A HIGH THROUGHPUT SOFT AGAR ASSAY ON A PANEL OF 60 TUMOR CELL LINES TO STUDY COMPOUNDS FOR POTENTIAL ANTI-ONCOGENIC PROPERTIES

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Introduction

Primary aim of cancer therapies is to target the malignant transformed phenotype of tumor cells. Therefore we were interested to establish a method to screen compounds on many cell types in parallel in an assay system indicative for cell transformation. The gold standard for in vitro assessment of cell transformation is the soft agar assay, indicative for anchorage independent growth. We here show analyses of the impact of substances Staurosporine and Actinomycin D on the soft agar growth of 60 different tumor cells lines.

Methods:

Soft agar assay:

Indicated tumor cells were seeded in 96 well plates in 50 µL 0,4% soft agar onto a 100 µL layer of 0,6% soft agar in adequate medium The day after, compounds were added and after 8-14 days each condition was photographed. Cell viability indicative for colony growth was determined by measurement of fluorescence upon Alamar Blue stain.

Proliferation assay:

Indicated tumor cells were seeded in 96 well plates in 150 µL of adequate medium. The day after, compounds were added and cell viability was determined after 3 days by measurement of fluorescence upon Alamar Blue stain.

Compounds:

In the screen, Staurosporine and Actinomycin D were tested in decalog dilution steps. Only the inner wells of the plate were used. On each plate, six high (solvent 0,1% DMSO) and low controls treated with 1E-5M staurosporine were generated to determine margin of detection and Z' factor.

Data analysis:

Raw data were converted into percent soft agar growth relative to high controls and low controls, which were set to 100% and 0%, respectively. IC50 calculation was performed using GraphPad Prism 5 software with a variable slope sigmoidal response fitting model using 0% soft agar growth as bottom constraint and 100% soft agar growth as top constraint.

Conclusion:

The established soft agar screening platform on 60 tumor cell lines has several advantages:

- Find transformation targets
- Longer treatment periods without confluency
- Closer to physiology due to 3D
- Allows profiling of compounds

The observed prooncogenic properties of Staurosporine on selected cell lines suggest that here inhibition as well as activity of a specific set of kinases causes morphological changes and enhanced growth.

Result

Cells transformed by a given oncogene aquire the capability to grow anchorage independent in soft agar.

We wanted to know, if such cells are sensitive to specific inhibitors in the same way in the proliferation and soft agar assay.

Our data show that transformed cells are more sensitive to transformation-specific inhibitors in the soft agar assay.

The results suggest that a soft agar assay

- would pick up compounds which would be missed in the proliferation assay
- would identify cell lines transformed but not addicted to the target of interest

We went to establish a 96 well format soft agar assay with various tumor cells to enable

A	determination of Soft Agar IC50 values
B	profiling of compounds on many cell lines

We show that

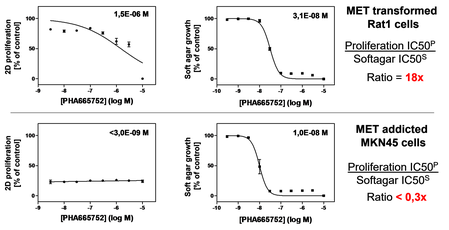
- 60 tumor cell lines were successfully established in the 96-well Soft agar assay in a reliable fashion with Z' factors > 0,4.
- IC50 values were determined for Actinomycin D (translation inhibitor) and Staurosporine (broad kinase inhibitor).
- On several cell lines, soft agar IC50 values were significantly more potent compared to proliferation IC50´s in a substance specific manner.

Surprisingly, Staurosporine displayed biphasic activity on soft agar growth of some cell lines.

To exclude artefacts, this observation was analysed more closely with zoomed titration.

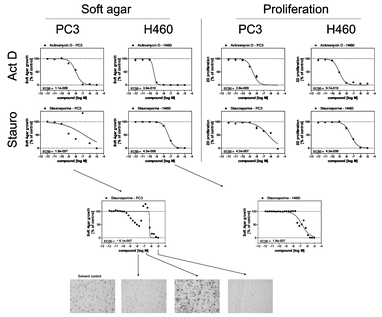
Our data show that

- Staurosporine has a predominant inhibitory impact on softagar growth
- With PC3 cells, Staurosporine inhibition is diminished and even stimulation observed at a concentration of 1E-7M.
- Stauro-stimulated colonies had a different morphology than control colonies.
- Zoomed titration revealed hidden stimulatory effects also in other cell lines.



Normal Rat1 cells proliferate but do not grow in soft agar. Introduction of active MET induces soft agar and tumor growth (MET transformed Rat1). MKN45 gastric cancer cells per se endogenously express active MET and are addicted to MET expression.

In the 2D proliferation assay, MET inhibitor PHA665752 shows potent inhibition of MKN45, but is hardly active on Rat1-MET cells. The soft agar assay reveals similar potent activity of PHA665752 on both cell lines.



In soft agar assays, Staurosporine reproducibly showed strongly enhanced growth stimulation at around 1E-7M on some cell lines such as PC3, while other cell lines such as H460 showed the typical sigmoidal inhibition curves. This effect was neither visible in proliferation assays, nor with Actinomycin D. To further characterize this observation, Soft agar assays were performed with Staurosporine titrated in 24 binary-log dilutions steps. Interestingly, a very narrow region of Staurosporine concentrations was detected, in which PC3 cells were stimulated. As visible from the images, PC3 at stimulatory Staurosporine dosage looked different from control PC3 cells. Surprisingly, also H460 cells showed a related response when analysing this zoomed-up resolution.

			Soft agar characteristics						Impact Staurosporine			Impact Actinomycin D		
Cell type	Entity	Medium	Cell-number	Average Z' factor	Average Margin	Days of incubation	Stauro Curve	Image	Prolif. Mean	SoftAgar Mean	Prolif SA	Prolif. Mean	SoftAgar Mean	Prolif SA	Cell type
A2058	skin	DMEM	10.000	0,78	26,4	7	---		2,E-08	3,E-08	0,8	8,E-10	4,E-10	1,9	A2058
A375	skin	DMEM	2.500	0,77	13,1	8	---		2,E-08	9,E-09	2,2	6,E-10	3,E-10	2,0	A375
A498	kidney	DMEM	10.000	0,76	12,3	8	---		5,E-07	1,E-08	47,7	3,E-09	1,E-08	0,3	A498
A549	lung	DMEM	5.000	0,94	17,5	8	---		4,E-08	3,E-09	12,0	5,E-09	8,E-10	5,9	A549
AsPC-1	pancreas	DMEM	5.000	0,69	11,1	8	---		2,E-06	7,E-09	292,6	2,E-07	1,E-09	150,0	AsPC-1
C33a	cervix	DMEM	10.000	0,80	11,2	11	---		1,E-07	4,E-08	2,4	5,E-10	3,E-10	1,4	C33a
C8161	melanoma	DMEM	2.500	0,86	17,7	8	---		1,E-07	4,E-10	256,8	2,E-09	3,E-10	5,6	C8161
Caki-2	kidney	DMEM	10.000	0,33	6,2	8	---		3,E-06	7,E-08	38,6	5,E-09	3,E-09	1,4	Caki-2
Calu-6	lung	DMEM	5.000	0,72	12,0	8	---		2,E-08	7,E-09	3,6	6,E-10	5,E-10	1,3	Calu-6
Colo 205	colon	DMEM	2.500	0,73	9,8	8	---		1,E-08	2,E-09	6,2	9,E-10	2,E-10	5,1	Colo 205
CX-1	colon	DMEM	5.000	0,72	7,4	8	---		3,E-08	3,E-08	0,7	6,E-09	7,E-10	7,9	CX-1
CXF-94	colon	DMEM	5.000	0,62	11,9	8	---		2,E-06	1,E-07	17,0	4,E-08	8,E-10	50,6	CXF-94
DLD-1	colon	DMEM	2.500	0,79	23,6	8	---		9,E-08	6,E-09	13,8	2,E-09	8,E-10	2,0	DLD-1
DU-145	prostate	DMEM	10.000	0,78	9,2	8	---		1,E-06	6,E-08	0,2	2,E-09	3,E-09	0,8	DU-145
H1299	lung	DMEM	10.000	0,88	23,0	8	---		2,E-08	1,E-09	12,6	8,E-10	4,E-10	2,1	H1299
H460	lung	DMEM	2.500	0,94	22,3	8	---		5,E-08	6,E-08	0,8	1,E-09	4,E-10	2,5	H460
HCC 1569	breast	RPMI-1640	10.000	0,88	14,1	11	---		6,E-07	1,E-08	47,4	7,E-09	3,E-10	21,0	HCC 1569
HCC827	lung	RPMI-1640	10.000	0,78	11,9	11	---		2,E-07	4,E-08	4,1	3,E-09	3,E-10	9,4	HCC827
HCT116	colon	DMEM	2.500	0,94	23,3	8	---		2,E-08	7,E-09	3,2	6,E-10	2,E-10	2,6	HCT116
HeLa	cervix	DMEM	2.500	0,94	23,8	8	---		2,E-08	7,E-09	2,2	1,E-09	2,E-10	5,2	HeLa
HepG2	liver	DMEM	2.500	0,56	4,4	14	---		2,E-07	3,E-09	75,6	3,E-10	1,E-10	2,6	HepG2
Hs 746T	stomach	DMEM	2.500	0,87	14,8	8	---		5,E-08	1,E-08	1,3	5,E-10	4,E-10	1,1	Hs 746T
HT-1080	sarcoma	DMEM	10.000	0,58	13,4	8	---		3,E-09	7,E-10	4,3	4,E-10	2,E-10	1,6	HT-1080
HT-29	colon	DMEM	2.500	0,52	7,1	8	---		1,E-08	3,E-08	0,3	2,E-09	1,E-09	1,5	HT-29
Hutu 80	colon	DMEM	10.000	0,15	8,2	8	---		2,E-07	3,E-10	633,6	2,E-10	8,E-11	2,3	Hutu 80
IGROV-1	ovary	RPMI-1640	10.000	0,75	13,6	8	---		8,E-09	4,E-09	1,9	6,E-10	3,E-10	1,9	IGROV-1
JIMT-1	breast	RPMI-1640	20.000	0,80	19,8	8	---		2,E-07	2,E-08	12,8	3,E-09	2,E-09	1,6	JIMT-1
LnCap	prostate	DMEM	2.500	0,62	11,3	8	---		9,E-08	3,E-07	0,3	1,E-09	5,E-10	2,2	LnCap
LOVO	colon	DMEM	2.500	0,91	20,4	8	---		3,E-08	2,E-08	1,5	1,E-09	5,E-10	2,1	LOVO
LS174T	colon	DMEM	10.000	0,89	14,7	8	---		2,E-07	4,E-07	0,4	5,E-10	5,E-10	1,0	LS174T
MCAS	ovary	MEM	5.000	0,83	11,3	8	---		5,E-07	2,E-09	282,5	5,E-10	7,E-10	0,8	MCAS
MCF-7	breast	DMEM	5.000	0,72	13,5	8	---		7,E-08	3,E-08	2,2	1,E-09	5,E-10	2,5	MCF-7
MDA MB 231	breast	DMEM	15.000	0,58	10,9	11	---		4,E-09	1,E-09	3,0	1,E-09	1,E-09	0,9	MDA MB 231
MDA MB 435	skini	RPMI-1640	2.500	0,82	19,3	9	---		2,E-07	8,E-08	2,1	3,E-09	6,E-10	6,0	MDA MB 435
MDA MB 468	breast	DMEM	5.000	0,65	19,5	8	---		2,E-08	2,E-09	6,7	4,E-07	5,E-08	7,3	MDA MB 468
Mia PaCa 2	pancreas	DMEM	5.000	0,92	22,2	8	---		5,E-08	2,E-08	2,8	8,E-10	9,E-11	9,0	Mia PaCa 2
MKN-1	stomach	RPMI-1640	5.000	0,87	20,8	9	---		1,E-07	1,E-08	13,8	3,E-09	8,E-10	4,1	MKN-1
MKN-45	stomach	DMEM	2.500	0,90	27,7	8	---		1,E-08	5,E-09	3,1	1,E-09	9,E-10	1,3	MKN-45
N417	lung	RPMI-1640	2.500	0,83	16,9	9	---		3,E-08	1,E-08	2,6	6,E-10	1,E-10	5,7	N417
NCI-ADR	ovary	RPMI-1640	2.500	0,95	22,0	9	---		3,E-08	5,E-09	5,8	3,E-07	2,E-08	10,9	NCI-ADR
NCI-H441	lung	RPMI-1640	10.000	0,75	7,4	11	---		2,E-07	1,E-08	11,5	2,E-09	5,E-10	2,9	NCI-H441
NCI-N87	stomach	DMEM	10.000	0,65	3,4	11	---		8,E-09	1,E-09	7,4	2,E-09	2,E-09	1,2	NCI-N87
PC3	prostate	DMEM	5.000	0,89	10,7	11	---		1,E-08	9,E-08	14,4	3,E-09	1,E-08	0,3	PC3
R30C	breast	DMEM	2.500	0,86	19,1	8	---		3,E-07	2,E-08	12,2	1,E-09	8,E-10	1,5	R30C
RKO	colon	DMEM	5.000	0,85	23,2	8	---		2,E-08	1,E-08	1,8	3,E-10	2,E-10	1,8	RKO
RL95-2	ovary	DMEM	10.000	0,85	11,0	10	---		2,E-07	3,E-09	50,7	2,E-09	5,E-10	3,4	RL95-2
SiHa	cervix	DMEM	2.500	0,82	24,7	11	---		2,E-08	3,E-09	8,1	2,E-09	3,E-10	5,7	SiHa
SK BR-3	breast	McCoys 5a	10.000	0,84	16,1	8	---		8,E-08	2,E-07	0,3	4,E-09	1,E-09	3,4	SK BR-3
SK-N-FI	brain	DMEM	5.000	0,72	15,3	11	---		7,E-08	4,E-08	1,7	6,E-09	1,E-09	6,5	SK-N-FI
SK-N-MC	bone	MEM-alpha	2.500	0,51	15,5	11	---		1,E-08	7,E-10	13,8	6,E-10	2,E-10	2,9	SK-N-MC
SK-N-SH	brain	DMEM	10.000	0,65	4,3	14	---		2,E-07	3,E-09	67,4	4,E-10	5,E-10	0,8	SK-N-SH
SK-OV3	ovary	DMEM	10.000	0,89	11,8	11	---		4,E-07	1,E-08	34,6	1,E-08	2,E-09	4,9	SK-OV3
SW480	colon	DMEM	10.000	0,54	10,0	7	---		1,E-07	5,E-08	2,6	5,E-10	2,E-10	2,3	SW480
SW620	colon	DMEM	1.000	0,70	8,6	11	---		1,E-08	1,E-09	9,0	6,E-10	8,E-11	6,8	SW620
SW707	colon	DMEM	5.000	0,82	12,2	8	---		1,E-08	2,E-09	6,8	2,E-09	1,E-09	1,5	SW707
SW948	colon	DMEM	2.500	0,72	15,3	8	---		1,E-08	4,E-08	1,7	6,E-09	1,E-09	6,5	SW948
T47D	breast	RPMI-1640 +insulin	5.000	0,84	11,3	9	---		4,E-07	1,E-07	3,5	1,E-08	5,E-10	21,3	T47D
U118MG	brain	DMEM	10.000	0,84	14,6	11	---		2,E-07	4,E-09	53,0	7,E-09	2,E-09	3,7	U118MG
U87MG	brain	DMEM	5.000	0,77	17,6	11	---		5,E-08	4,E-09	12,2	6,E-10	5,E-10	1,3	U87MG
ZR-75-1	breast	DMEM	5.000	0,81	7,6	11	---		5,E-07	6,E-07	0,9	2,E-09	2,E-09	0,9	ZR-75-1
															* ratio > 0,2x

Soft agar analysis in 96 well format revealed 60 cell lines, that yielded margins of detection above 4 fold and Z' factors above 0,5. Images show that tumor cell lines significantly differ in colony size, number and morphology, which was taken into account by different incubation periods and seeding densities.

IC50 values were determined for substances Staurosporine (broad kinase inhibitor) and Actinomycin D (transciption inhibitor) in comparison to the standard 2D proliferation assay. Interestingly, the Softagar analysis in many cell lines revealed more potent IC50 values than the proliferation assay (more than 5x more potent = green ratio fields), This effect was substance specific. The inverse case (5x less potent) was not observed in this study. Surprisingly, we observed not only inhibitory, but also stimulatory activity of Staurosporine.