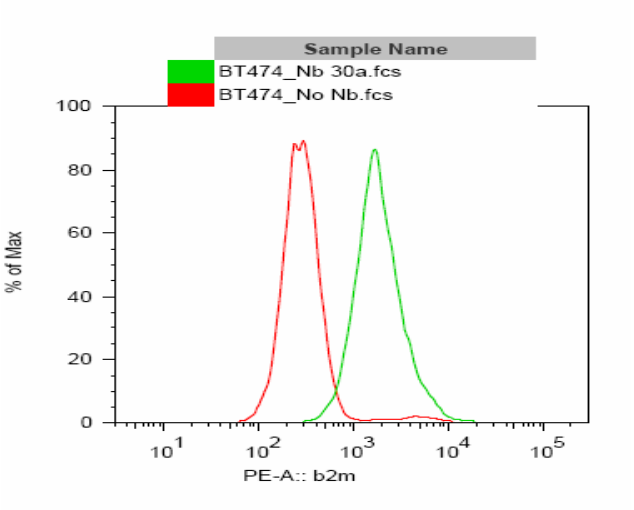
β2m:nb

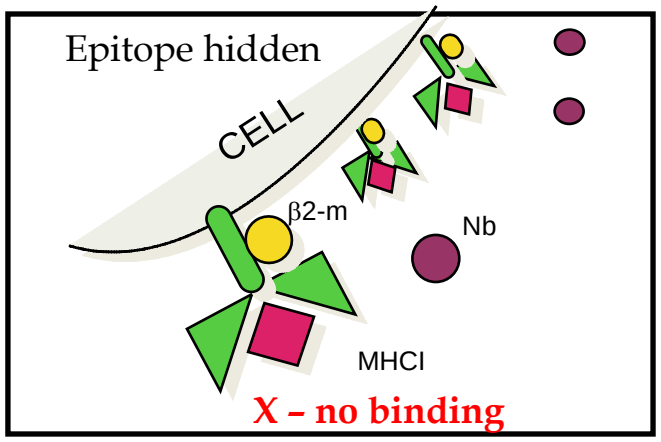
1:0

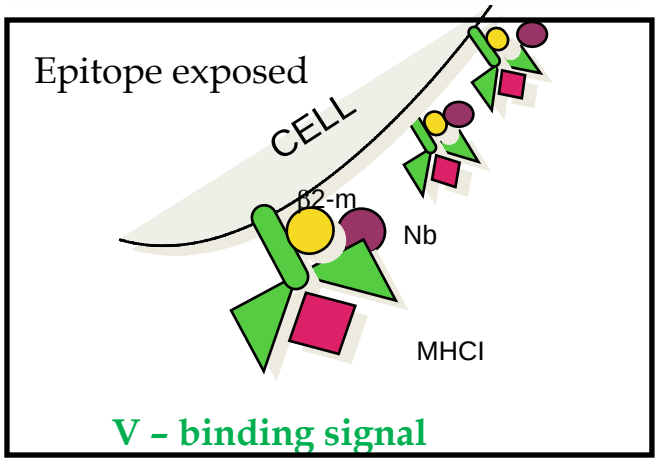
1:1





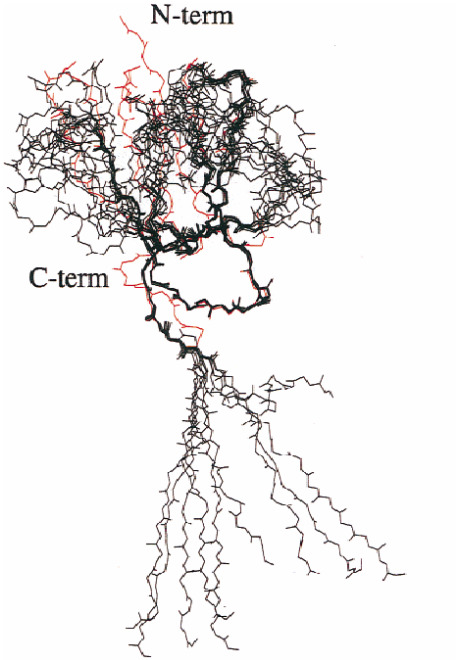




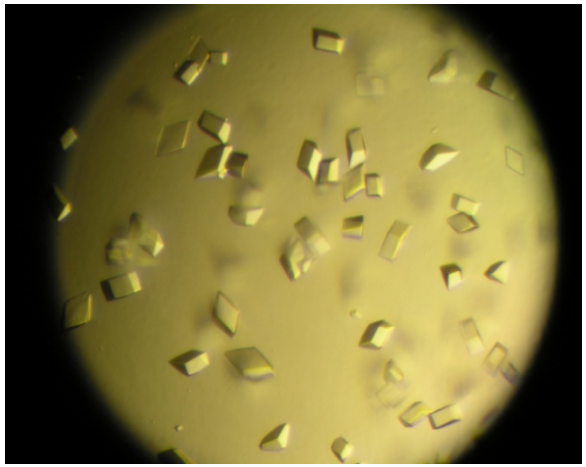


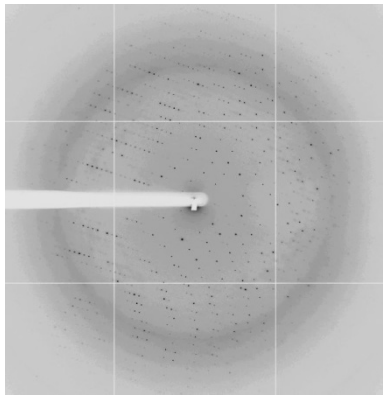
	Cell line	Nb20	Nb22	Nb23	Nb24	Nb30a	Nb30b	Nb31	Nb272	Nb273
1	BT 474	x	x	x	x	V	V	x	x	V
2	MDA-MB 435D	x	x	x	x	x	x	x	x	x
3	SKBR3	x	x	x	x	V	V	x	x	V

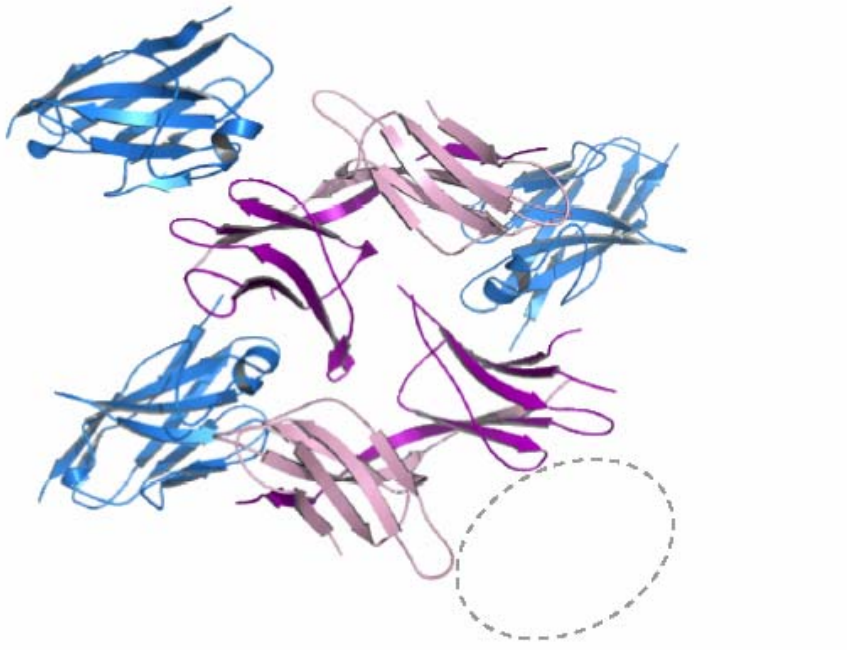
X-ray crystallography

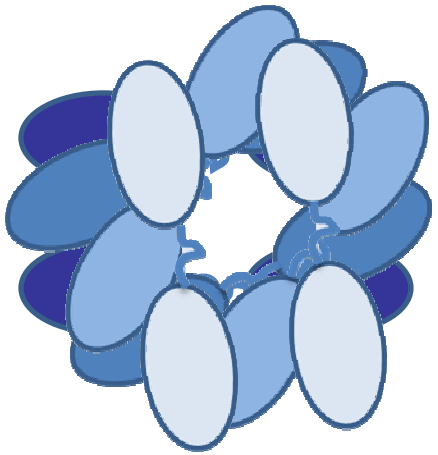


Esposito et. al Pro Sci 9:831-45 (2000)









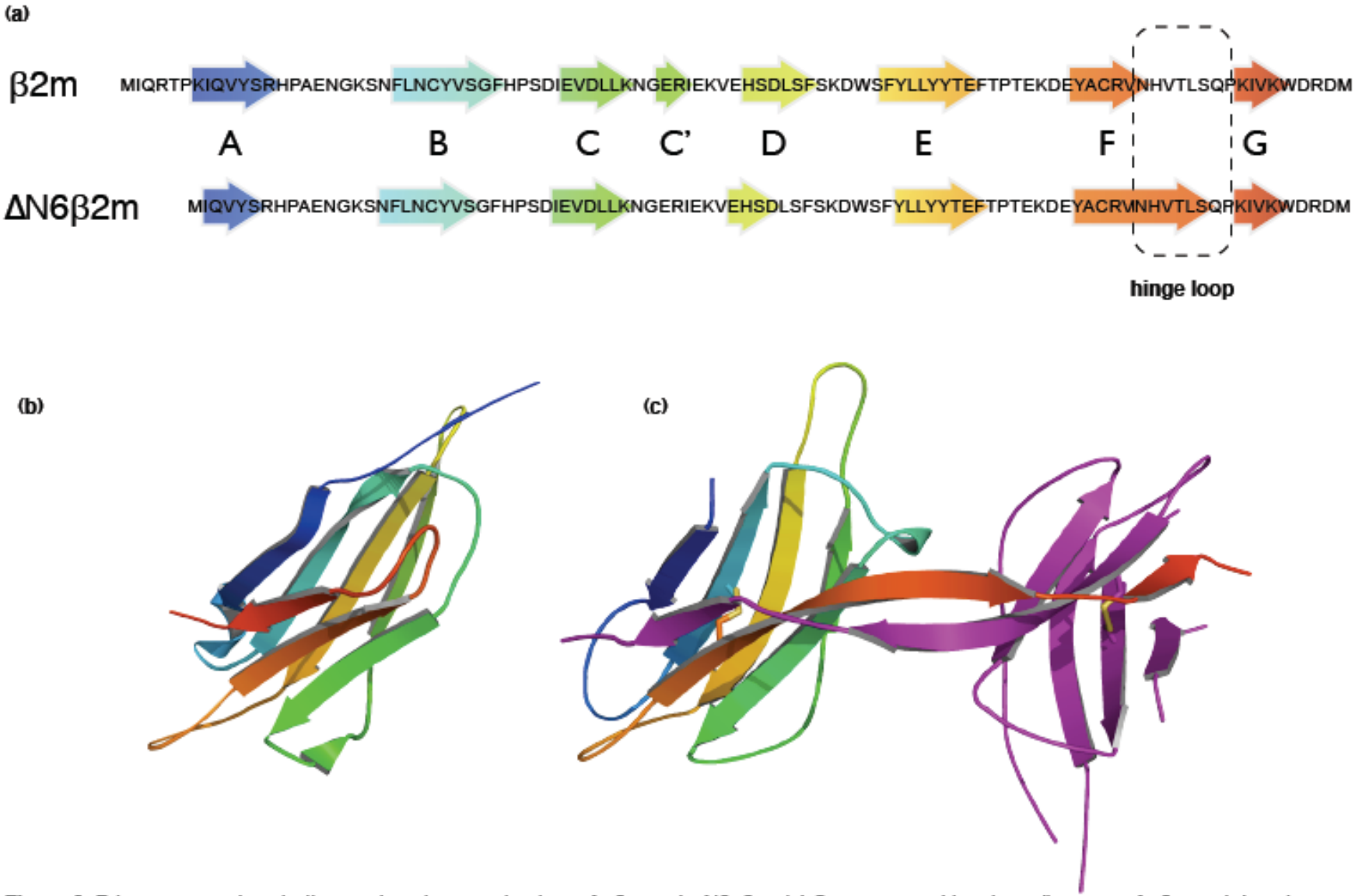


Figure 3. Primary, secondary, tertiary and quaternary structure of β2m and ΔN6β2m. **(a)** Sequence and topology diagrams of β2m and domain-swapped ΔN6β2m. The hingle loop is included in a dashed box. **(b)** The tertiary structure of the β2m monomer (1LDS) and **(c)** of the quaternary structure of the domain-swapped dimer of ΔN6β2m (this paper). β-strands are colored according to panel a. The single disulfide bond that bridges the two central sheets in the monomer and the swapped dimer are given in stick representation.

Conclusion

Nanobodies we have produced and characterized are usefull tool in basic and translational research

Molecular characterization of the highly toxic species ΔN6β2-m was awaited by many years and finally solved in the complex with a specific nanobody

Nanobodies against β2-m can be exploited in preparing devices capable to clear the β2-m excess in haemodialysis and those specific for ΔN6β2-m might have an in vivo therapeutic translation

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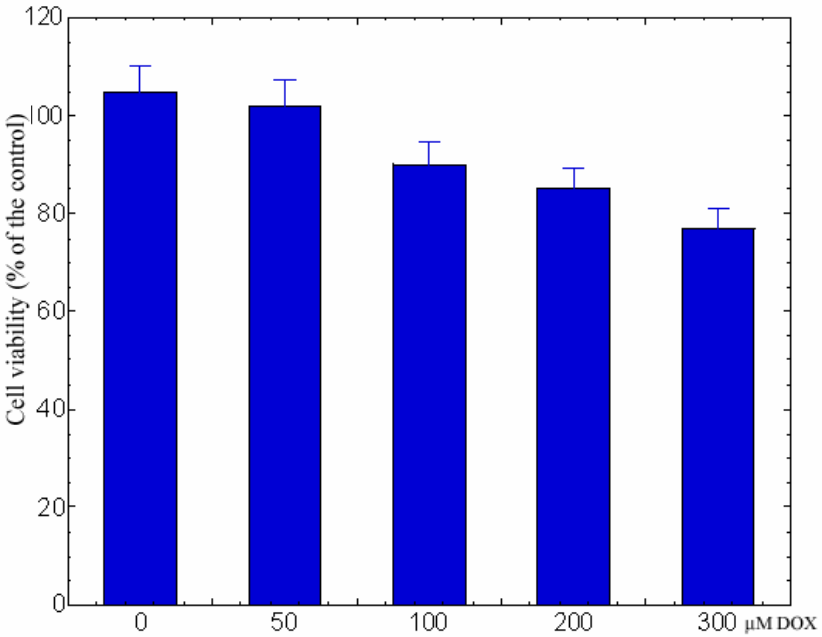
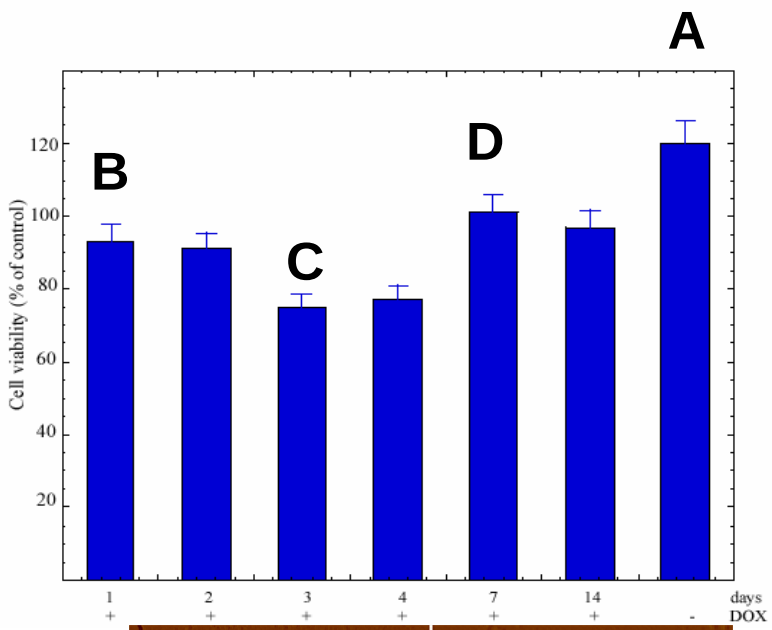
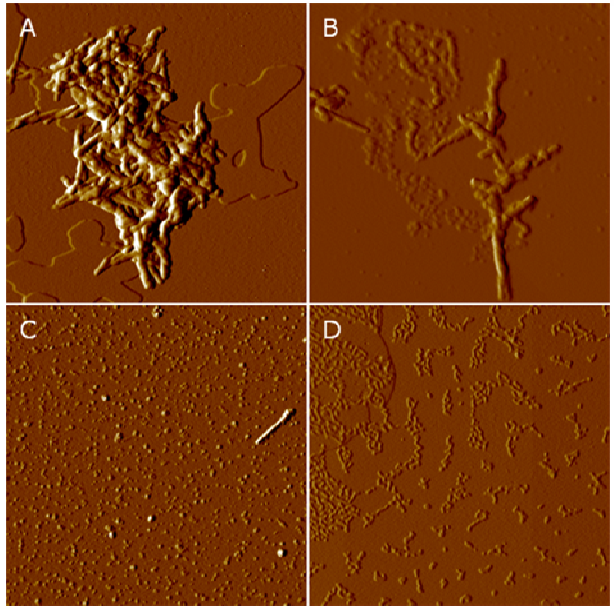
Cell viability valued on SHSY-5Y treated for 24 h with preformed fibrils of b2-m treated for 72 h with Doxicycline

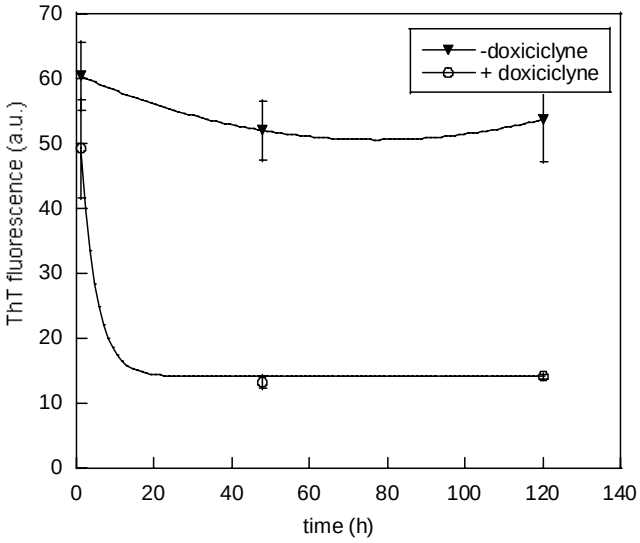


Viability valued on SHSY-5Y cells exposed for 24 h to 20μM of b2-m fibrils upon their treatment for different time with Doxicycline (300μM)





"Ex vivo "fibrils



"In vitro" fibrils