Cell-penetrating peptides as carriers

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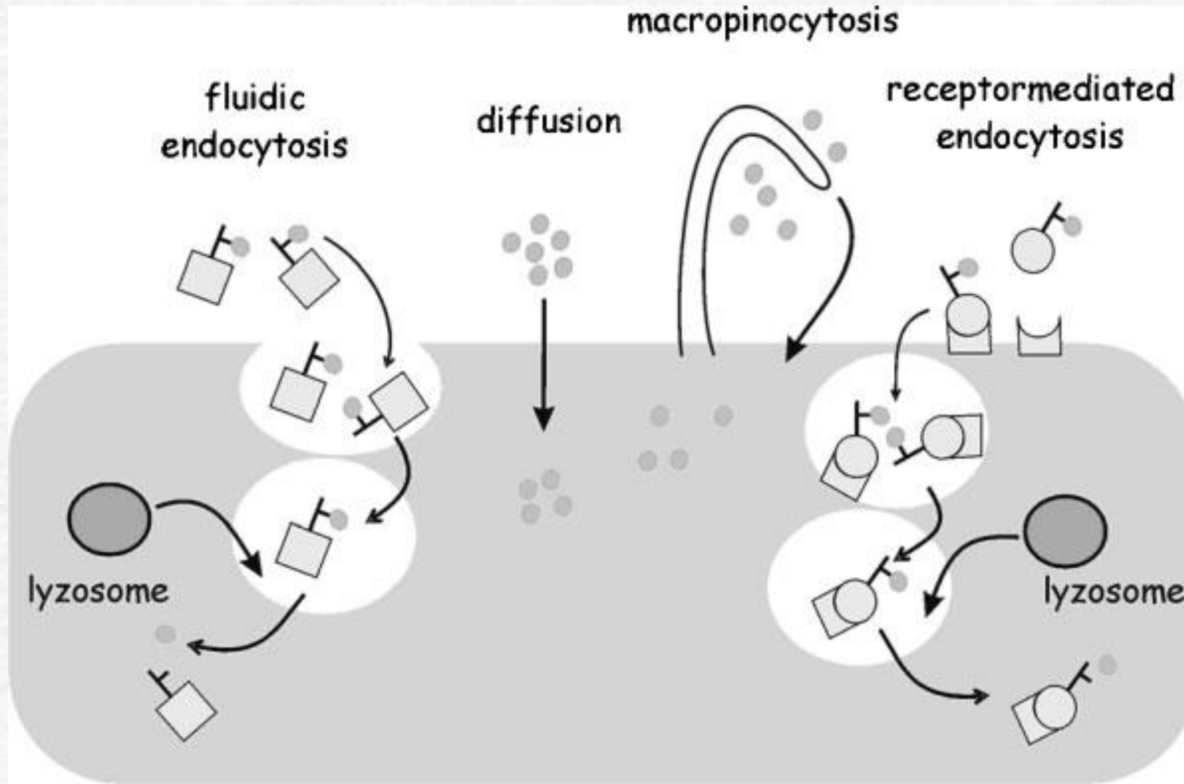
Cellular uptake of compounds



Transporter proteins/chanels

The first observations

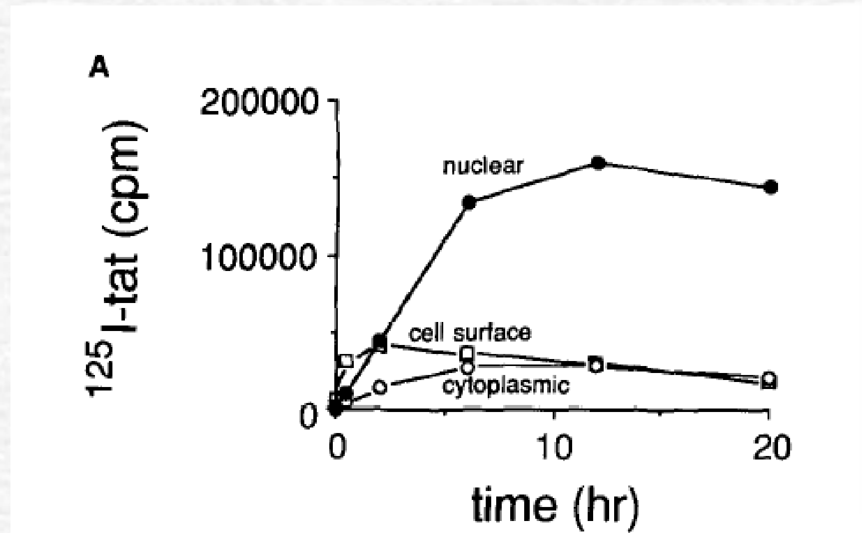
Tat (transactivator of HIV transcription)

Tat-86 protein - internalisation

- localisation in nucleus

Frankel AD, Pabo CO. *Cell* 1988, 55, 1189–1193.

^{48}GRKKRRQRRRPPQ^{60}

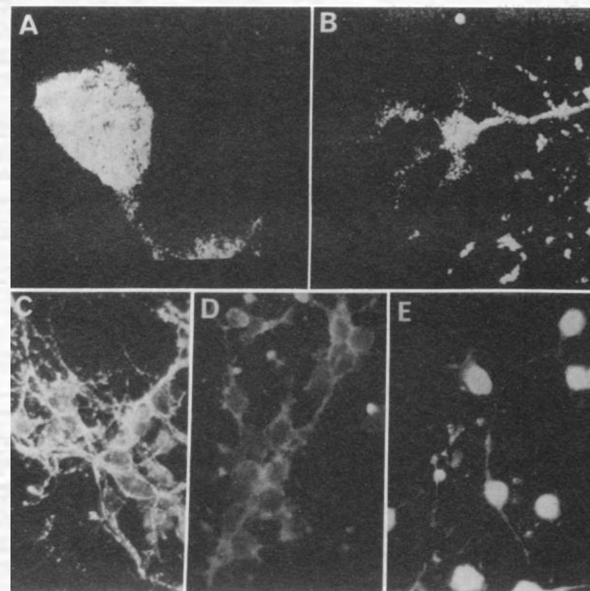


Homeodomain of Drosophila Antennapedia protein

- goes through the membrane of nervous cells
- localisation in nucleus

Joliot A, et al. *Proc Natl Acad Sci USA* 1991, 88, 1864–1868.

^{43}RQIKIWFQNRRMKWKK^{58}



Cell-penetrating peptides

Short peptides - with cell-internalisation ability - can transport the covalently attached cargo (protein, ODN, PNA, drugs) into cells upto 30 kDa. The mechanism of penetration is not clear.

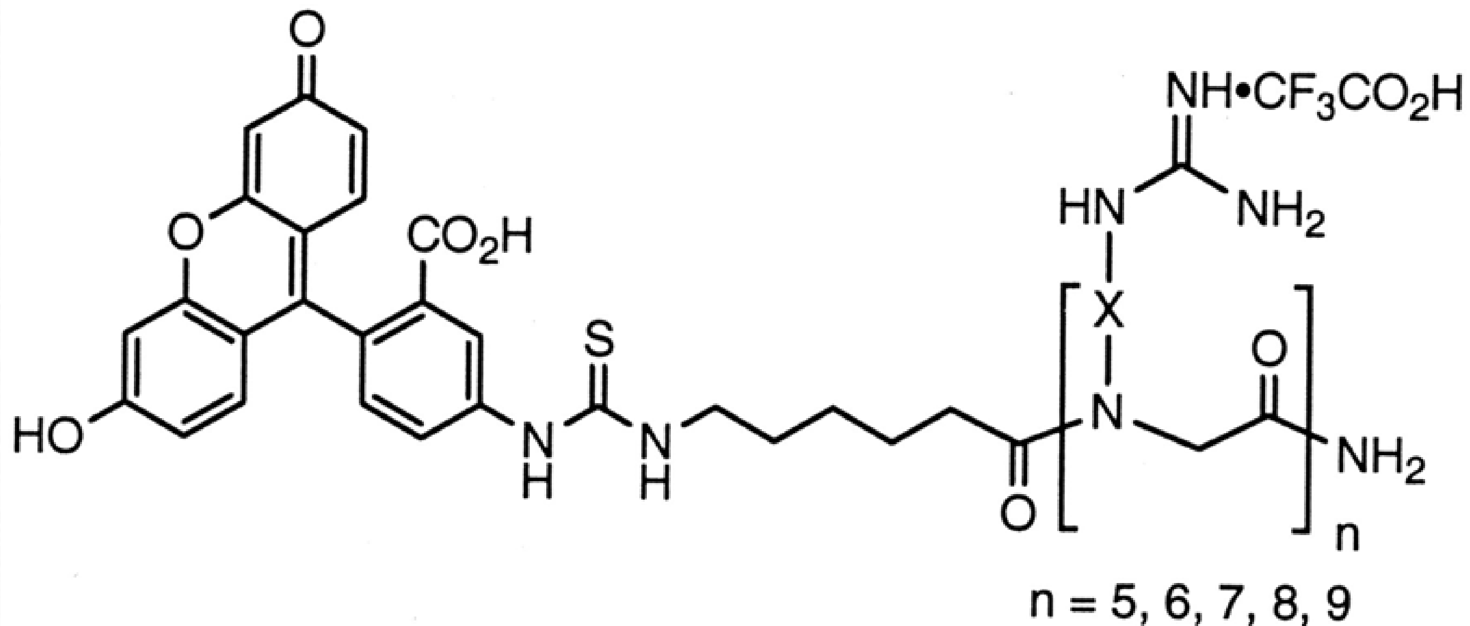
Peptide	Sequence (protein)	Origin
Tat (48–60)	^{48}GRKKRRQRRRPPQ^{60}	HIV-1 Tat protein
Penetratin	^{43}RQIKIWFQNRRMKWKK^{58}	*Drosophila* Antennapedia protein
Signal sequence peptide	AAVALLPAVLLALLAP	Kaposi fibroblast growth factor (K-FGF).
	EILLPNNYESYKYPGMFIALSK	Kaposi fibroblast growth factor (K-FGF).
	VQRKRQKLMP	NF-kB p50 transkription factor
Hydrophobic peptides	MGLGLHLLVLAAALQGA	Caïman crocodylus Ig(v)
	MGLGLHLLVLAAALQGAKKKRKV.	chimera peptide (Ig(v)-SV40 T-antigen)
Virial peptides/proteins	VP22 protein	herpes simplex virus-1
	^{1}AVGAIGALFLGFLGAAG^{17}	HIV gp41 glikoprotein, ^{8}Met→Leu
	GLFEAIAGFIENGWEGMIDGGGYC	Influenza hemaglutinin,
Substance P	RPKPQQFFGLM	neuropeptide
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	chimera peptide, galanin-mastoparan

F. Hudecz et al., *Med Res Rev.* 2005, 25, 679-736.

De novo designed peptides

Oligopeptides (Lys, Arg, Orn, His)

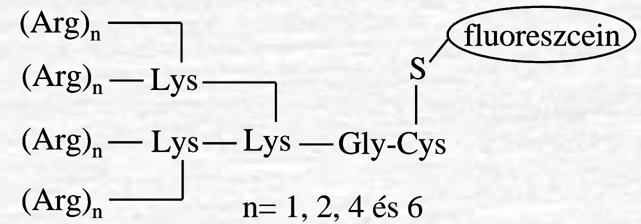
- Hexaarginine*
- peptoid transporters**
    - distance between the Arg residues
    - distance of guanidino group



* Mitchell DJ, et al. *J. Pept. Res.* 2000, 56, 318–325.
**Wender PA, et al. *Proc. Natl. Acad. Sci. USA* 2000, 97, 13003–13008.

Oligoarginines

- hexa- and octaarginine*
- arginine tree**



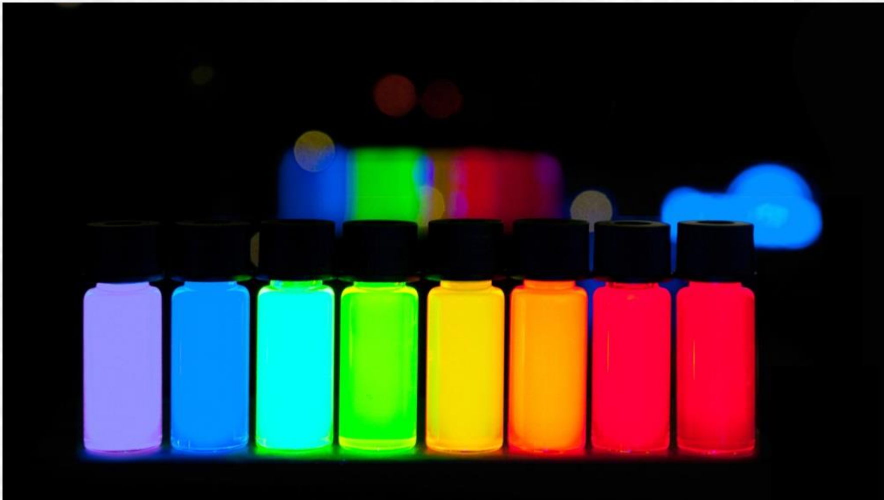
* Futaki S, et al. *J. Biol. Chem.* 2001, 276,5836-5840.
**Futaki S, et al. *Biochemistry* 2002, 41, 7925-7930.

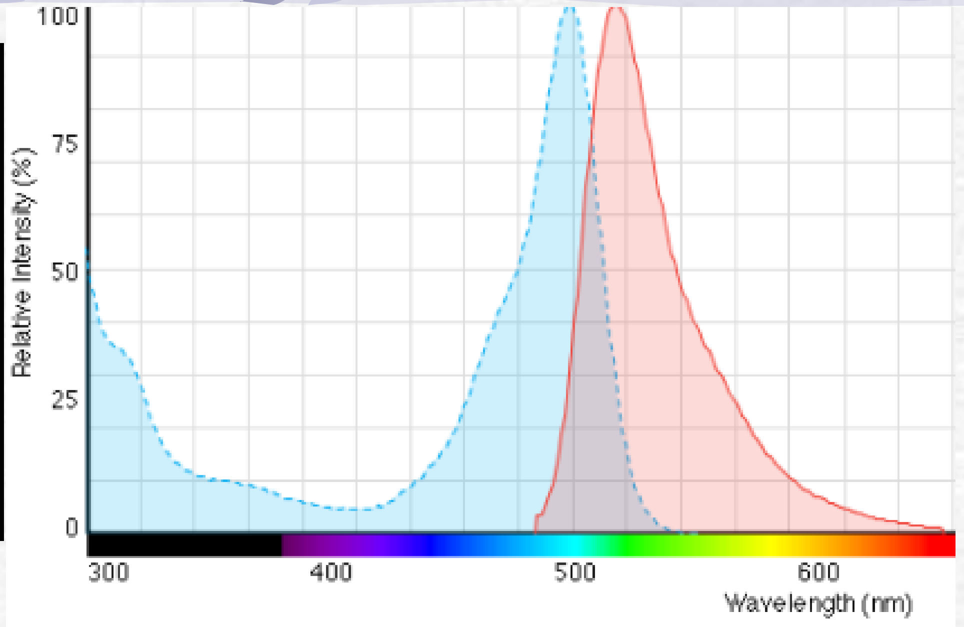
Model peptides

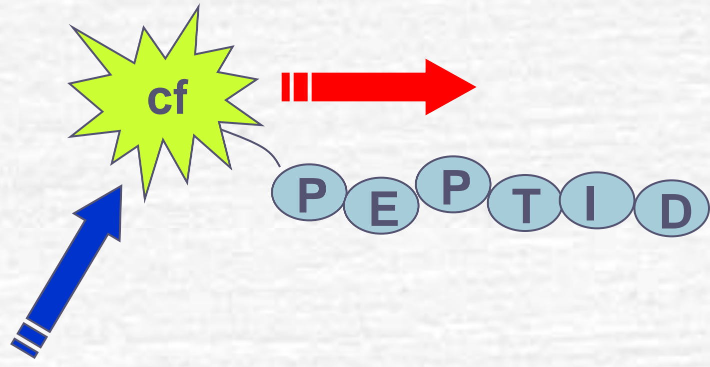
- KLA model peptide#
KLALKLALKALKAALKLA
- Model peptide##
KETWWETWWTEWSQPKKKRKV

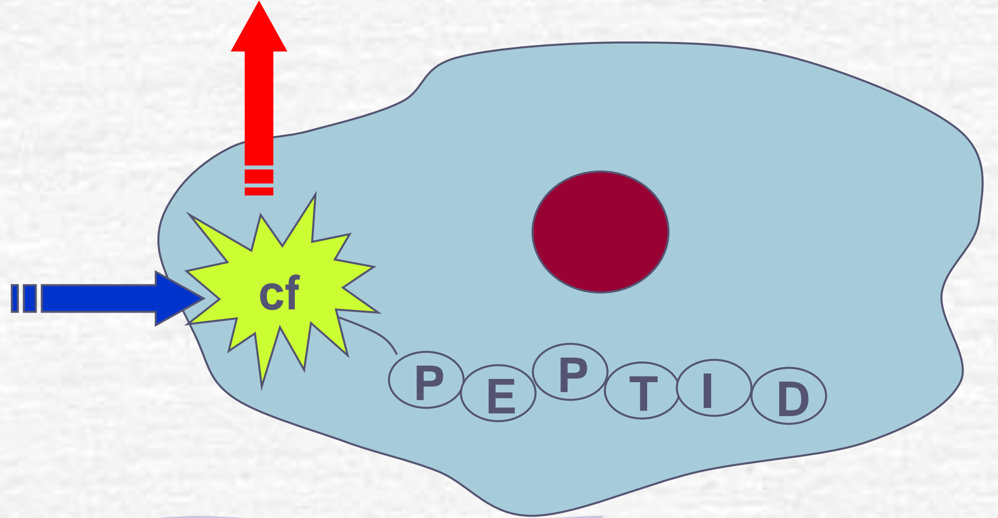
Scheller A, et al. *J. Pept. Sci.* 1999, 5, 185-194.
##Morris MC, et al. *Nat. Biotechnol.* 2001, 19, 1173-1176.

Cellular uptake studies using fluorescence labelling

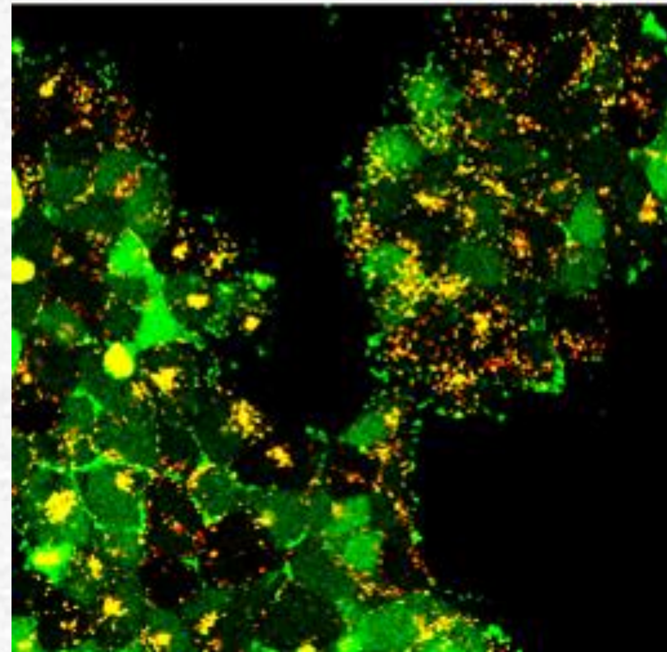


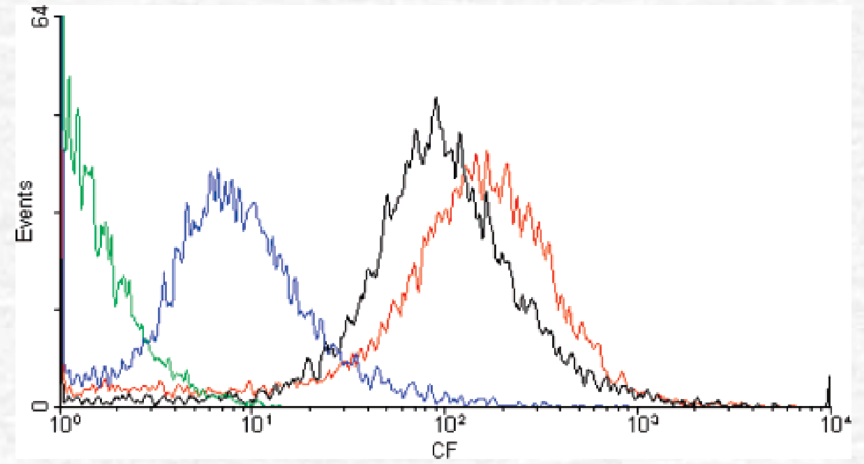




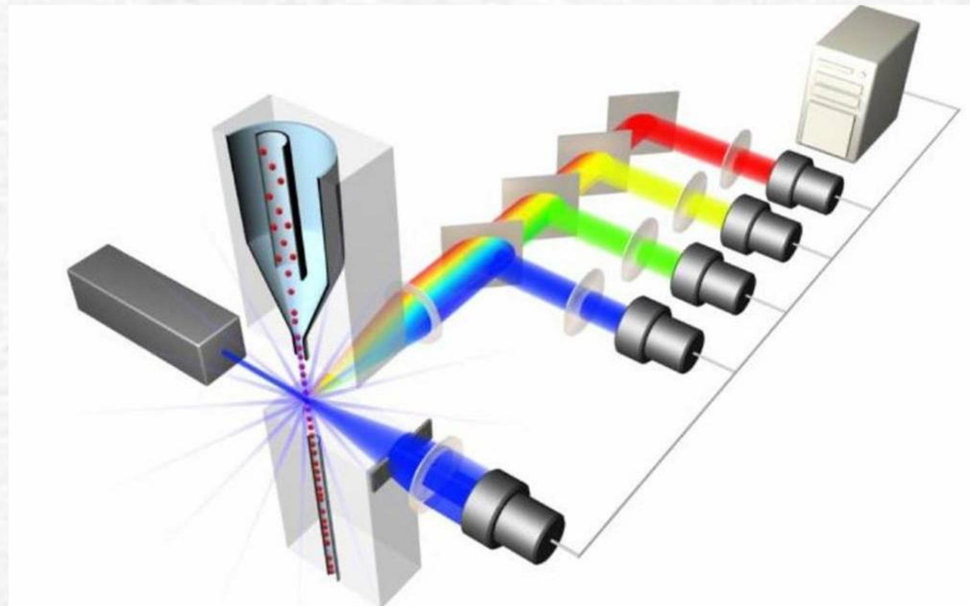


Olympus CKX41 fluorescence microscope



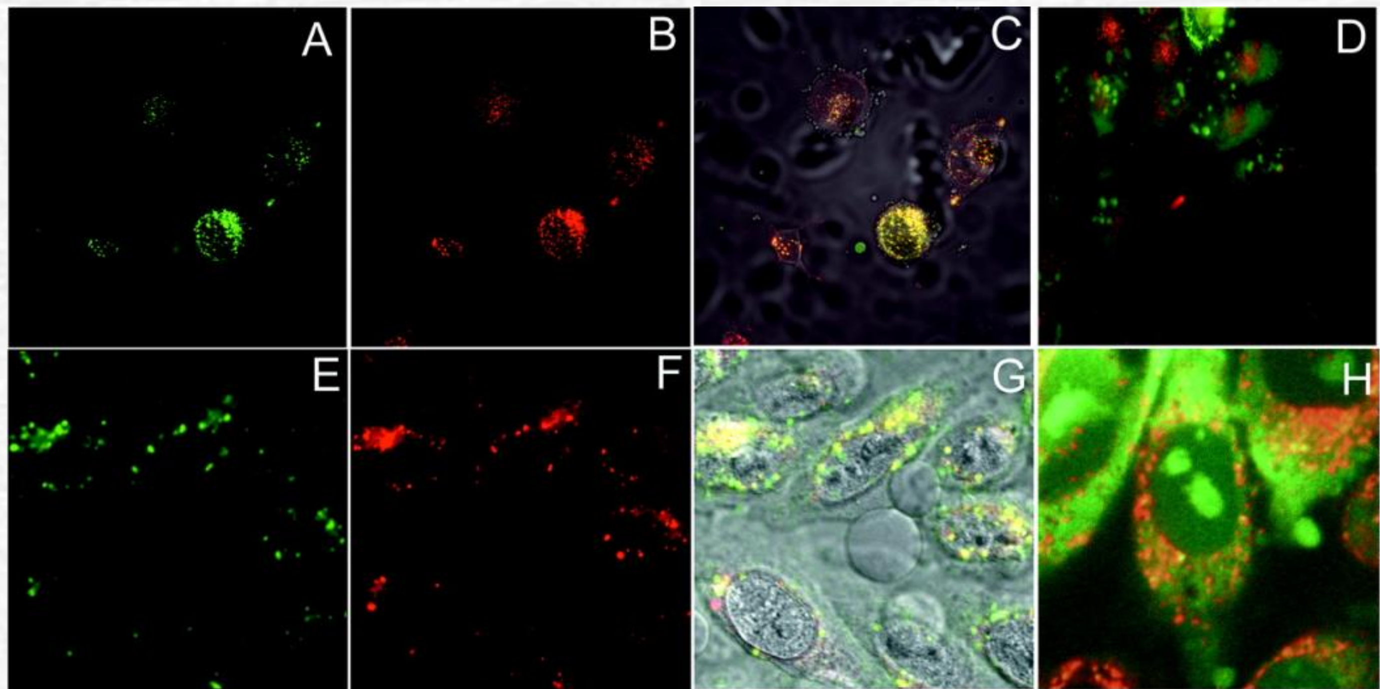


BD LSR II flow cytometer



First experiments

- fluorescence microscopy
- fixation
- 4 °C
- r_n



Richard P.J. et al. *J. Biol. Chem.* 2003, 278, 585-590.

Fluorescence detection – microscopy, flow cytometry

- without fixation
- remove the membrane bound peptides
    - digestion by trypsin
    - washing with heparin
- using inhibitors

ATP depletion: NaN_3 and deoxyglucose

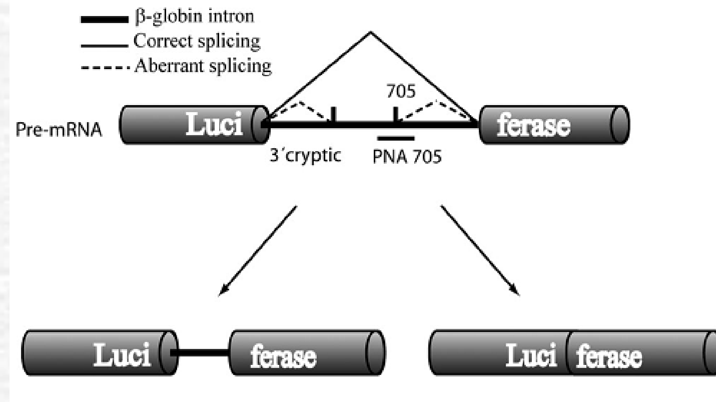
endocytosis inhibitors: methyl-β-cyclodextrin

chloropromazine,

Cellular uptake studies II.

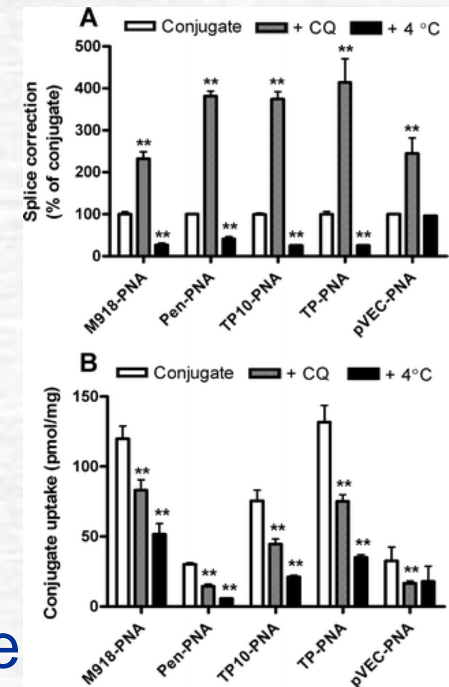
Functional studies

- luciferase splice correction test



Lundin P. et al. *Bioconj. Chem*, 2008, 19, 2535-2542

- inhibition of the expression of luciferase



RP-HPLC with fluorescence detector

Palm C, et al. *Peptides* 2006, 27 1710–1716.

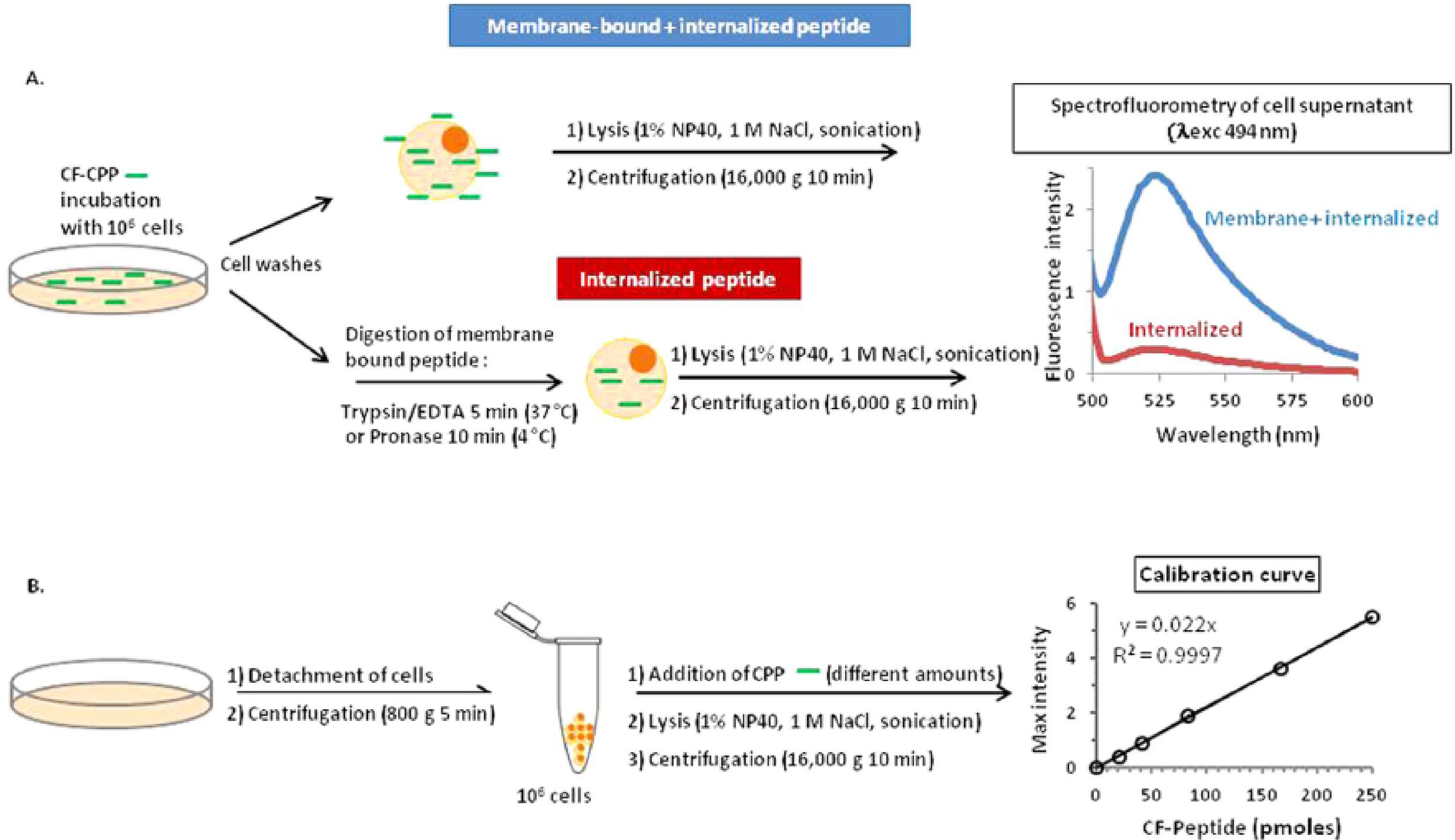
Mass spectrometry

Burlina F, et al. *Angewandte Chemie* 2005, 44, 4244-4247.

Cellular uptake studies III.

Using fluorimeter

Illien F, et al. *Sci Rep.* 2016; 6, 36938.



Mechanism of internalisation

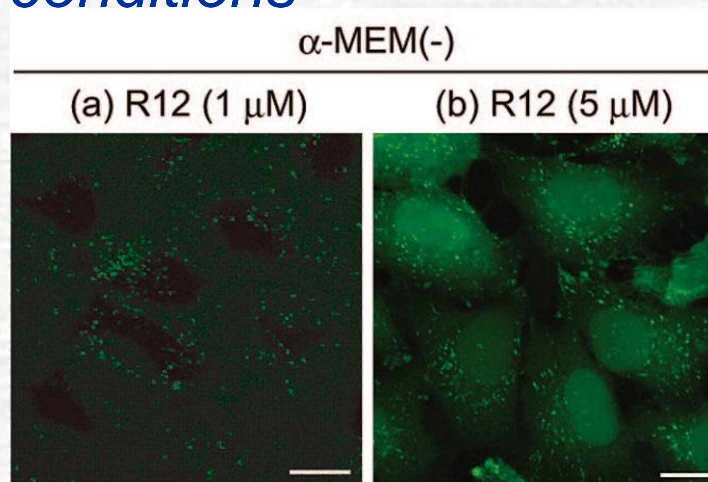
Is there receptor?

- r_n is more effective than R_n

More than one mechanism

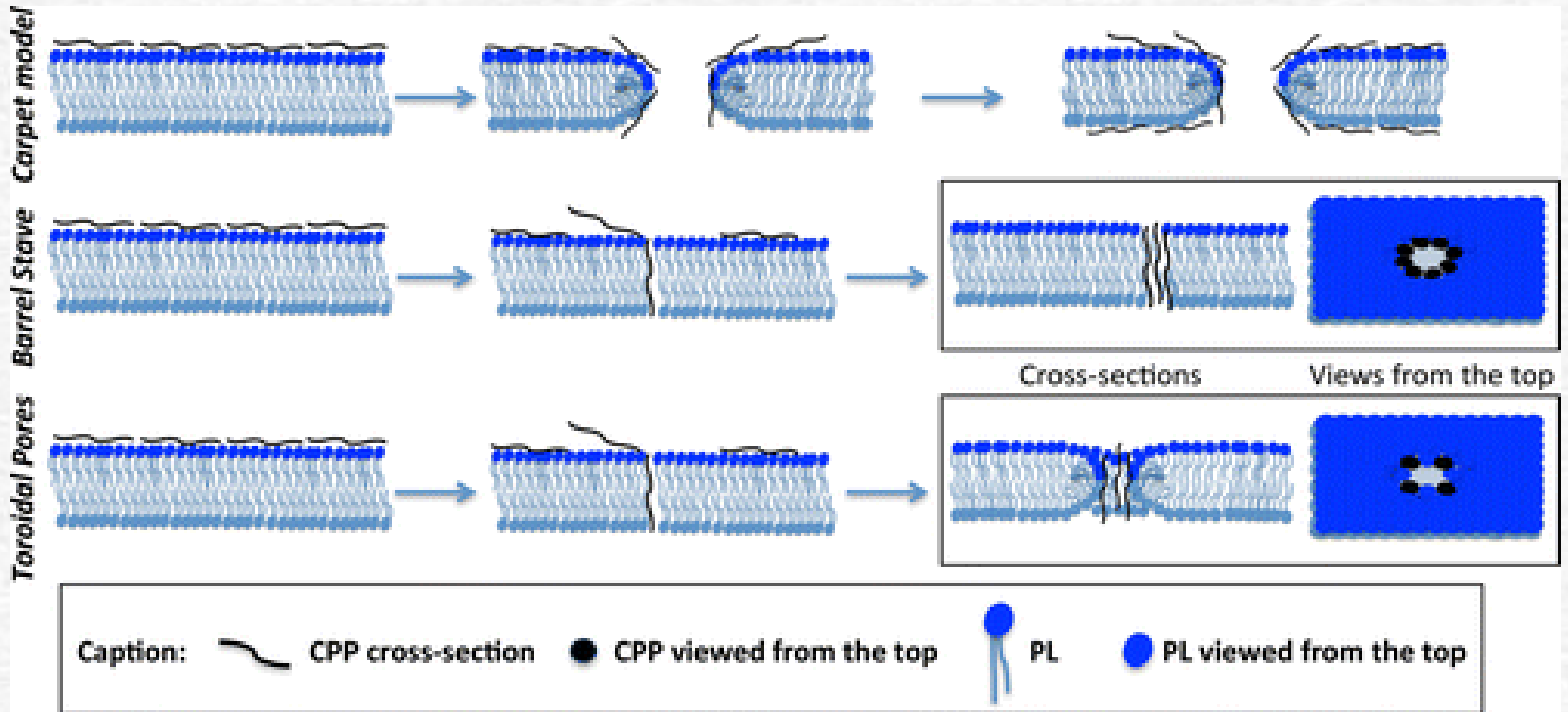
- endocytosis
- direct penetration above a threshold concentration

Depending on the conditions



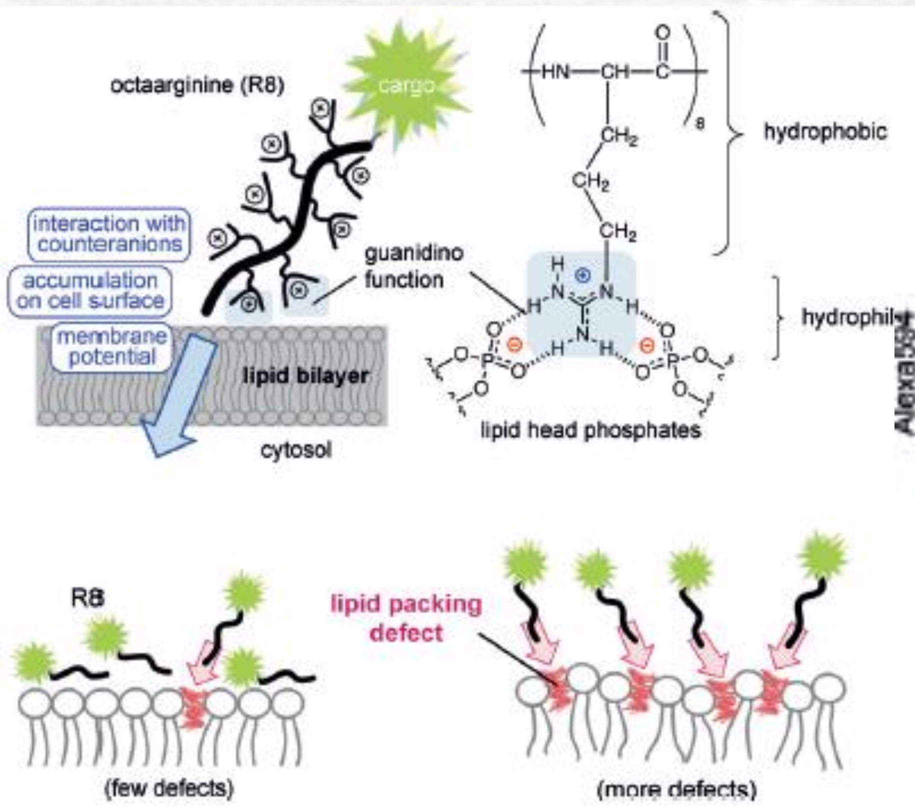
Kosuge M. et al. *Bioconj. Chem.* 2008, 19, 656-664

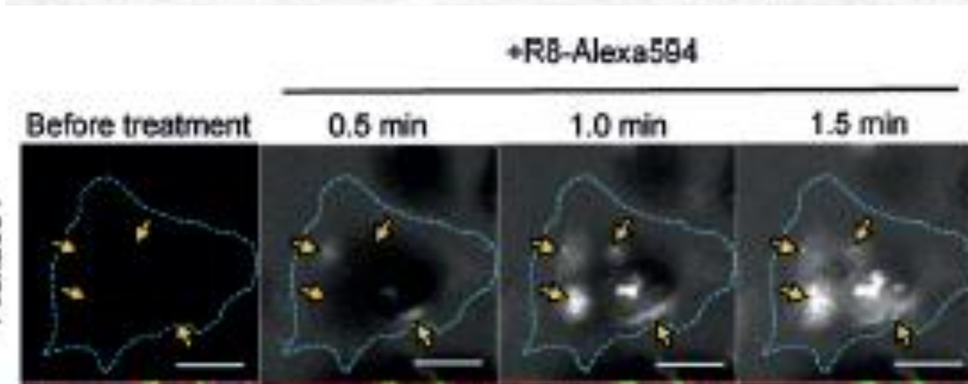
Direct internalisation – how?



Di Pisa M. *Biochemistry*, 2015, 54, pp 194–207.

Internalisation of oligoarginines

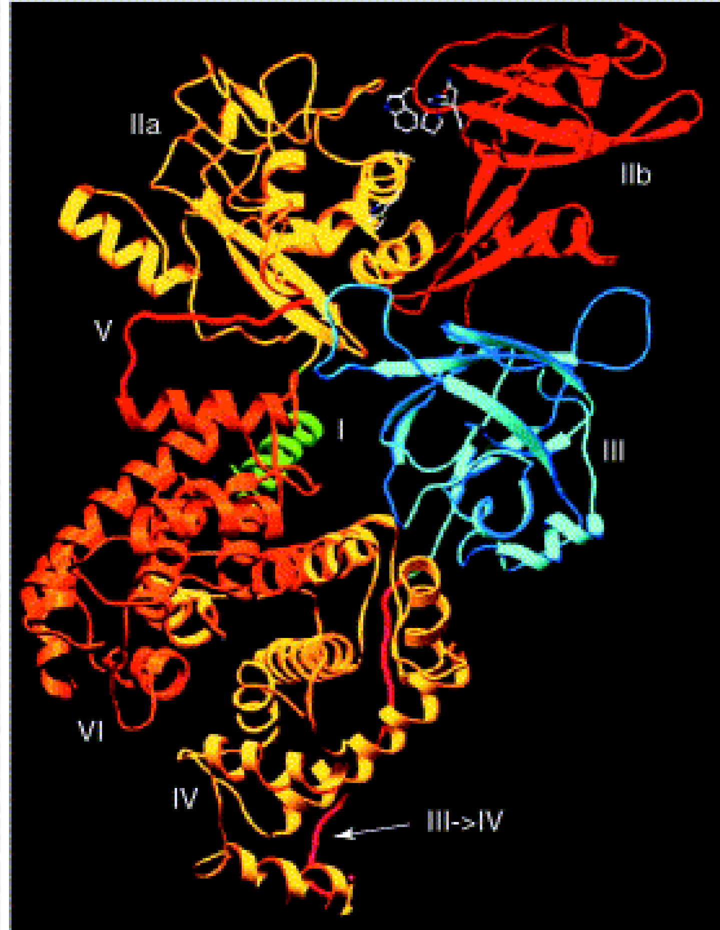




Murayama T, et al., *Angew. Chem. Int. Ed.* 2017, 56, 7644 –7647

Calpain activator conjugates

Calpains



Superfamily of Ca^{2+} dependent cysteine proteases.

Ca^{2+} signal induced cleavage of specific proteins involved in signaling cascades.

In mammals m-calpain and μ-calpain are constitutively expressed in all tissues.

Perrin J.B., et al., *Int. J. Biochem. Cell Biol.* 2002, 34, 722-725.

Heterodimer of non-activated m-calpain
Strobl S., et al., *Proc. Natl. Acad. Sci. U. S. A.* 2000, 97, 588-592.

Structure of conjugates

Penetratin-Calpastatin A (C)

***N*-terminal**

X-RQIKIWFQQNRRMKWKKC-NH_2

***C*-terminal**

H-CSGKSGMDAALDDLIDTLGG-NH_2

(AcCSKPIGPDDAIDALSSDFTS-NH_2)

X-RQIKIWFQQNRRMKWKKC-NH_2

CH_2-CO-SGKSGMDAALDDLIDTLGG-NH_2

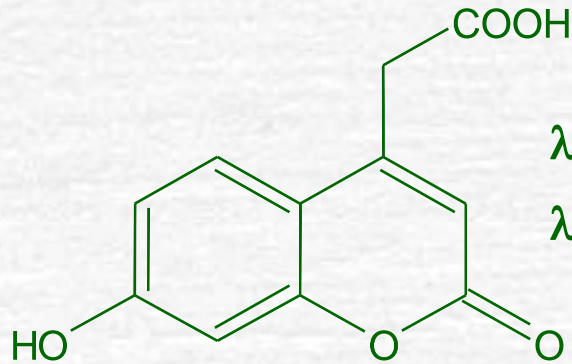
(SKPIGPDDAIDALSSDFTS-NH_2)

X-RQIKIWFQQNRRMKWKKSGKSGMDAALDDLIDTLGG-NH_2

(SKPIGPDDAIDALSSDFTS-NH_2)

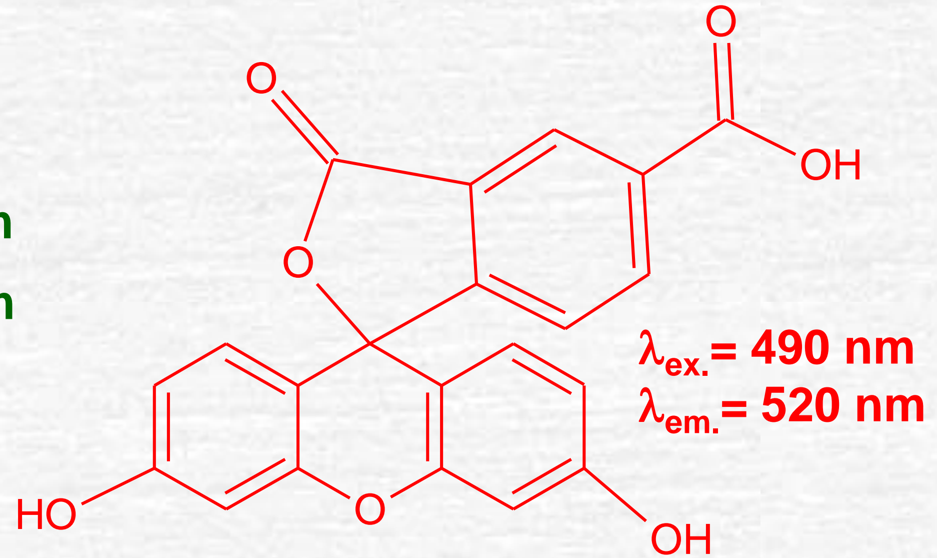
X: H or Hca

Conjugates labelled by two fluorophores



λ_{ex} = 360 nm

λ_{em} = 480 nm



$\lambda_{ex.}$ = 490 nm

$\lambda_{em.}$ = 520 nm

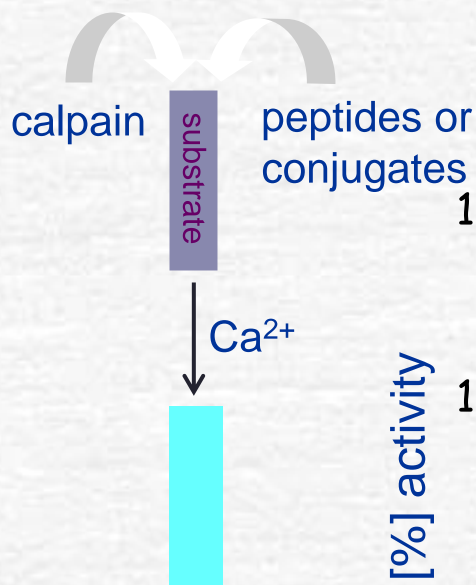
4-(7-hydroxycoumaryl) acetic acid (Hca)

5-carboxyfluorescein (Flu)

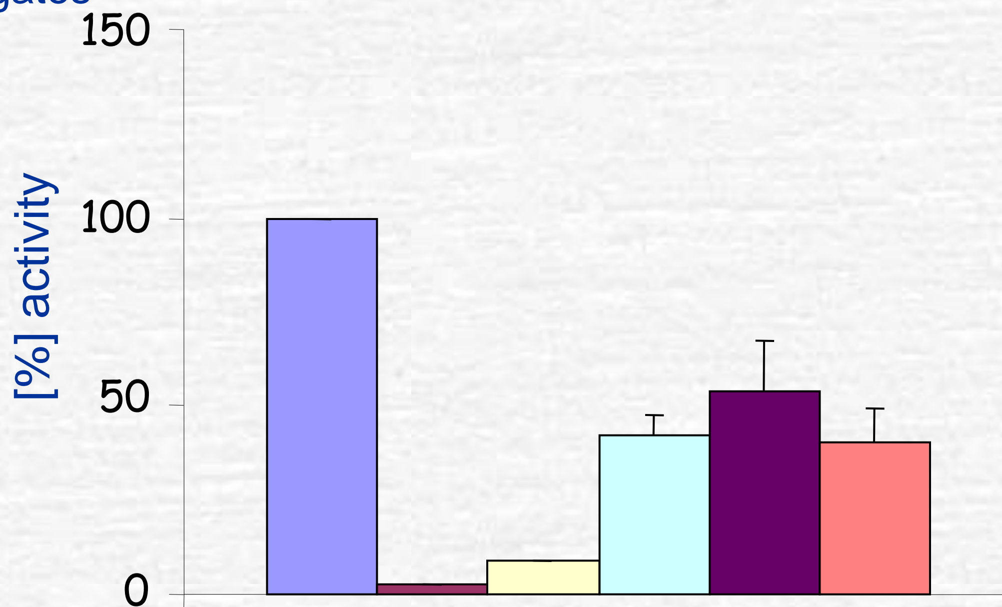
Hca-RQIKIWFQQNRRMKWKKC-NH_2

Ac-CSK(Flu)PIGPDDAIDALSSDFTS-NH_2

Activation of isolated m-calpain



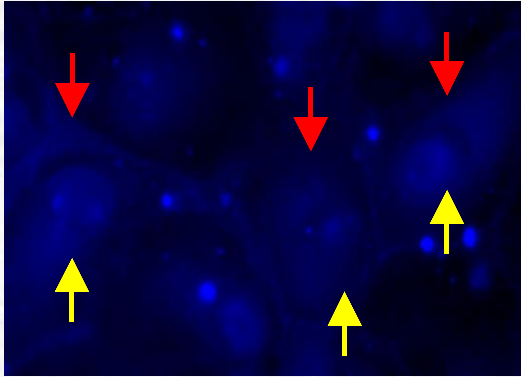
The effect of conjugation (c = 75 μM)



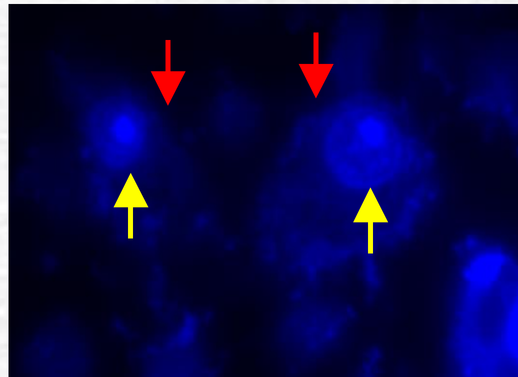
5 mM $CaCl_2$
0.1 mM $CaCl_2$
KalpA + KalpC (0.1 mM)
PenKalpA + PenKalpC S-S (0.1 mM)
PenKalpA + PenKalpC amide (0.1 mM)
PenKalpA + PenKalpC thio (0.1 mM)

Bánóczi Z. et al. *Bioconjugate Chem.* 2007, 18, 130-137.

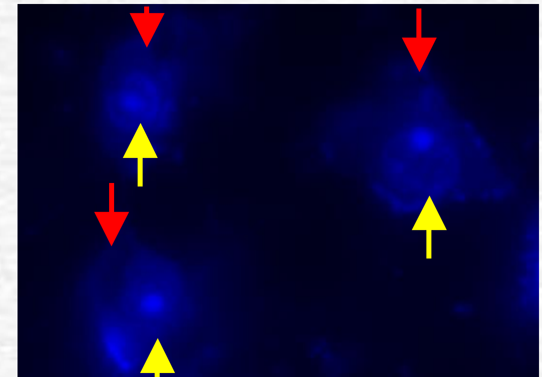
Uptake of Hca-penetratin-calpastatin peptide conjugates by COS-7 cells with (A,B) or without (C) fixation.



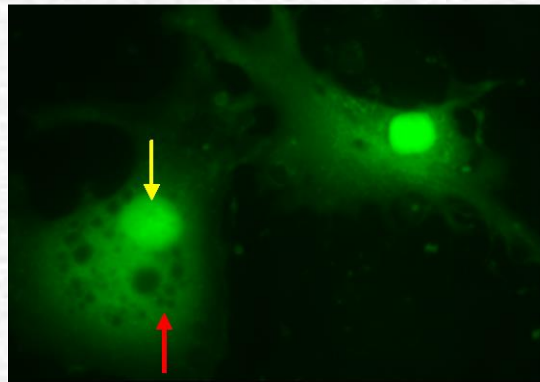
A) HcaPenKalpA amide

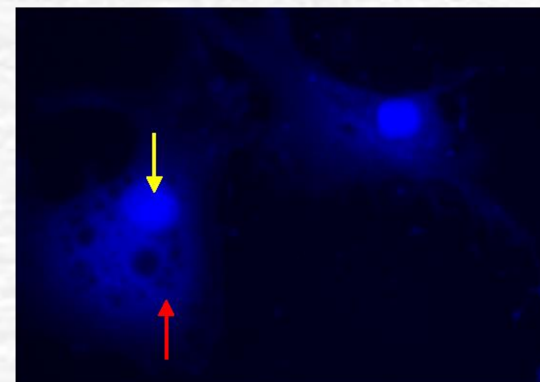


B) HcaPenKalpC thioether



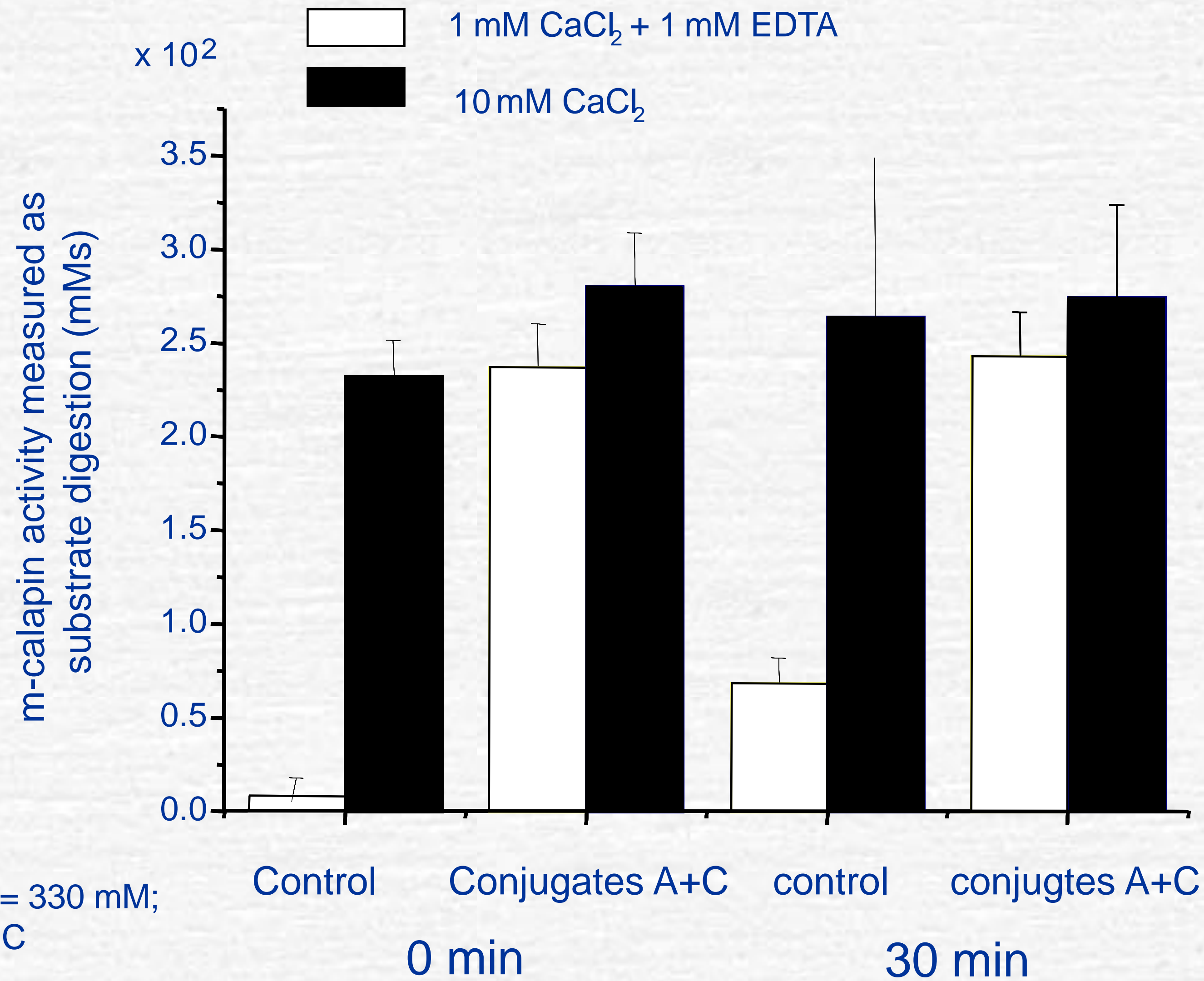
C) HcaPenKalpC thioether





HcaPenCalp(Flu)C conjugate with disulfide bond

Calpain activity in COS-7 cell lysate



Incubation: c= 330 mM;
for 4 h at 37°C

Detection of intracellular calpain
activity

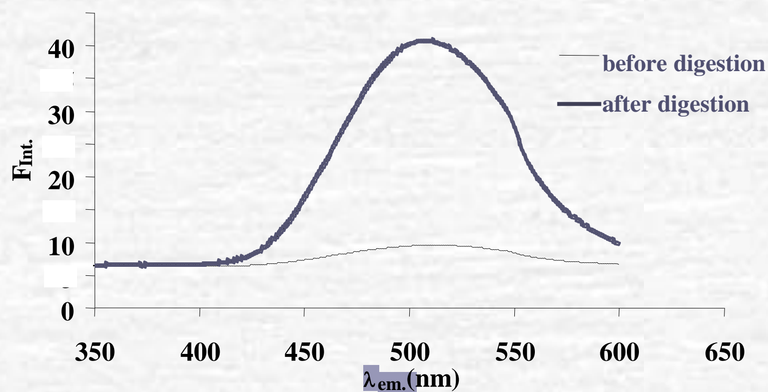
Calpain substrate

A novel and optimized FRET (fluorescence resonance energy transfer) substrate was designed and prepared from the preference matrix of calpain cleavage sites.

Suitable for *in vitro* measurements: the fluorescence intensity depends only on the calpain activity.

Tompa P., et al. *J. Biol. Chem.* 2004, 279, 20775-20785.

Calpain cleavage of FRET substrate – heptaarginine conjugate



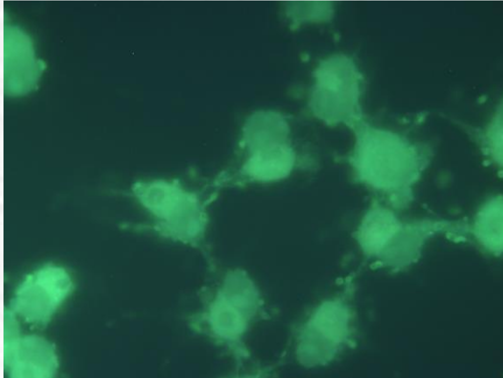
$c_{substrate}$= 200 μM, $c_{CalpainB}$= 0.5 μM
λ_{ex}=320 nm, λ_{em}= 480 nm

Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
DABCYL- TPLKSPPPSPR-*EDANS*	250	0.2	680
DABCYL-TPLKPPPSPRE(*EDANS*)RRRRRRR-*NH_2*	40	0.17	5000

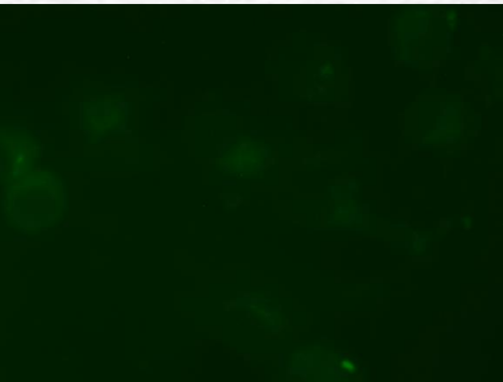
Bánóczi Z. et al. *Bioconjugate Chem.* 2008, 19, 1375-1381.

Penetration of FRET substrate-heptaargine conjugate and substrate peptide into COS-7 cells

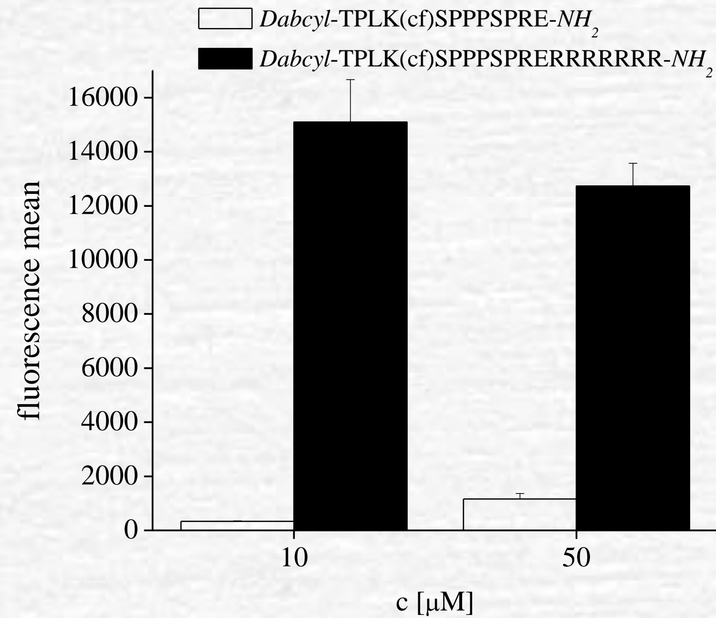
a)



b)

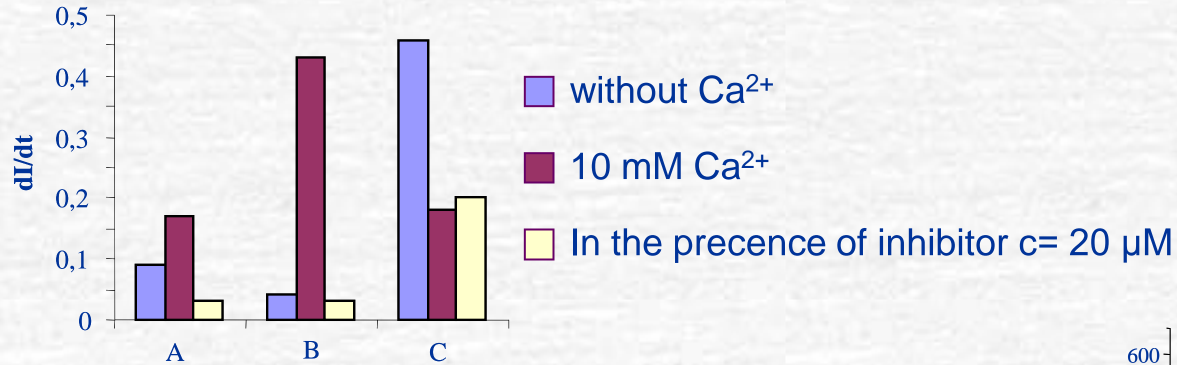


COS7 cells were incubated at 330 μM for 4 h



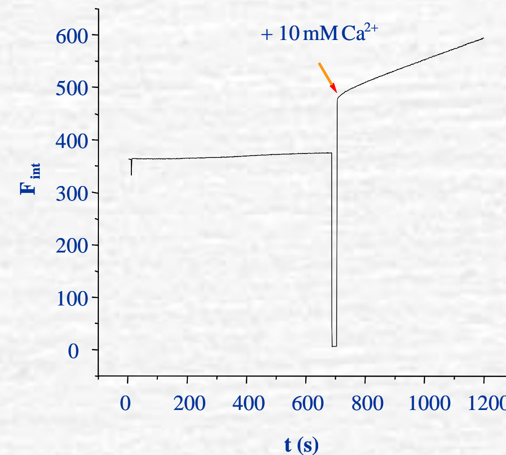
COS7 cells were incubated for 3 h and were treated by trypsin

Calpain activity in cell-lysate



A) Substrate is in lysate of S2 cell
B) Substrate is in lysate of S2 cell overexpressing calpain
C) LY-AMC is in lysate of S2 cell overexpressing calpain

$c_{LY\text{-}AMC}$= 1 mM or $c_{substrate}$= 100 μM



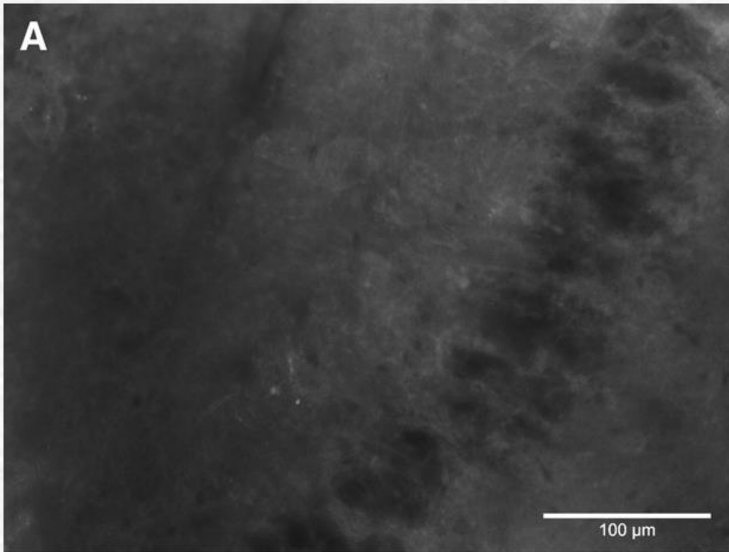
S2 cells were incubated with 150 μM substrate at 25-28ºC for 20 h and were Lysed.

LY-AMC:

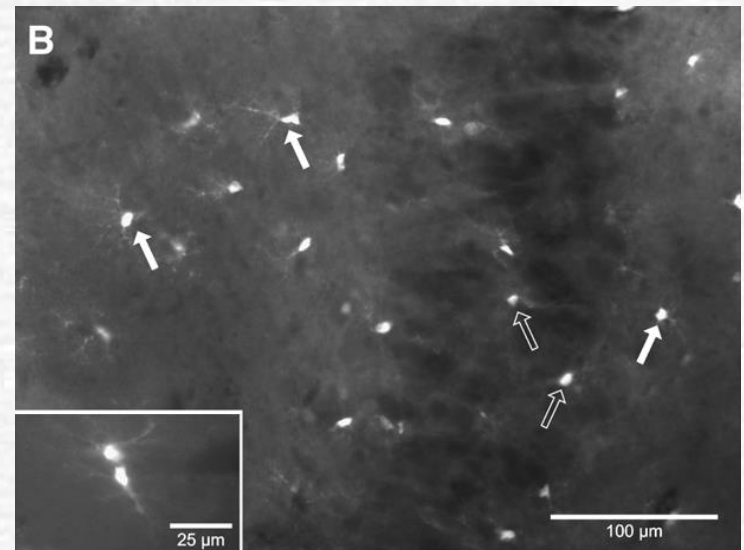
$\lambda_{ex.}$=380 nm, $\lambda_{em.}$= 460 nm

Substrate:

$\lambda_{ex.}$=320 nm, $\lambda_{em.}$= 480 nm



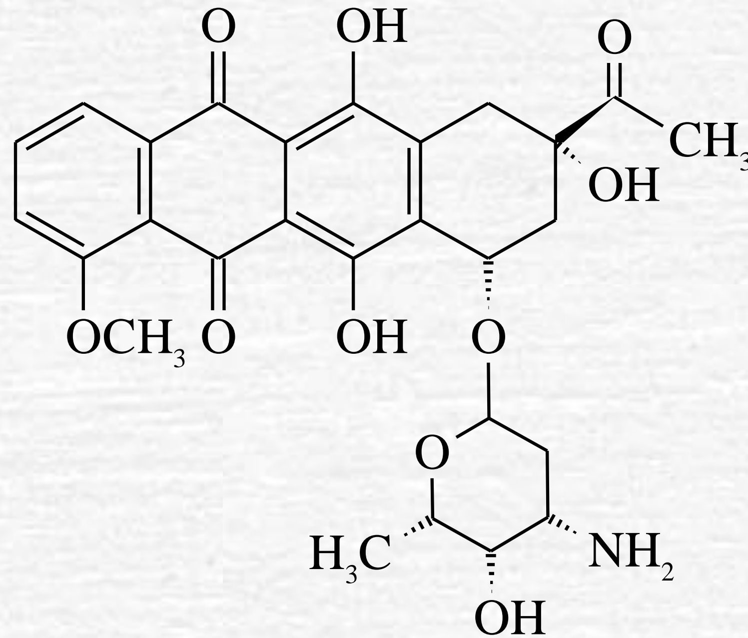
Hippocampal slices slices were treated only with the 50 µM cell–penetrating substrate for 5 min.



Slices treated with the 50 µM cell–penetrating substrate for 5 min and then 5 µM A+C conjugates was mixed into the solution and incubation was followed for further 15 min. Pyramidal cell layer (empty arrows) and also in other regions (filled arrows)

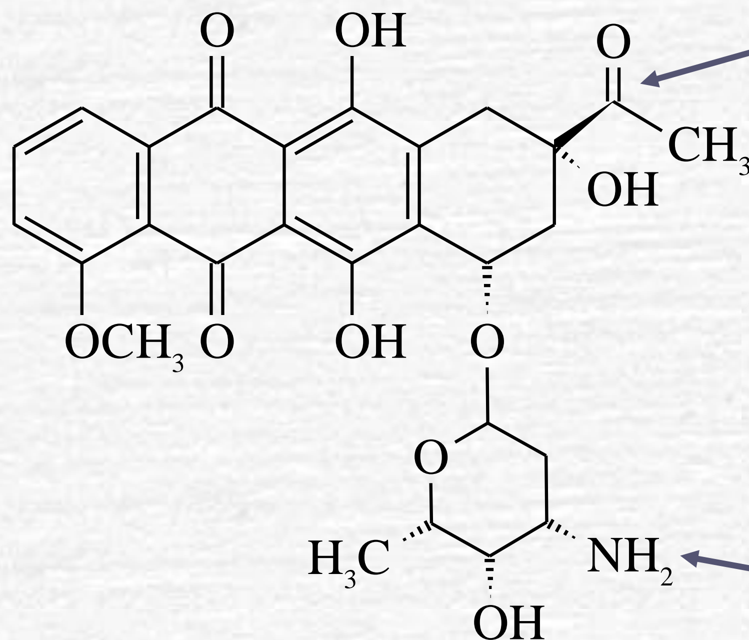
Drug-Cell penetrating peptide
conjugates

Daunomycin



- antitumor drug used in cancer treatment
- side-effects; cardiotoxicity, immunsupression
- development of resistance

Conjugation sites



Alkyl-hydrazine

Langer, M. et al., *J. Med. Chem.*, 2001, 44, 1341.

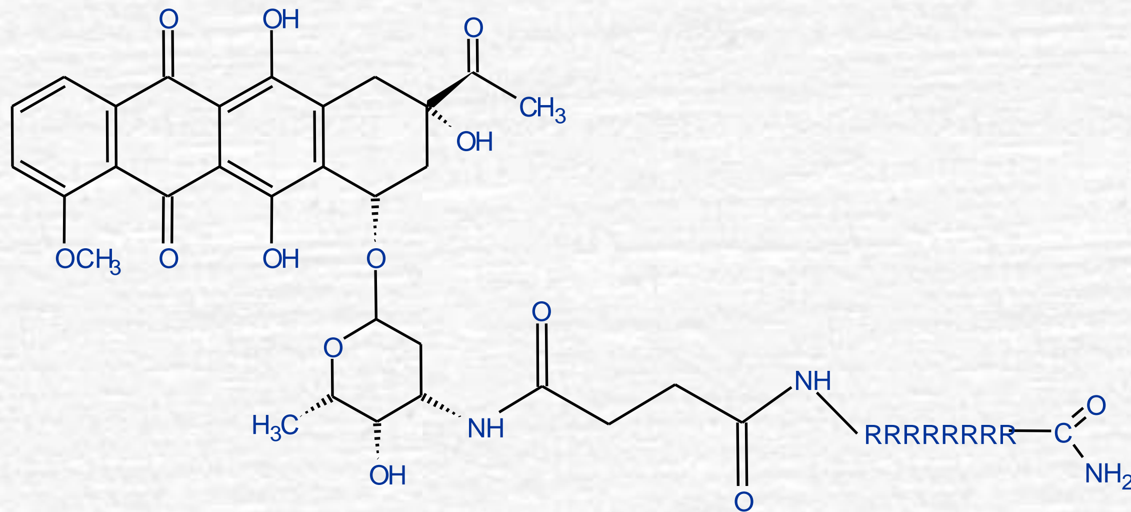
O-alkyl hydroxylamine

Ingallinella, P. et al., *Bioorg. Med. Chem. Lett.*, 2001, 11, 1343.

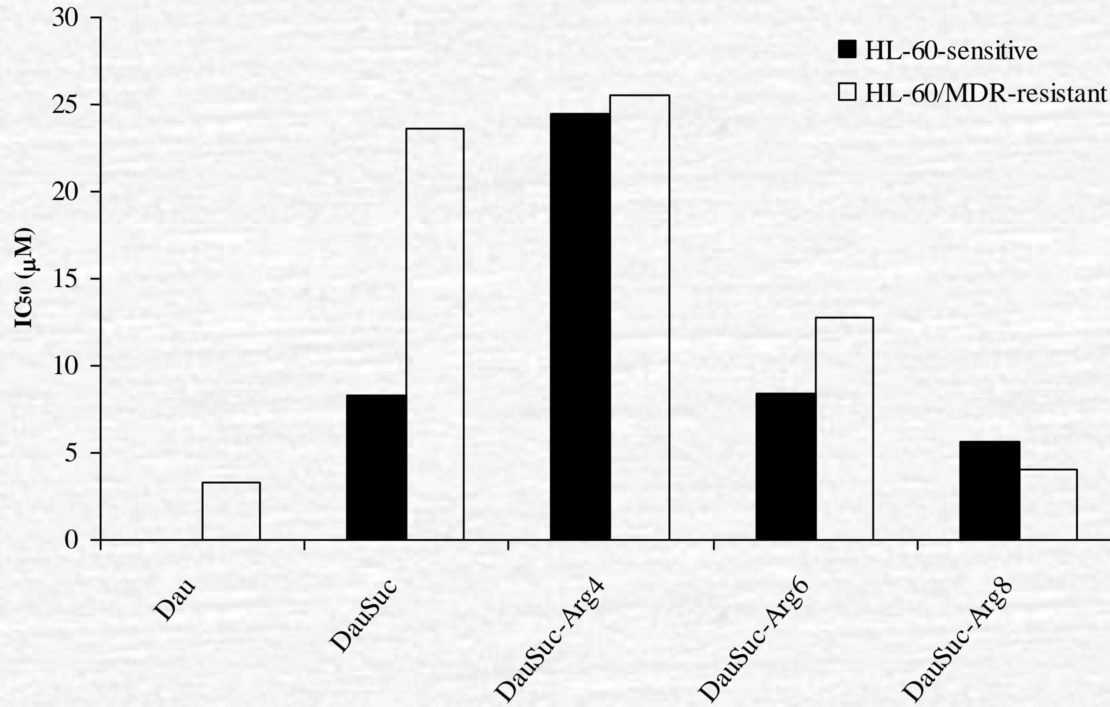
Carboxylic acids

Yamamoto, K. et al., *J. Med. Chem.*, 1972, 15, 872.

Structure of conjugates

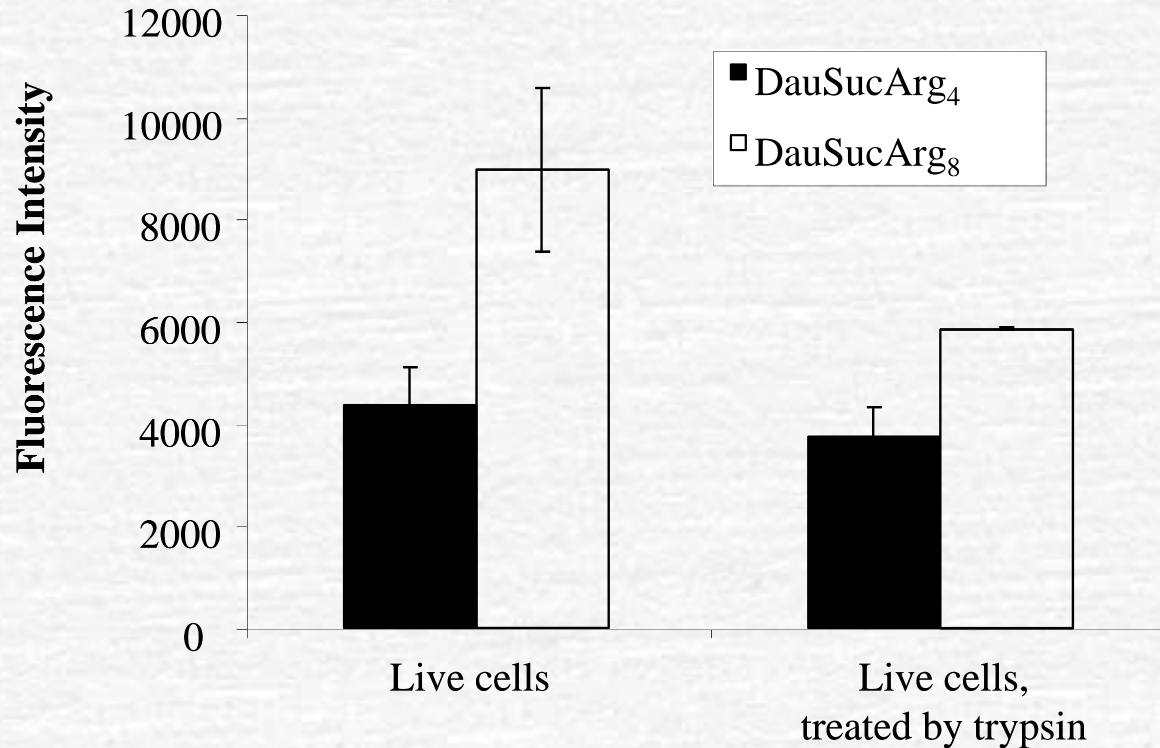


Cytostatic effect of conjugates



- Cells were treated by the conjugates solution at concentration 2.6×10^{-4}-10^{2} μM.
- The IC_{50} values were determined by MTT assay.

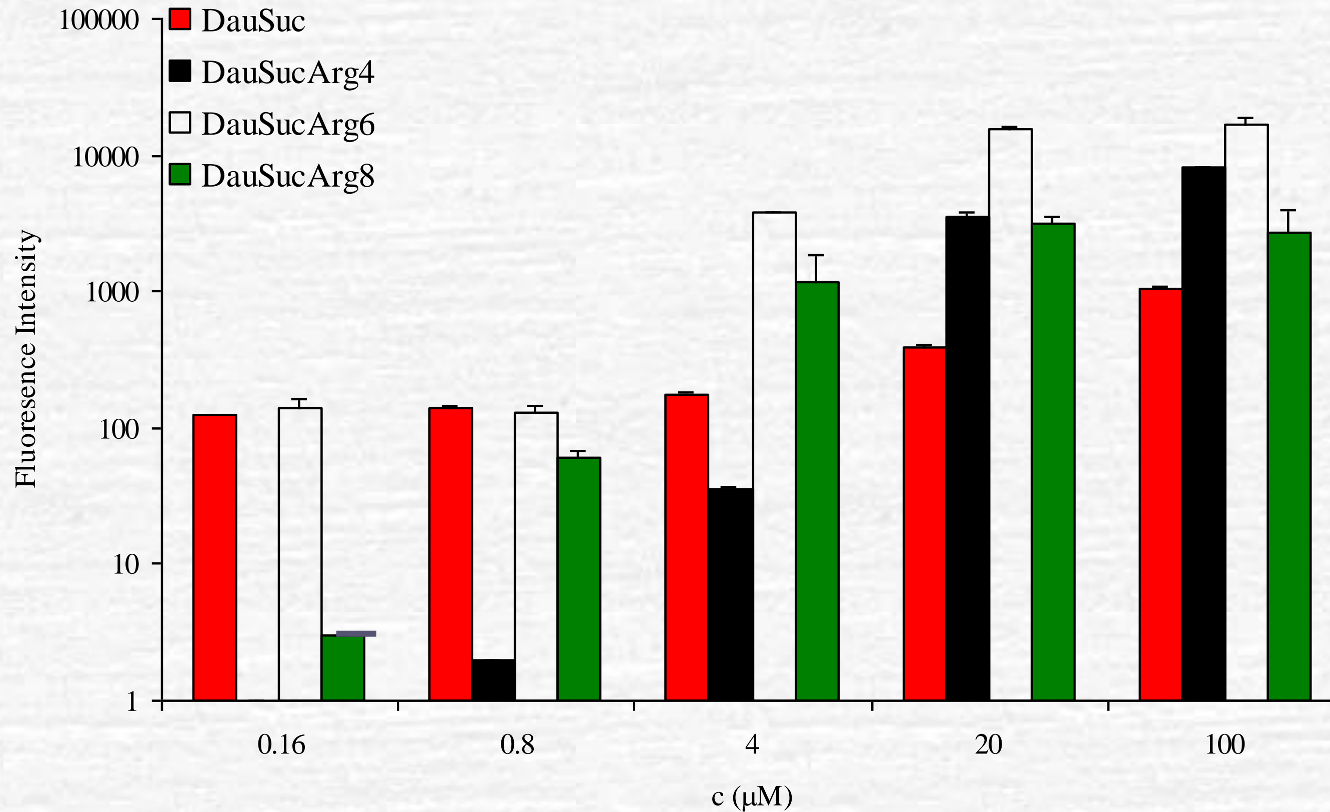
The effect of trypsin treatment



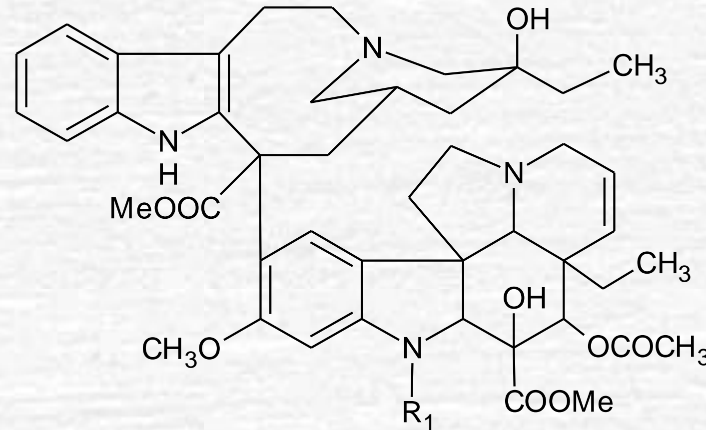
HL-60 cells were treated with the solution of conjugates (c= 30 μM, 90 min), then with/without trypsin treatment the fluorescence intensity of cells was studied by flow cytometry.

Cellular uptake by HL-60 cells

Concentration dependence



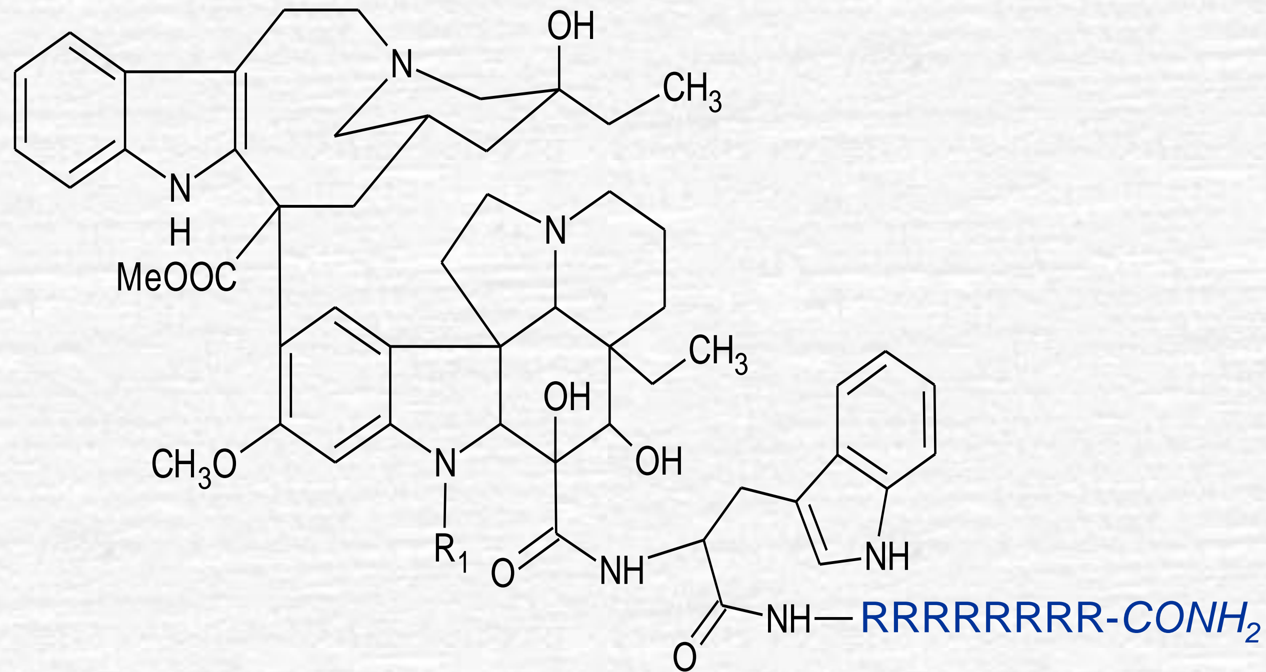
Vinblastin



	R_1
vincristin	CHO
vinblastin	CH_3

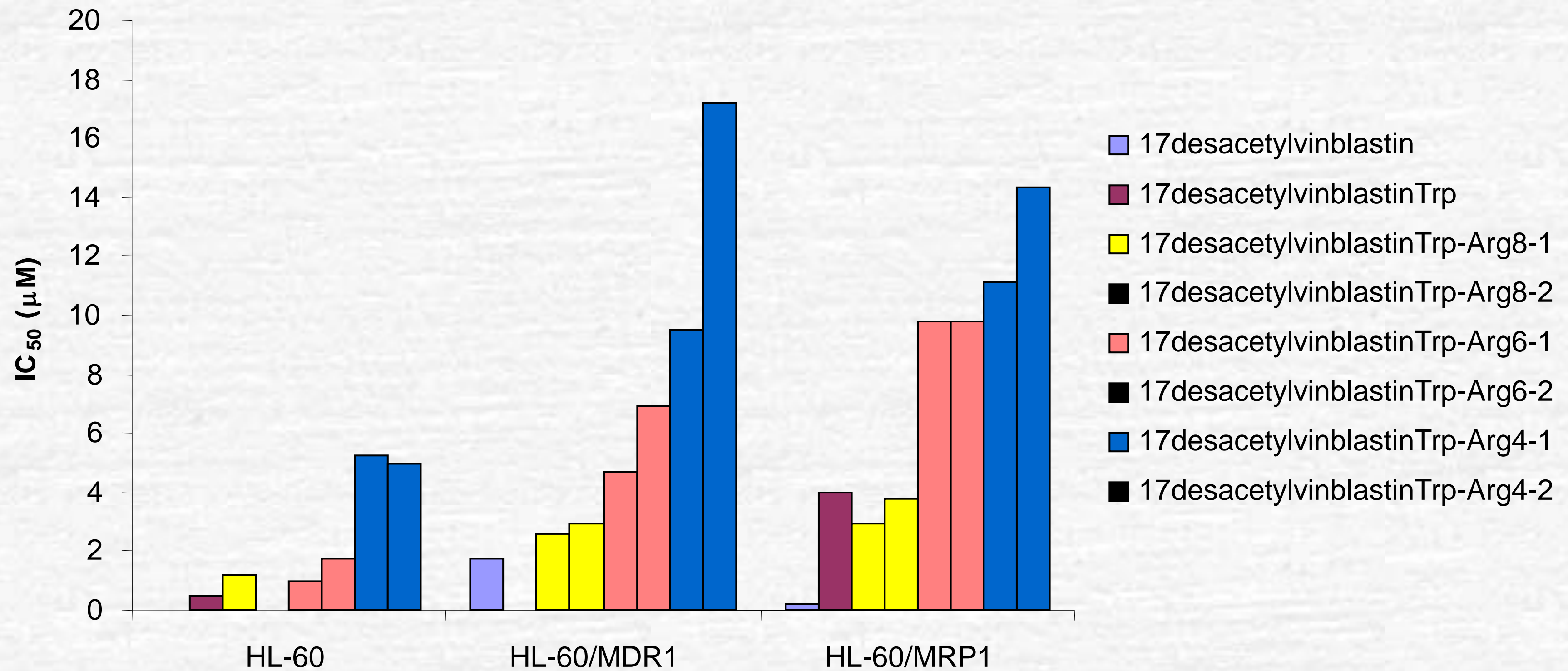
- *vinca* alkaloids (vincristin, vinblastin)- bisindol alkaloids
- vinblastin is used in chemotherapy
- destroy the microtubular system
- side effects, e.g. leucopenia

Structure of conjugate

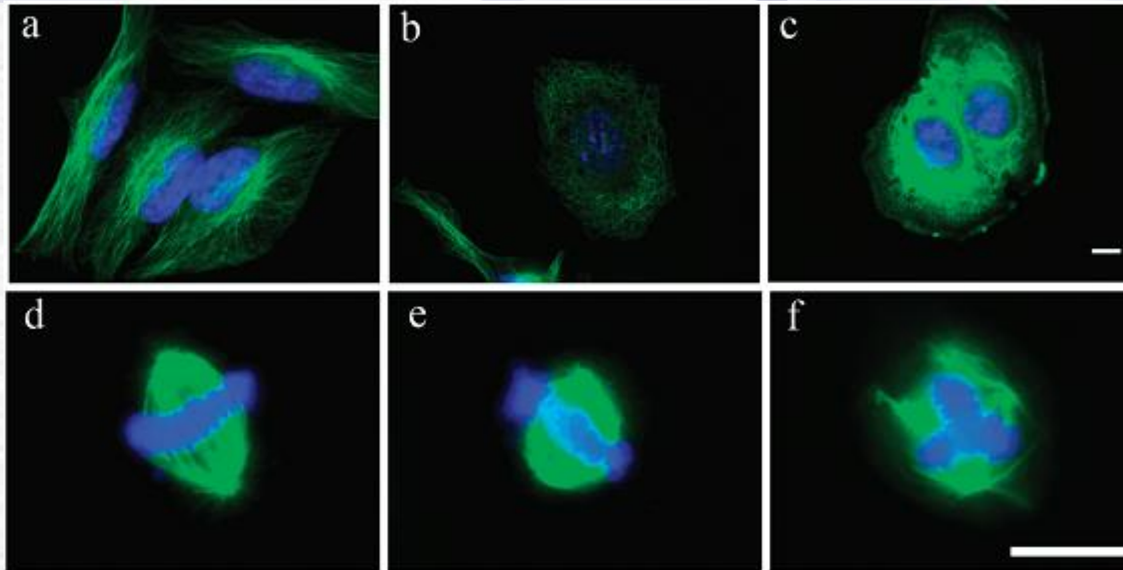


two isomers: L- or D-Trp

Cytostatic activity of conjugates

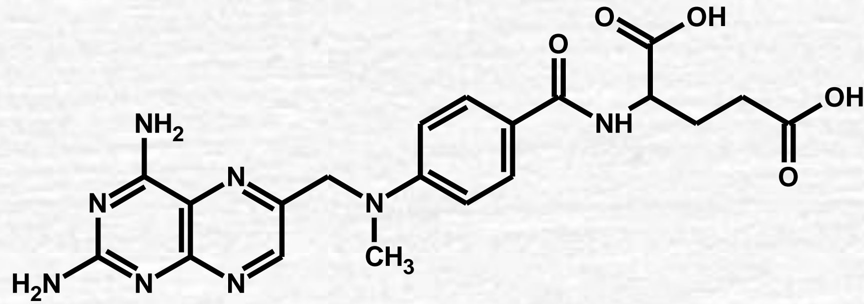


Depolymerisation of microtubular system



	vinblastin		17-dezacetylvinblTrp		17-dezacetylvinblTrpArg$_8$-2		17-dezacetylvinblTrpArg$_8$-1	
	aberant mitozis (%)	*interfase microtubuless*	*Aberant mitozis (%)*	*interfase microtubules*	*Aberant mitozis (%)*	*interfase microtubules*	*Aberant mitozis (%)*	*interfase microtubules*
control	2	normal	2	Normal	2	normal	2	normal
0,25μM	100	Depolymerised	47	normal	22	normal	n. d.	n. d.
1μM	100	Depolymerised	100	fragmented	75	normal	45	normal
2,5μM	100	Depolymerised	100	fragmented	98	fragmented	75	normal
5μM	100	Depolymerised	100	depolymerise	100	fragmented	100	normal

Methotrexate



Well-known antitumor agent.

Antifolate. Inhibit dihydrofolate reductase and thymidylate synthase.

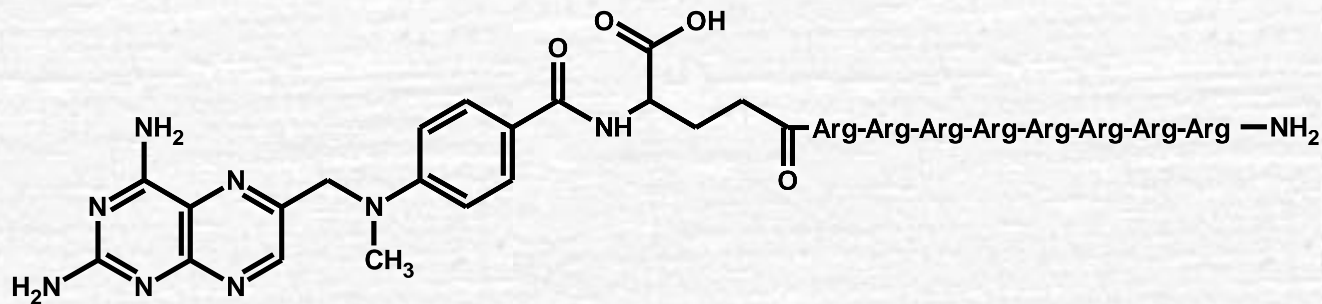
Important step is the polyglutamilation.

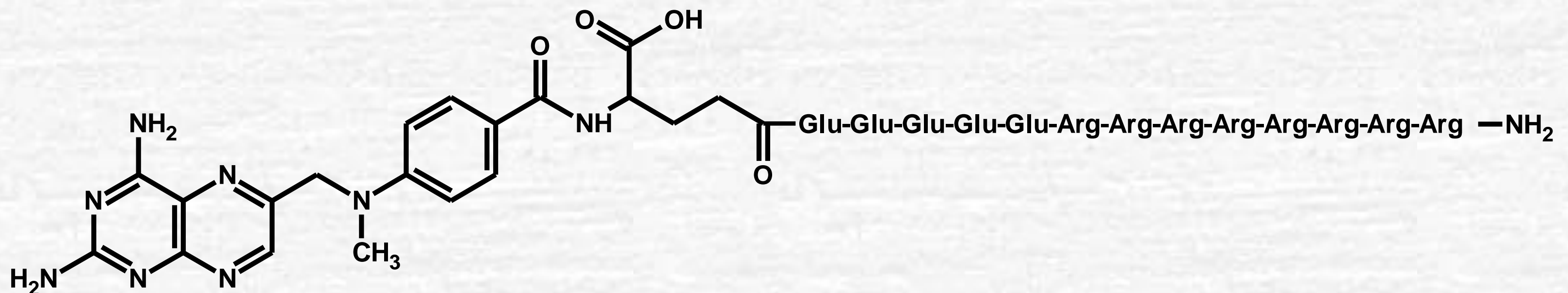
Immunosuppressive and anti-inflammatory effect.

B. J. Perrin et al., Int. *J. Biochem. Cell Biol.* 2002, 34, 722-725.

Structure of conjugates

Our aim was to use conjugates containing free and pentaglutamilated methotrexate and cell-penetrating peptide against resistant tumor cells.





Cellular uptake of conjugates

HL-60 cells

Compound	F_{mean} (sd)			Fluorescent cells % (sd)		
	1 μ M	10 μ M	50 μ M	1 μ M	10 μ M	50 μ M
Cf-Arg_8	2569(35)	185413(25267)	259163(545)	100 (0)	100 (0)	100 (0)
Cf-Glu_5-Arg_8	55(2)	493(31)	3333(689)	3 (0)	92 (2)	100 (0)
Cf-Glu_5-Gly_3-Arg_9	335 (21)	2881 (105)	-	62.8 (5.2)	100 (0)	-
Cf-PenC($desMet^{12}$)	4129(744)	22421(863)	48957(10221)	100 (0)	100 (0)	100 (0)
Cf-Glu_5-Pen($desMet^{12}$)	172(27)	3450(336)	9646(268)	13 (2)	100 (0)	100 (0)
Cf-Glu_5-Gly_3-Pen($desMet^{12}$)	343 (12)	3540 (372)	-	73.9 (2)	100 (0)	-

HL-60 cells were treated for 90 min. After washing and trypsin treatment the fluorescence intensity of cells was measured by flow cytometry on a BD LSR II cytometer.

Cytostatic effect of MTX-conjugates

MCF-7 and MDA-MB-231 cells

Compounds	IC_{50} (sd) (μM)	
	MCF-7	MDA-MB-231
Penetratin	> 100	> 100
MTX	0.56 (0.57)	> 100
MTX-Pen(desMet^{12}) (1)	>100	82.5 (13.9)
MTX-Pen(desMet^{12}) (2)	50.4 (34.3)	11.9 (5.4)
MTX-Glu_5-Pen(desMet^{12})	> 100	0.1 (0.1)
MTX-Arg_8	> 100	> 100
MTX-Glu_5-Arg_8	> 100	> 100

MCF-7 and MDA-MB-231 cells were treated for 3 hrs at 2.56×10^{-4} – 100 μM concentration range. After 3 days at 37° C, MTT-assay was carried out. (2 parallel measurements)