A MODULAR NEUROPILIN–HSV DERIVED RF–TUFTSIN PLATFORM FOR TARGETED DELIVERY: VALIDATED IN SPHEROIDS AND EPITHELIAL BARRIER MODELS

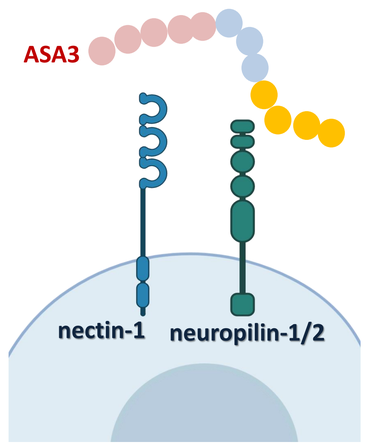
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MODULAR TANDEM DESIGN

QRTVAVYSLRIAGFHG [1]	LINKER [5,6]	TUFTSIN [2-4]



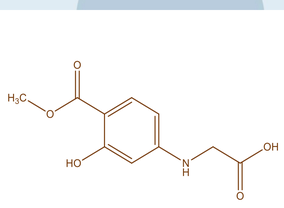
TANDEM ARCHITECTURE/RECEPTOR PROXIMITY MODEL

HSV-gD-RF — **BASED ON HSV-1 gD SERVING AS DERIVED PRIMARY ENTRY SEQUENCE[1]**

TUFTSIN — **NEUROPILIN-ASSOCIATED TARGETING**

SGSG/H12 LINKER — **TUNABLE DISTANCE, NET CHARGE, HYDROPHILICITY/logP VALUES PHYSICOCHEMICAL VERSATILITY**

Cf/ASA3 CARGO — **FOR UPTAKE AND DELIVERY EVALUATION**



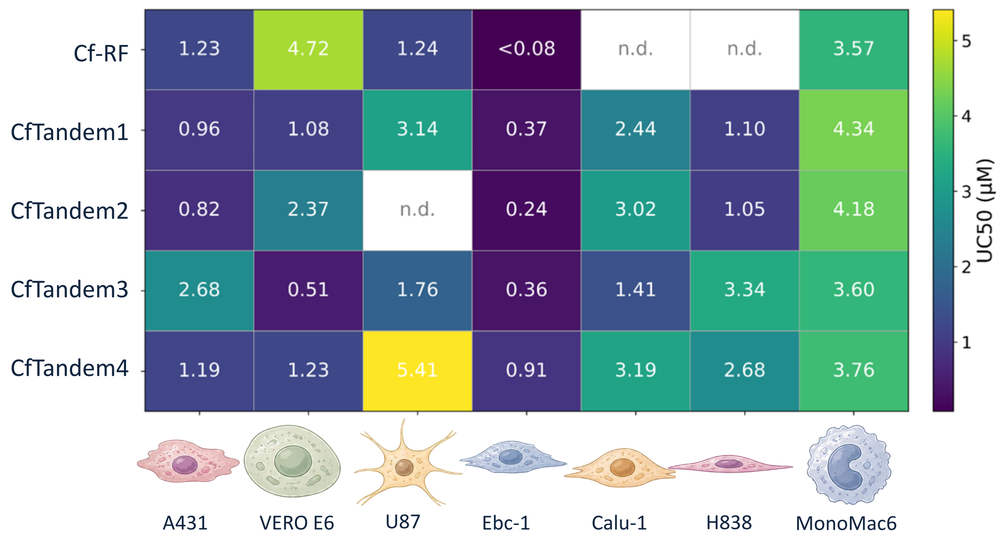
ASA3[2] : (3-hydroxy-4-(methoxycarbonyl)phenyl)glycine

	Code	Mw. calc.	Mw. meas.	Net charge	logP
Cf-RF	Cf-RF *	2132.36	2132.17	2.52	-5.847
Cf-RF-OT5(P)	Cf-RF-OT5(P) *	2712.10	2710.81	4.52	-9.718
CfTandem1	Cf-RF-H12-OT5(P) *	3859.31	3857.33	4.54	-19.552
CfTandem2	Cf-RF-H12-OT5 *	3791.23	3790.98	4.54	-20.159
CfTandem3	Ac-RF-K(Cf)-SGSG-OT5(P) *	3170.49	3169.44	4.52	-15.945
CfTandem4	Ac-RF-K(Cf)-SGSG-OT5 *	3102.41	3101.13	4.52	-16.552
Tandem1	Ac-RF-H12-OT5(P) #	3543.01	3542.65	4.11	-22.863
Tandem2	Ac-RF-H12-OT5 #	3474.93	3474.58	4.11	-22.848
Tandem3	Ac-RF-K(Ac)-SGSG-OT5(P) #	2854.24	2852.51	4.11	-19.256
Tandem4	Ac-RF-K(Ac)-SGSG-OT5 #	2786.16	2786.11	4.11	-19.241
Cf-M3	ASA3-RF-K(Cf)-SGSG-OT5(P) §	3335.65	3335.17	4.03	-14.345
Cf-M4	ASA3-RF-K(Cf)-SGSG-OT5 §	3266.71	3267.57	4.02	-14.947
M2	ASA3-RF-H12-OT5 ‡	3640.07	3639.60	4.35	-21.954
M3	ASA3-RF-K(Ac)-SGSG-OT5(P) #‡	3019.38	3019.01	4.03	-18.061
M4	ASA3-RF-K(Ac)-SGSG-OT5 #‡	2951.31	2950.15	4.10	-17.917

OT5(P): TKPPR, OT5: TKPKG. Mw: Bruker Amazon SL ESI MS, Net charge (pH: 7.4) and logP calculated by www.chemicalize.com/ChemAxon.[4,7,8].
* Fluorescently labeled carriers for uptake and penetration studies # Acetylated carriers for NMR ‡ Drug conjugates for therapeutic evaluation, § Fluorescently labeled drug conjugates for uptake and penetration

CELLULAR AND MECHANISTIC PROFILING & THERAPEUTIC PROOF OF CONCEPT

Figure 1. *In vitro* cellular internalization of Cf-peptides represented by UC_{50} value [1] (concentration value Cf positivity in 50 % of live cells). Treatment: 3h. Quantified by flow cytometry.



EBC-1: **HIGHEST UPTAKE**
H838: **FAVORABLE UPTAKE FOR H12 TANDEMS**
A431 / VERO E6: **LINKER-DEPENDENT DIFFERENCES**
MM6: **GOOD DETECTOR CELLS IN TRANSWELL SYSTEM**

ENERGY-DEPENDENT UPTAKE AND LYSOSOMAL LOCALIZATION

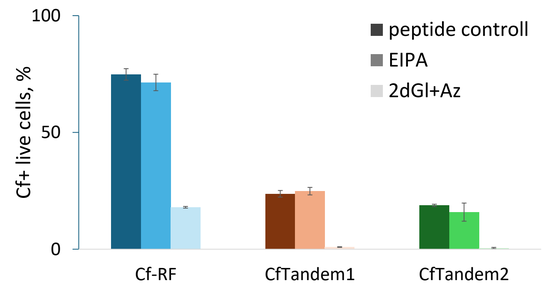
ANTIPROLIFERATIVE ASA3-CONJUGATES

TANDEM DIMINISHED ASA3'S HEMOLYTIC ACTIVITY

BASED ON NMR
TANDEMS MAINLY DISORDERED

ASA3 HAS NO INFLUENCE ON STRUCTURE

Figure 2. Inhibition of internalization[9] with EIPA and ATP depletion, A431 cells

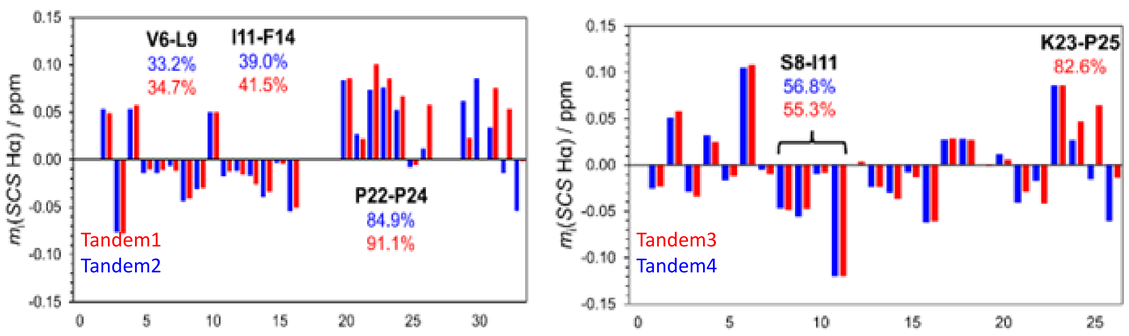


Antiproliferative and hemolytic activity of tamdem conjugates *vs* free ASA3

code	IC_{50} (μM)		HC_{50} (μM)
	U87	Ebc-1	RBC
ASA3	> 100	> 100	3.8
M2	73.0	6.1	> 100
M3	>100	18.6	> 100
M4	>100	23.8	> 100

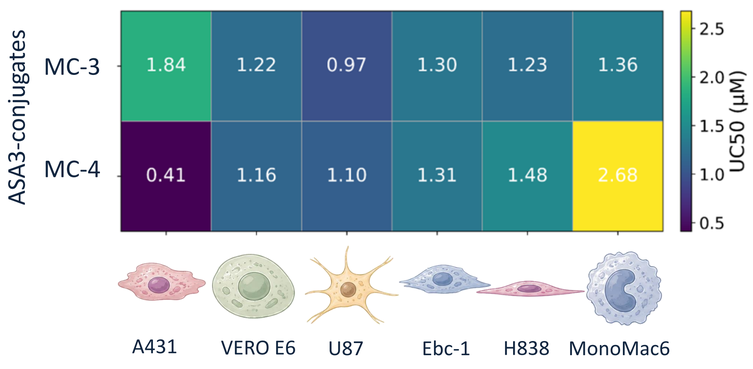
Cytostatic activity was determined by end-point MTT
Hemolytic activity, 1 v/v % RBC: red blood cells, 4 h, 37 °C

Figure 3. Average $^1H^{\alpha}$ secondary chemical shifts (SCS $\delta=\delta_{measured}$ – RCCS of a given atom), ^1H,^{15}N SOFAST-HMQC spectra, 298K, 700MHz. Structural Propensity Identification [10] >95% (– helical, + extended).



VALIDATION IN TISSUE MODELING BARRIER SYSTEMS AND SPHEROIDS

Figure 4. *In vitro* cellular internalization of ASA3-Cf-conjugates represented by UC_{50} value (concentration value Cf positivity in 50 % of live cells). Treatment: 3h. Quantified by flow cytometry.



HIGH TANDEM TRANSPORT ACROSS BRONCHIAL AND ALVEOLAR BARRIER MODELS

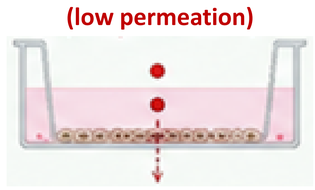
HIGHEST ACCUMULATION IN EBC-1 SPHEROIDS AS LUNG TISSUE MODEL

H12-TANDEMS PROMOTED DEEPER, MORE EFFICIENT PENETRATION

ASA3-CONJUGATES RETAINED UPTAKE AND SPHEROID PENETRATION

Figure 5. Transwell permeation[2-4], comparison on Caco-2, Calu-1 and H838

Caco-2 / MM6 (low permeation)



Calu-1, H838 / MM6 (high permeation)

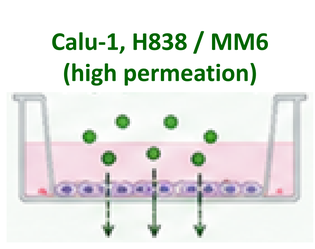
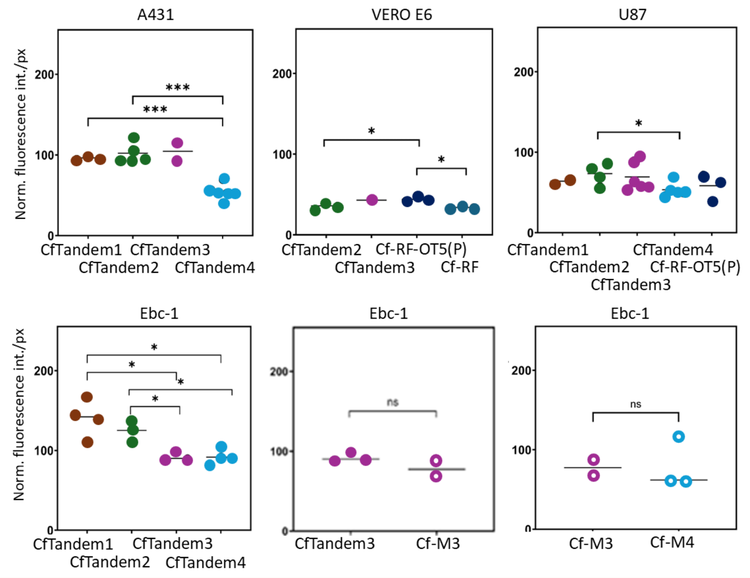
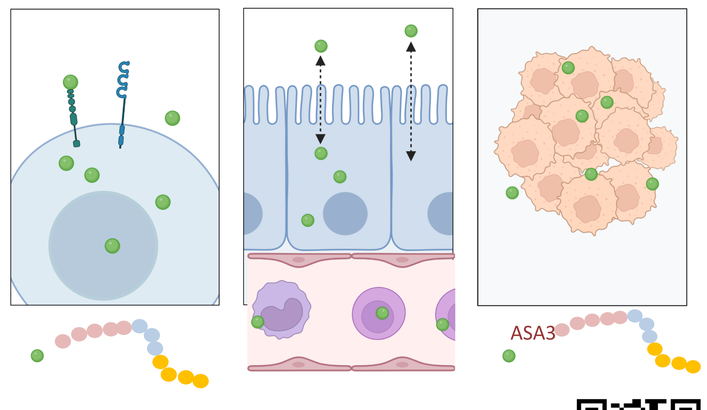


Figure 6. Penetration profile of tamdem derivatives on spheroids [2-4,11]



KEY FINDINGS AND CONCLUSION

1. **TANDEMS SHOWED ROBUST, CELL-DEPENDENT UPTAKE FAVORING EBC-1 AND H838 CELLS**
2. **EMPLOYING LINKER TUNED UPTAKE AND INTRACELLULAR TRAFFICKING**
3. **TANDEMS EXHIBITED ENERGY-DEPENDENT INTERNALIZATION UNLIKE CYTOPLASMIC PARENT RF**
4. **OT5(P)-TANDEMS PERFORMED BEST IN CALU-1 BARRIERS**
5. **H12-TANDEMS SHOWED DEEPER SPHEROID PENETRATION**
6. **TANDEMS DELIVERED ASA3 INTO TUMOR CELLS AND MADE THE CARGO EFFECTIVE**
7. **ASA3-CONJUGATES PRESERVED UPTAKE, PENETRATION AND CONFORMATIONAL FEATURES**



Acknowledgements: NNKFIH K142904, K137940, PD146764, 150838; VEKOP-2.3.3-15-2017-00020, 2.3.3-15-2017-00016; ELTE Excellence Fund EKA 2023/071-P085-1, National Cardiovascular Laboratory Program RRF-2.3.121-2022-00003, Therapeutic Thematic Programme TKP2021-EGA-23, EU HORIZON WIDERA 2023 IDP2Biomed 101160233.

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