Research Paper

Safety and Efficacy of Suicide Gene Therapy with Adenosine Deaminase 5-Fluorocytosine Silmutaneously in *in Vitro* Cultures of Melanoma and Retinal Cell Lines

Antonios Sakkas[1], Paul Zarogoulidis[1✉], Kalliopi Domvri[1], Wolfgang Hohenforst-Schmidt[2], Dimitris Bougiouklis[3], Stylianos Kakolyris[4], Thomas Zarampoukas[1], Ioannis Kioumis[1], Georgia Pitsiou[1], Haidong Huang[5], Qiang Li[5], Soultana Meditskou [6], Theodora Tsiouda[7], Nikolaos Pezirkianidis[8], Konstantinos Zarogoulidis[1]

1. Pulmonary Department-Oncology Unit, ``G. Papanikolaou`` General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece
2. II Medical Department, ``Coburg`` Regional Clinic, Coburg, Germany.
3. Gene and Cell Therapy Center, Hematology-BMT Unit, ``G. Papanikolaou`` Hospital, Thessaloniki, Greece.
4. Oncology Department, University General Hospital of Alexandroupolis, Alexandroupolis, Greece.
5. Department of Respiratory Diseases, Changhai Hospital/First Affiliated Hospital of the Second Military Medical University, Shanghai, People's Republic of China.
6. Laboratory of Histology, Embryology and Anthropology, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece
7. Internal Medicine Department, ``Theiageneio`` Anticancer Hospital, Thessaloniki, Greece.
8. Surgery Department, Private Cabinet, Serres, Greece.

✉ Corresponding author: Paul Zarogoulidis, Pulmonary Department-Oncology Unit, ``G. Papanikolaou`` General Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece. Tel: +306977271974, Fax: +302310992433, e-mail: pzarog@hotmail.com.

Abstract

Local treatment as a treatment modality is gaining increased general acceptance over time. Novel drugs and methodologies of local administration are being investigated in an effort to achieve disease local control. Suicide gene therapy is a method that has been investigated as a local treatment with simultaneously distant disease control. In our current experiment we purchased HTB-70 (melanoma cell line, derived from metastatic axillary node) and CRL-2302 (human retinal epithelium) were from ATCC LGC Standards and Ancotil®, 2.5 g/250 ml (1 g/00ml) (5-Flucytosine) MEDA; Pharmaceuticals Ltd. UK. Adenosine Cytosine Deaminase (Ad.CD) was also used in order to convert the pro-drug 5-Flucytosine to the active 5-Fluoracil. Three different concentrations of 5-Flucytosine (5-FC) were administered (0.2ml, 0.8ml and 1.2ml). At indicated time-points (4h, 8h and 24h) cell viability and apoptosis were measured. Our concept was to investigate whether suicide gene therapy with Ad. CD-5-FC could be used with safety and efficiency as a future local treatment for melanoma located in the eye cavity. Indeed, our results indicated that in every 5-FC administration had mild cytotoxicity for the retinal cells, while increased apoptosis was observed for the melanoma cell line.

Key words: suicide gene therapy, 5-fluorocytosine, melanoma, retinal.

Introduction

Melanoma is a malignant tumor of melanocytes.[1] Melanocytes produce the dark pigment, melanin, which is responsible for the color of skin. These cells can be found in skin, but also in other parts of the body, including the bowel and the eye. Uveal melanoma is also an entity that has been reported.[2,3] Melanoma can originate in any part of the body that contains melanocytes. Melanoma is less common than

other skin cancers, however, it is much more aggressive if it is not treated early.[1] It causes the majority (75%) of deaths related to skin cancer. The treatment of choice if it is discovered early is surgical removal of the tumor.[4] Surgical treatment is successful while it is still small and thin, and if it is completely removed, then the chance of cure is high. The likelihood of relapse or spreading depends on how deeply it has gone into the layers of the skin. For melanoma that relapses or spreads, additional treatments include chemo- and immunotherapy, or radiation therapy.[5-8] Chemotherapy and radiotherapy have adverse effects and in several situations the patients` treatment has to be postponed. [9] Moreover, severe bone marrow suppression in many cases requires hospitalization with additional costs for the national health system. [10,11] Novel routes of drug administration are being investigated in an effort to reduce the adverse effects in many types of cancer with different strategies, with the main concept being the local treatment. [12-22] Additional investigation of cancer pathways revealed underlying mechanisms that could be utilized to block chemotherapy resistance and sensitize tumors to chemotherapy and radiotherapy. [23] A major breakthrough has been achieved with so called: ``suicide gene therapy`` modality. The introduction of a therapeutic gene encoding, enzyme capable of transforming a nontoxic pro-drug into a cell toxin enhances the cytotoxic effect for cancer cells and protects the healthy cells.[15-17] Suicide gene therapy, utilizing the cytosine deaminase/5-fluorocytosine (CD/5-FC) system, is an efficient methodology for targeted therapy in cancer research with favorable results in previously published studies.[24-28] In specific the cytosine deaminase (CD) enzyme converts the anti-fungal agent 5-Fluorocytosine (5-FC) into its antimetabolite 5-Fluoracile (5-FU), thereby killing tumor cells. Most suicide genes under investigation mediate sensitivity by encoding viral or bacterial enzymes that convert inactive forms of a drug, into toxic metabolites capable of inhibiting nucleic acid synthesis. [29,30] The second suicide gene therapy methodology that has been extensively investigated is the herpes simplex virus thymidine kinase gene (HSV-tk), which converts ganciclovir (GCV) to ganciclovir monophosphate and inconsequence inside the cancer cell the enzymes covert it to ganciclovir triphosphate .

The bystander effect, through which the Ad.CD system applies, has to do with the fact that a pro-drug is converted into an antineoplastic agent in only a percentage of the target cells expressing the drug activating enzyme. These cells are killed as a result of this expression, thus releasing the newly formed anticancer agent to the tumor microenvironment killing also adjacent cells.[29-38] The suicide gene therapy has been investigated in several cancer types; a) colon [16,39,40], b) lung [41,42], c) liver [17,43,44], d) medulloblastomas [45], e) spinal cord tumors [46], f) neuroendocrine [47], g) prostate [48], h) breast [49,50], i) bladder [51], j) head and neck [52], k) brain [53], l) gliomas [54-56], m) sarcomas [57] and n) melanoma (HSV-tk- GCV)[58]. The suicide gene modality has been also investigated as; a) anti-vascular endothelial treatment [59,60], b) interleukin-12 (IL-12) [61] and c) immune stimulation with interleukin- 7 (IL-7).[62] (Table 1.) Moreover, suicide gene therapy has been proven to be efficient in chemotherapy resistant cancer cell lines [63] and to enhance radiotherapy treatment modality. [64] Additional control of micrometastasis has also been observed in suicide gene therapy studies.[65] In the current *in vitro* study we investigated the safety and efficiency of ad.CD-5-FC in melanoma and uveal cell lines and proposed a future method of local administration for primary or metastatic uveal melanoma treatment methodology.

Table 1. Suicide Gene Therapy Studies.

Author	Cells lines	Design	Result	Transport	Ref
Michaelsen S. R. et al.	GLC-14, GLC-16, GLC-19, NCI-H69, H69-VP, H69-CPR, H69-DAU, H69-BCNU	*In Vitro* *In Vivo*	Effective both in chemosensitive and chemoresistant cell lines	INSM1 promoter-driven SG	63
Mader R.M. et al.	CCL227 (with low and Intermediate phenotypes)	*In Vitro*	Effective with 100% Activation	Adenoviral cosmids	16
Bondanza A. et al.	Leukemia (mouse)	*In Vitro* *In Vivo*	Effective with IL-7 receptor expression (HA-1-, H-Y-)	Herpes simplex virus Thymidine kinase (*tk*)	62
Xu Y. et al.	Lewis Lung Cancer A549	*In Vitro* *In Vivo*	Combination IL-12 and suicide gene therapy enhances the antitumor effect as a factor modifying the tumor microenvironment	AdCMV(-), AdhTERTHRP, AdCMVmIL-12	61
Sia KC. et al.	HCC 26-1004	*In Vitro* *In Vivo*	Effective HSV-1 amplicon viral vector and 5-FU administration	HSV-1 amplicon viral Vector coupled with yCD	17
Li S. et al.	C17.2 NSC line	*In Vitro* *In Vivo*	ATRA enhanced the HSV-tk/GCV	HSVtk/GCV	45
Finocchiaro	sarcoma	*In Vivo*	Effective	Lipid-complexed plasmid	57

M.E.L. et al.			Microenvironment Control and Distant metastasis	Bearing IFN-β and suicide genes co-administered with ganciclovir (ISG)	
Leng A. et. al.	Human colon carcinoma (Lovo) cell line	*In Vitro* *In Vivo*	Anti-VEGF-A- Suicide gene therapy	5-FC, CPNP-shVEGF-CDTK	59
Liu T. et. al.	SGC7901 human gastric Cancer cell line	*In vitro* *In Vivo*	Anti-VEGF- Suicide gene therapy	5-FC, triple gene vector Expressing VEGF-shRNA and fusion suicide gene yCDglyTK delivered by CPNPs	60
Finzi et. al.	Human HT29 and murine DHDK12 pro-b	*In Vitro* *In Vivo*	MTX, aphidicolin and ara-C. The rate of apoptosis increased two-fold in MTX-treated DHDK12 cells after treatment with GCV.	HSVtk-GCV	40
Niu H. et al.	VX2 liver cancer	*In Vivo*	Effective with lipiodol embolism and WTp53	TK/CD plus intraperitoneal Injection of GCV at 100mg/(kg.d) and 5-FC at 500mg/(kg.d)	43
Marukawa Y. et. al.	HCC	*In vitro* *In Vivo*	Effective Mac-1, CD4, CD8a-positive and TNF increase	-HSV-tk/GCV and MCP-1 -rAd harboring human *MCP-1* and the membrane-spanning domain of the tumor cell surface	44
Kosaka H. t. al.	9L rat glioma cells and 293 cells	*In vitro* *In Vivo*	MSC-EGFP or MSC-CD-5-FC resulted in significant prolongation of survival	AdexCAEGFP AdexCACD	55
Schmidt M. et. al.	Head and Neck squamous carcinoma cell line FADU	*In Vitro*	Effective with deletion Mutant of ETA as a Target gene	Gene Switch System	52
Cottin S. et. al.	Glioblastoma	*In Vitro*	Effective against Cx43 cytoplasmic localization	Lentiviral delivery of HSV-tk/GCV	56
Kakinoki K. et. al.	HCC	*In vitro* *In Vivo*	Effective against metastasis and control of microenvironment	CCL2/MCP-1 HSV-tk/GCV	65
Sun X. et. al.	R3327-AT rat prostate Carcinoma cells	*In Vitro* *In Vivo*	Effective against hypoxic cells	Bifunctional cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT) with 5-FC and radiotherapy	64
Amano S. et. al.	C6 glioma cells	*In Vitro* *In Vivo*	Safety evaluation of the Stem cell therapy in brain tissue	Rat MSCtk/GCV	67
Zhao Y. et. al.	U87 glioma and H4 cells	*In Vitro* *In Vivo*	Effective as cellular Vehicle for targeted suicide gene therapy	Tumor-tropic neural stem cells, HSV-tk/GCV	75
Wang C. et. al.	NCI-H460-GFP cells	*In Vitro* *In Vivo*	Effective brain metastasis treatment	NSC line expressing CD and TK	53
Yin X. et. al.	Bladder cancer with N-methyl-nitrosourea perfusion	*In Vitro* *In Vivo*	Effective both in extrisinc and intrisinc papoptosis pathways	BI-HSV-tk/GCV	51
Cramer F. et. al.	SCLC: GLC16, DMS53 and NCI-H69 and NSCLC cancer lines: H1299 and A549	*In Vitro*	Improved plasmid nuclear delivery	NFnB-DTS in an YCD-YUPRT (SCD)	41
Duan X. et. al.	C-26	*In Vitro* *In Vivo*	DMP Delivered Survivin-T34A gene DMP/S-T34A) which induced apoptosis	DOTAP and MPEG-PCL hybrid micelles (DMP)	71
Zarogoulidis P. et. al.	Lewis lung cancer, SCLC, NSCLC patients	Animals Humans	Survival and malignant pleural effusion control with higher efficiency observed for SCLC.	Ad.CD+5-FC	14
Yi B. et. al.	Review	Review	Review	Review	50
Qiu Y. et. al.	A549, 16HBE, SPC-A-1 And NCI-H520	*In Vitro*	Specific CA-positive Target gene expression	CEA promoter and double suicide genes TK and CD. pCEA-TK/CD	42
Won Y. et. al.	C6, U87, F98 and 9L	*In Vitro* *In Vivo*	Tumor growth Suppression and locomotor function maintenance	rPOA/HSV-tk/GCV	46
Akerstrom V. et. al.	Neuroendocrine tumors: NCI-H69, NCI-H1155, NCI-H727, DMS53, U87MG, IMR-32, S-N-SH, SK-N-BE(2), Y79, WERI-Rb1, HeLa, ANC-1, BEAS, RIN, D283 Med, HepG2	*In Vitro* *In Vivo*	Enhanced antitumor activity over the RSV control	INSM1 promoter, HSV-tk to generate Ad-K5 virus	47

Author	Cancer/Cell line	Type	Result	Therapy/Vector	Ref
Lu M. et. al.	Prostate	Human	Initiated and recruiting at the time of publication	Replication-Competent Adenovirus- mediated suicide gene therapy	48
Ma S. et. al.	MCF-7 and MDA-MB-231 Breast cell lines	*In Vitro* *In Vivo*	Effective antitumor control	*Drosophila melanogaster* (Dm-dNK)	49
Preuss E. et. al.	G62 human glioblastoma cell Line, A549 human lung Carcinoma, SW620 human Colorectal adenocarcinoma Cell line and IPC298 Human Melanoma cell line	*In Vitro* *In Vivo*	Continuous complete remission	TK.007 novel suicide gene	86
Ahn Y. et. al.	CT26 murine colon adenocarcinoma cells and AGS human gastric adenocarcinoma cells	*In Vitro* *In Vivo*	Effective combination Suicide immune therapy	shRNA-lentivirus and Ad5.CMV.HSV.tk	39
Gruber C. et. al.	SCC	*In Vitro* *In Vivo*	Efficient transfection of RDEB SCC	SLO=PTM	87
Luo X. et. al.	SGC7901 human gastric Cancer cell lines	*In Vitro* *In Vivo*	Higher efficiency with double suicide gene therapy CD/TK	Double suicide gene therapy Ad-survivin/GFP and Ad-survivin/CD/TK	70
Freytag S. O. et. al.	Prostate cancer	Human	Transgene expression up to 3 weeks, PSA decline, Acute urinary and gastrointestinal toxicities	Cytosine deaminasa(CD)/herpes simplexvirus thymidine kinase (HSV-1 TK) and 3D-CRT	81
Pandha H. S. et. al.	Breast cancer	Human	Efficient selectivity against *erb*-2	Therapeutic cassette that contains the *Escherichia coli* cytosine deaminase gene drivan by the tumor-specific *erb*-2 promoter	82
Li N. et. al.	HCC cancer	Human	Recurrence free survival	Adjuvant ADV-TK	80
Voges J. et. al.	Glioblastoma	Human	Inhomogeneity of tissue formulation distribution	HSV-1-tk liposomal vector	77
Nasu Y. et. al.	Prostate	Human	No serum cytokine changes after treatment, decreased PSA values, Increased CD8+/HLA-DR+ This study confirmed the safety profile at the surrogate marker of HSV-tk gene therapy.	Ad.HSV-tk/GCV	78
Rainov N.G. et. al.	Glioblastoma	Human	Surgical resection and Radiotherapy or standard therapy plus adjuvant gene therapy during surgery. Progression-free median survival in the gene group was 180 days compared with 1 83 days of control group	RV-HSV-tk/GCV	76
Xu F. et. al.	Head and Neck	Human	Local response	Intratumoral RV-HSV-tk/GCV	79
Nemunaitis J. et. al.	Refractory cancer patients	Human	*Salmonella bacterium* can be utilized as a delivery vehicle of the cytosine deaminase gene to malignant tissue with low dose 3×10^7 CFU/m²efficiently.	TAPET-CD	84
Freytag S. O. et. al.	Pancreas	Human	Augments radiotherapy treatment of pancreatic cancer without excessive toxicity	Ad5-yCD/*mut*TK$_{SR39}$*rep*-ADP HSV-1 TK $_{SR39}$	83

INSM1; insulinoma-associated 1 gene, **IFN-β;** Interferon-β, **GCV;** ganciclovir, **CEA;** Carcinoembryonic antigen, **ELISA;** Enzyme-linked immuno sorbent assay, **IL-7;** Interleukin-7, **wtCPE;** wild type *Clostridium perfringens enterotoxin,* **optCPE;** translation-optimised *Clostridium perfringens enterotoxin,* **IL-12;** interleukin-12, **HSV-1;** herpes simplex virus-1, **5-FC;** 5-Fluorocytosine, **VEGF;** vascular endothelial growth factor, **CPNPs;** bioresorbable calcium phosphate nanoparticles, **HSV-tk;** herpes simplex virus-thymidine kinase, **yCDglyTK;** fusion gene therapy of cytosine deaminase and thymidine kinase, **Mac-1;** Macrophage-1 antigen, **CD4+;** T lymphocytes, referring to those that carry the CD4 antigen, **CD8+;** T lymphocytes, referring to those that carry the CD8 antigen, **CCl2;** chemokine (C-C motif) ligand 2 [Homo sapiens (human), **rAd;** recombinant adenovirus, **MCP-1;** Monocyte chemoattractant protein-1, **ETA;** ETA receprtors, **WTp53;** wild type p53, **Cx43;** integral membrane protein of the connexin family, alpha-type (group II) subfamily, **MSC;** mesenchymal stem cells, **AdexCAEGFP;** MSC- adenovirus carrying either enhanced green fluorescent protein gene, **AdexCACD;** MSC- cytosine deaminase gene, **UPRT;** uracil phosphoribosyltransferase, **NSCs;** neural stem cells, **hTERT;** human telomerase reverse transcriptase, **HRP;** expressing horseradich peroxidase, **IAA;** idole-3-acetic acid, **CArG;** Smooth muscle alpha-actin CArG elements, **BI;** *Bifidobacterium infantis,* **VSV;** vesicular stomatitis virus, **NFnB;** nuclear factor B, **DOTAP;** N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate, **MPEG-PCL;** monomethoxy poly(ethylene glycol)–poly(3-caprolactone , **DMP;** DOTAP–MPEG–PCL, **KDR;** kinase insert domain receptor, **AFP;** α-fetoprotein, **rPOA;** poly (oligo-D-arginine), **DM;** *Drosophila melanogaster,*PET; positronemission tomography, **Ad5.CMV.HSV.tk;** adenoviruse 5 harboring the herpes simplex virus thymidine kinase gene, **SLO=PTM;** toxin Streptolysin O-3` pre-*trans*-splicing molecules, **RDEB-SCC;** recessive dystrophic epidermolysis bullosa squamous cell carcinoma, **PTM screen;** 3` pre-*trans*-splicing molecules, **PSA;** prostate specific antigen, **GDEPT;** gene-directed enzyme pro-drug therapy, **TAPET-CD;** pCVD442-*msb* B-VNP20009

Results

Results of cell viability and apoptosis analysis

Flow cytometry showed that ad.CD-5-FC + ancotil treatment induced apoptosis in both cell lines after 4h and 8h as determined by 7-AAD and Annexin V staining (Table 3). At these time-points, sensitivity to 0.2, 0.8 and 1.2mg ancotil was similar in both cell lines. However, the 24h measurement for cell cytometry for all the doses revealed that cell viability was increased for retinal cell line, whereas ad.CD-5-FC + ancotil treatment continued to induce apoptosis for melanoma cell line. The results of 7-AAD and Annexin V staining were also confirmed by trypan blue assay. (Table 2.) Comparison among the time-points revealed that 1.2 ml of ancotil increased the number of viable cells by 87% after 8h to 95% after 24h in retinal cell line, whereas in melanoma cell line viable cells were decreased by 78% after 8h to 75% after 24h. Similar observations were revealed for the other doses of ancotil.

Table 2: cell viability by trypan blue counting.

Concentrations/Time points	Melanoma cell line			Retinal cell line		
	4h	8h	24h	4h	8	24h
0.2ml ancotil	80%	80%	78%	82%	85%	93%
0.8ml ancotil	75%	75%	75%	80%	85%	90%
1.2ml ancotil	78%	78%	75%	80%	87%	95%

Table 3. Cell viability with 7-AAD and Annexin V/PI.

		7-AAD					
		4H		8H		24H	
		CELLS%	VIABILITY%	CELLS%	VIABILITY%	CELLS%	VIABILITY%
R CELLS	0.2	36.7	78.9	50.7	74.7	85.1	89.8
	0.8	20	71.4	56.4	70.1	65.4	82.1
	1.2	31.1	69.3	55	77.9	82.5	86.1
M CELLS	0.2	53.7	82.6	81.3	79.5	76.6	89.3
	0.8	56.3	80.1	79.5	85.7	83	85.9
	1.2	55.5	79.5	79.2	83.9	75.9	83.9

		ANNEXIN V/PI							
		4H		8H			24H		
		APOPTOTIC	VIABILITY%	CELLS%	APOPTOTIC	VIABILITY%	CELLS%	APOPTOTIC	VIABILITY%
R CELLS	0.2	20.7	58.2	43.7	33	57.5	65.4	20.3	72.7
	0.8	30.1	48.7	28.7	43.9	41.8	42.3	36.8	44.6
	1.2	38.5	46	36.4	38.2	50.2	57	28.3	62.9
M CELLS	0.2	5.1	66.3	83.4	85.2	0.7	75.7	1.6	80.7
	0.8	3.3	65	59.6	3	78.5	74.4	0.9	82.2
	1.2	3.7	72.8	55.5	2.9	73.3	73.9	1.9	75.1

Discussion

Currently there is need for more systems activating more pro-drugs. Therefore the thymidine-active mutant of dCK, dCK.DM.S74E was created which activates multiple pro-drugs such as; BVdU, LdUNAs and LdT. This system has the ability to sensitize and re-sensitize tumors to chemotherapeutic agents. Moreover, it can silmutaneously activate more than one drug and prevents multi drug resistence. [66] Previous studies have investigated suicide gene therapy as a local treatment to the tumor site without any remarkable histological adverse effects in lung cancer patients, and in glioma cancer cell lines [14,67]. Recently suicide gene therapy was applied for melanoma with (HSV-tk), which converts ganciclovir (GCV).[58] However, loco-regional administration is not always possible and therefore the ``Trojan horse`` approach has been investigated. In the study by Zhao Y. et. al. (2012) the tumor-tropic neural stem cells (NSCs) derived from HES1 human embryonic stem cell line had the ability to migrate from the injection site (vein systemic administration) or intracranial to the intracranial glioma xenografts. A baculovirus vector was used to insert the HSV-tk suicide gene into the cells. A concentration of ganciclovir was also administered in order for an amount of the drug to be present locally for the suicide system to act. A prolonged transgene expression was observed for three weeks. This study presented where a sustain release system of suicide gene therapy could be used as a future concept.[54] The same concept has been also applied with MSC in a hepatocellular (HCC) model [68]. Additionally, Wang C. et al. [69] investigated NSCs (F3)

as dual suicide gene therapy with cytosine deaminase (CD) and Thymidine Kinase (TK) creating the NSC-F3.CD-TK. Enhanced antitumor activity was observed against lung cancer metastasis in comparison to single suicide gene therapy. Dual suicide gene therapy was also used in lung cancer cell lines with a carcinoembryonic antigen (CEA) promoter with TK and CD constructing the pCEA-TK/CD [42]. Dual suicide gene therapy was also investigated with surviving promoter with Ad-survivin/GFP and Ad-survivin/CD/TK.[70] A very important parameter that has to be presented is the fact that the pro-drug has to be already diffused within the target tissue prior the administration of the adenovirus in order for the therapy to be efficient. Further investigation of transporting vehicles has led to the development of nanoparticles.[12] In the study by Duan X. et al.[71] the novel gene transfection cationic self-assembled DOTAP and MPEG-PCL hybrid micelles (DMP) was investigated. Less toxicity was observed when compared to the polymer Polyethyleneimine (PEI) with 25kDa. The DMP delivered efficiently the urvivin-T34 gene (S-T34A) to treat C-26 colon cancer cell lines.

Currently there are very few clinical studies in patients with suicide gene therapy and therefore every effort is welcomed.[14,72-85] Recently the first clinical trial design for early prostate cancer was published and another one with extensive stage has already been initiated. [48,81]

Moreover, novel suicide genes such as the TK.007, have already been introduced and demonstrated efficiency in several cancer cell lines (G62 human glioblastoma cell line, A549 human lung carcinoma, SW620 human colorectal adenocarcinoma cell line and IPC298 human melanoma cell line) [86]. In the study by Gruber C. et al. [87] the efficiency of *3` pre-trans-splicing molecules* (PTM) was investigated and high efficiency was observed against highly malignant tumors. In the study by Di Stasi et al. [88], the inducible caspase 9 (iCasp9) gene was investigated. It was applied to children who developed graft-vs.-host disease (GVHD) by donor lymphocytes; it was observed that the process was reversed with the novel suicide gene therapy.

Using promoters as a method to target specific overexpressed pathways has been also used for; a) carcino-embryonic antigen (CEA) [42], b) EGFR [89], c) prostate specific antigen (PSA) [90], e) transferrin receptor (TfR) [91], d) cyclooxygenase (Cox) [92], f) Telomerase-hTERT [93] and g) Cytokeratin 18 and 19 [94]. We suggest that a future method of application could be made with local injections or instillation with eye droplets.

Conclusions

Suicide gene therapy with ad.CD-5-FC could be used as a local treatment for primary or metastatic melanoma. We observed safety for the therapeutic dosages of 5-FC from 0.2 mg up to 1.2 mg for the normal retinal cells lines while the same dosages were lethal for the human melanoma cell lines. Future studies in animals and clinical trials remain to elicit the *in vivo* safety and efficiency of this therapeutic application.

Materials and Methods

Adenosine Cytosine Deaminase

The Ad.CD used in this study was kindly donated by Dr. A.B. Deisseroth (*Yale University School of Medicine, New Haven, US)*. This vector is a replication-incompetent recombinant adenoviral vector that contained the *Escherichia coli* CD gene in a L-plastine promoter-driven transcription unit[95] Ad.CD was propagated in 293 cells (ATCC, Teddington, UK) and recovered 36 hours after infection by five cycles of freezing/thawing of the infected cells. All viral preparations were purified by CsCl density centrifugation, dialyzed, and stored in dialysis buffer (10 mM Tris pH 7.8, 150 mM NaCL, 10mM MgCl2, 10% glycerol) at -70°C before use. Titers of the viral stocks were determined by plaque assay using 293 cells by standard methods.[96]

Cell cultures and reagents

HTB-70 (melanoma cell line, derived from metastatic axillary node) and CRL-2302 (human retinal epithelium) were purchased from ATCC LGC Standards. HTB-70 cells were isolated from a 24 year old female patient and CRL-2302 cells from a 19 year old male (http://www.lgcstandards-atcc.org). HTB-70 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) culture medium (ATCC-30-2003) supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom S0115). CRL-2302 cells were cultured in DMEM (ATCC-30-2006) supplemented with 10% FBS. Both cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2.[97,98] Cell lines were cultured in Coming's tissue culture flasks (25 and 75 cm²) according to the manufacturer's protocol. After cultures reached confluence, cells were detached with trypsin (1:250) 2.5 % (Biochrom L2133) and passaged. The indicated cell lines were seeded in 25 cm² flasks at a seeding density of 0.7×10^6 cells. At confluence, (approximately 2.8×10^6 cells), adenovirus (85µl of Crude viral lysate –CVL, approximately 1-10pfu/cell) was added in both cell lines. The adenovirus vector was provided by Prof. A. Deisseroth, Yale University School of Medicine, and cultured in the research la-

boratory of the Lung Tumor Research Section of the Pulmonary Dept. Aristotle Univ. Medical School. Cytopathic effect was observed only in melanoma cell lines after 36h and then Ancotil® 2.5 g/250 ml (1 g/00ml) (5-Flucytosine) MEDA; Pharmaceuticals Ltd. UK was added in both cell lines at different concentrations (0.2ml, 0.8ml and 1.2ml). At indicated time-points (4h, 8h and 24h) cell viability and apoptosis was measured.

Trypan Blue Assay

Trypan blue assay was applied to measure cell viability. Trypan blue dye can penetrate only porous, permeable membranes of lethally damaged (dead) cells, which is clearly detectable under optical microscopy.[99] After adenovirus infection and ancotil treatment, both cell lines were trypsinized and collected, washed with PBS and suspended in complete culture medium. Then, 50µl of this cell suspension were added to 50 µL of 0.04% trypan blue dye (Sigma Aldrich Corp.). This solution was maintained in room temperature for 2 minutes to allow trypan blue penetration and then viable and dead cells were counted in the hemocytometer under an inverted light microscope (Zeiss, West Germany). Cell viability was calculated by deducting the number of nonviable cells from the number of total cells. The number of cells obtained in the counting corresponded to n × 104 cells permilliliter of suspension. (Table 2.) (Figure 1.)

Flow Cytometry

Separation of dead and alive cells with 7-AAD staining (7-amino-actinomycin D)

7-Aminoactinomycin D (7-AAD) is a fluorescent chemical compound with a strong affinity for DNA. It is used as a fluorescent marker for DNA in fluores-

cence microscopy and flow cytometry. 7-AAD staining was purchased from Immunostep company (Spain) and the analysis of the samples was performed using BD FACSCalibur 4 colors, with CELLQUEST software (BECTON-DICKINSON USA). After adenovirus infection and ancotil treatment, both cell lines were trypsinized and collected, washed with PBS and suspended in complete culture medium. 100 µL of cell suspension (concentration 3000 to 5000 cells/ µL) were added to 5 µL of 7AAD staining. This solution was incubated for 10 min in a dark place at room temperature. Then it was diluted with 0.5 ml of PBS and analyzed in the flow cytometer at indicated time-points (after 4h, 8h and 24h). (Table 3.) (Figures 2-10)
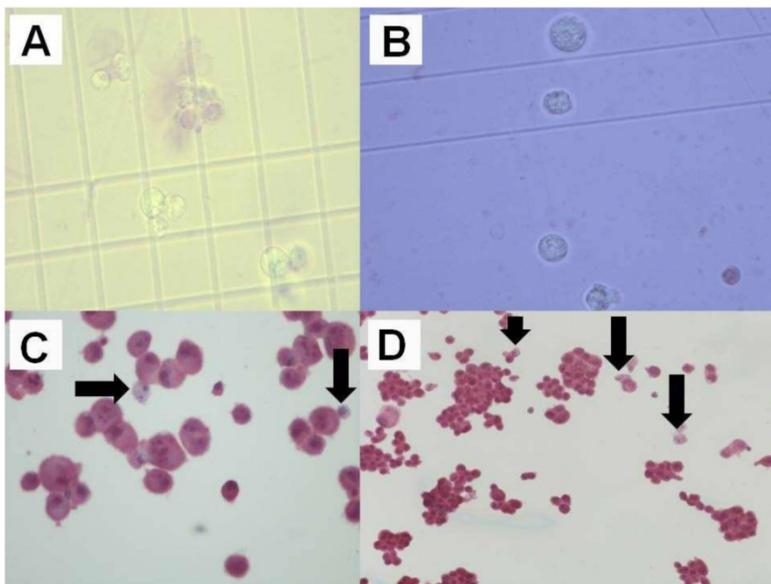
Analysis of the apoptotic cells with ANNEXIN V/ PI

Annexin V staining is used as a probe to detect cells that have expressed phosphatidylserine (PS) on the cell surface, an event found in apoptosis as well as other forms of cell death. Propidium iodide (PI) is used as a DNA stain for both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis[100], and microscopy to visualize the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. The Annexin V kit used in this study was purchased from Immunostep company (Spain) and the analysis of the samples were performed in BD FACSCalibur 4 colors, with CELLQUEST software (BECTON-DICKINSON USA). After adenovirus infection and ancotil treatment, both cell lines were trypsinized and collected, washed with PBS and suspended in complete culture medium. 100 µl of cell suspension (concentration 3000 to 5000 cells/ µL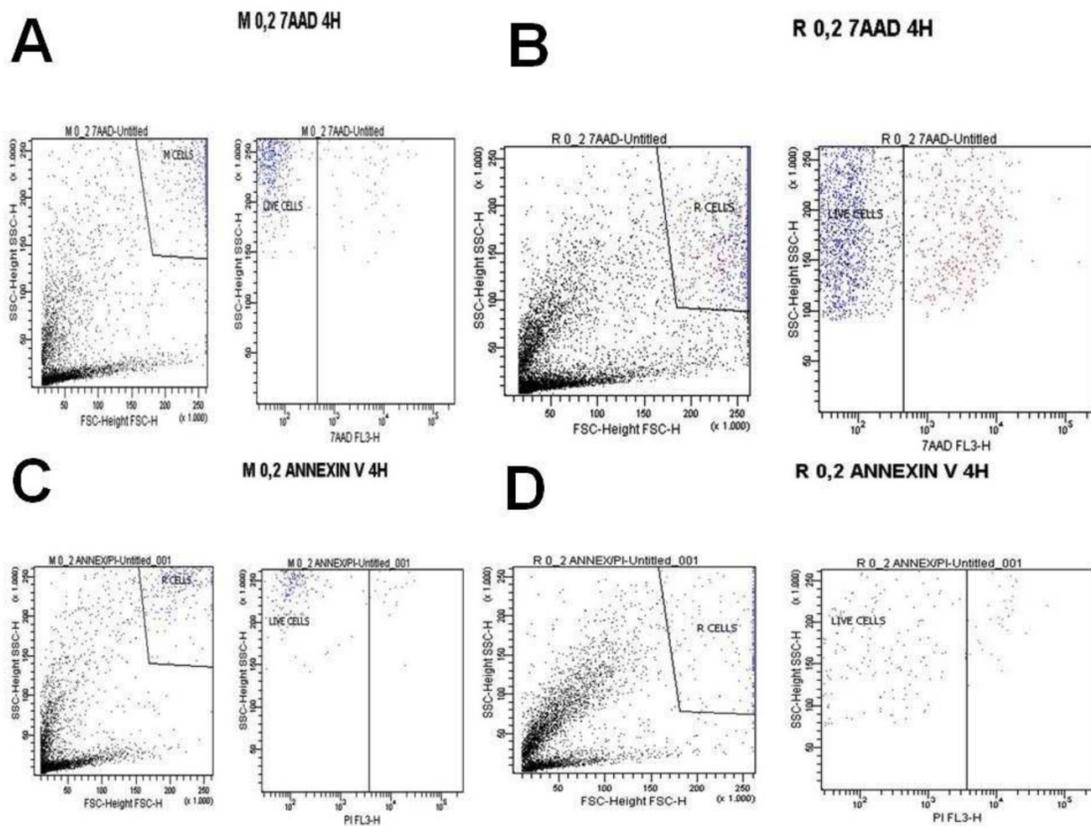) were added to 500 µL of Annexin binding buffer. Then 5 µL of Annexin V and 5 µL PI were added to this solution and it was incubated for 15 min in a dark place at room temperature. Then it was analyzed in the flow cytometer at indicated time-points (after 4h, 8h and 24h). (Table 3.) (Figures 2-10)
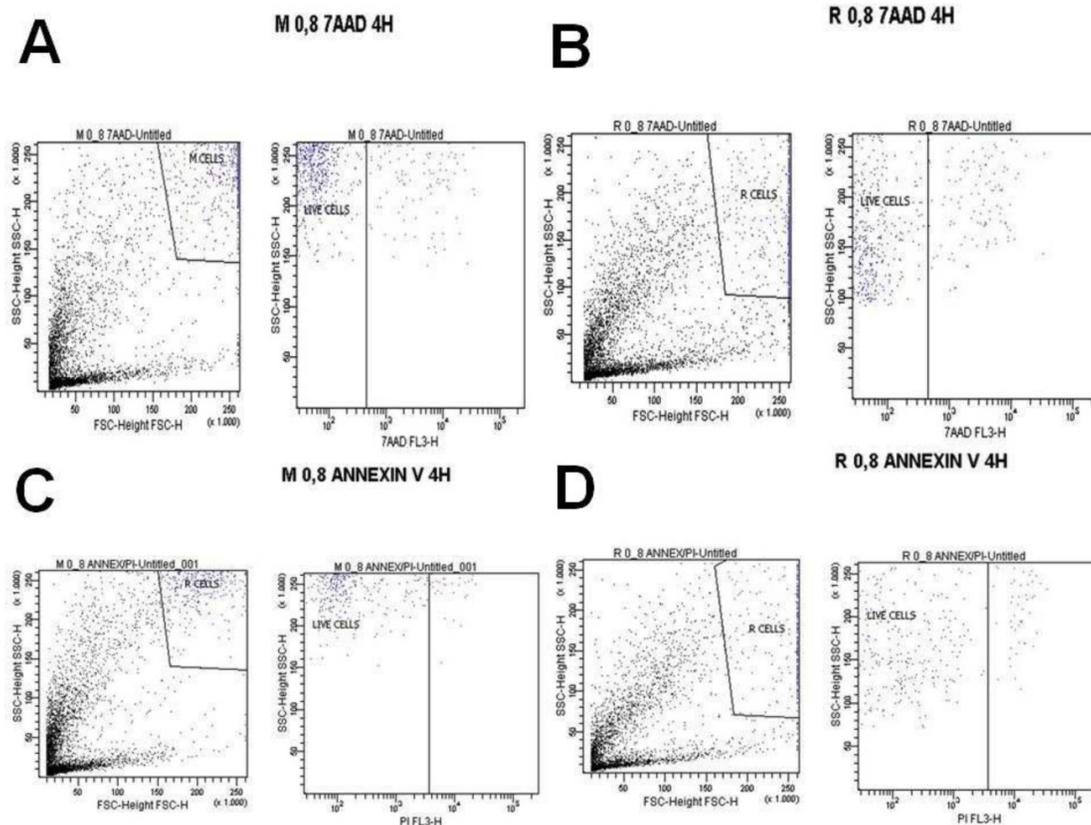
5-Fluorocytosine

The Ancotil® 2.5 g/250 ml (1 g/00ml) (5-Flucytosine) MEDA; Pharmaceuticals Ltd. UK was purchased and used for the experiment.
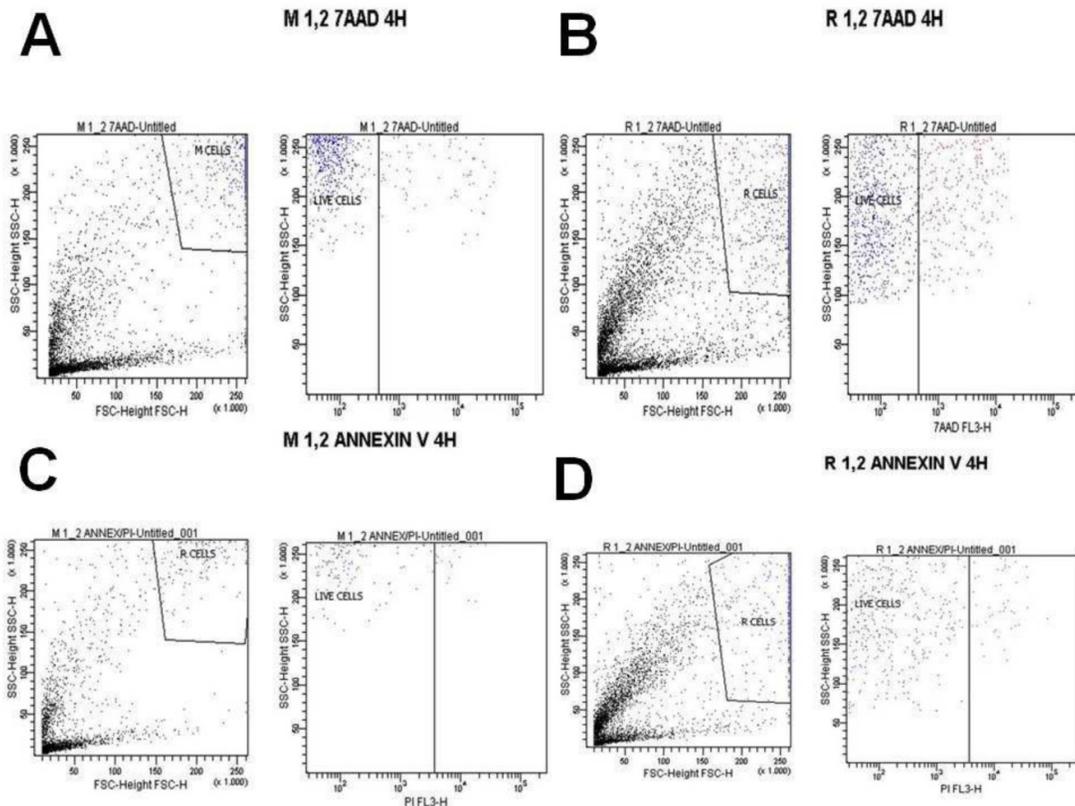

Figure 1. A) Melanoma trypan blue x 400, B) Retinal Trypan Blue x 400, C) Melanoma cells plus adenovirus x 400 (black arrows indicate the Ad.CD), D) Retinal cells plus adenovirus x 400 (black arrows indicate the Ad.CD).
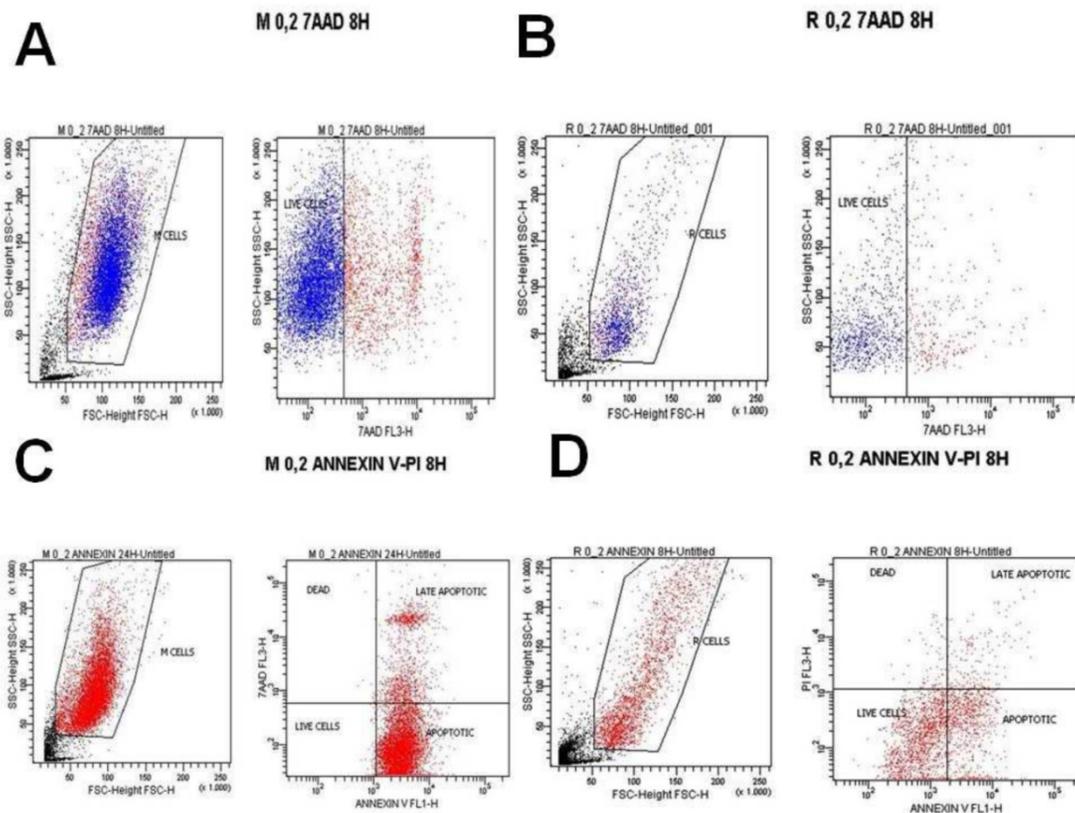
Figure 2. A) Melanoma cells 0.2 mg ancotil and viability at 4 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 4 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 4 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 4 hours with annexin.
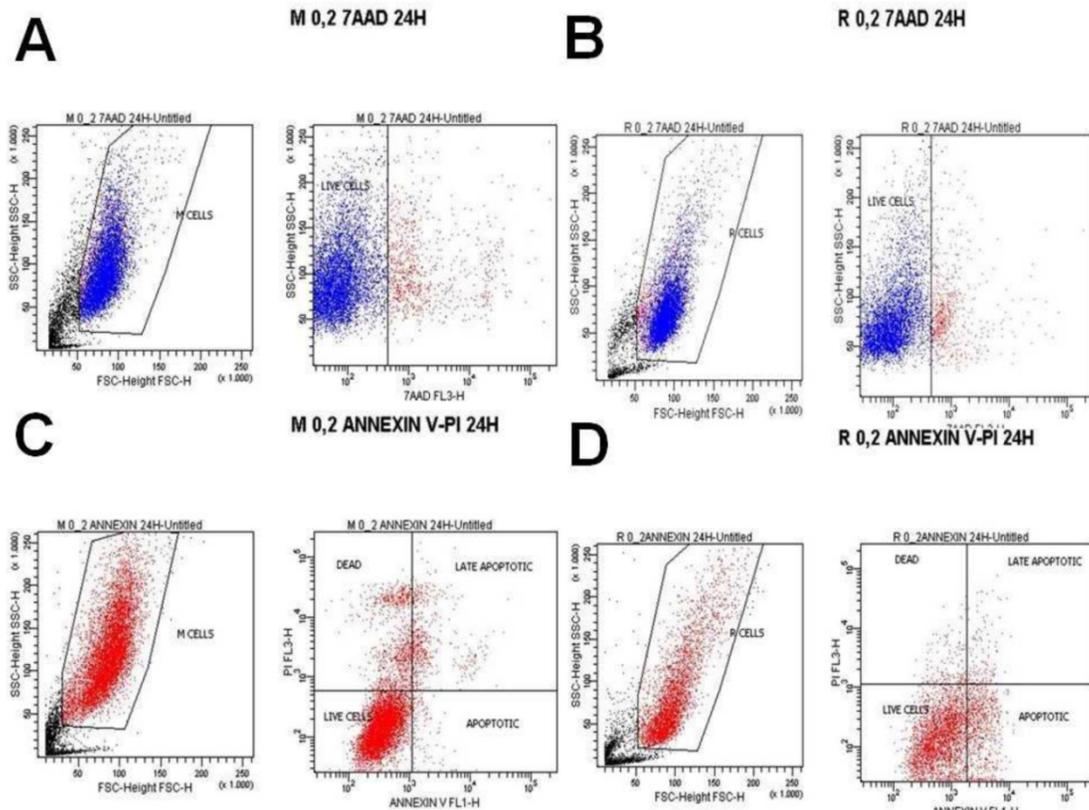

Figure 3. A) Melanoma cells 0.8 mg ancotil and viability at 4 hours with 7-AAD, B) Retinal cells 0.8 mg ancotil and viability at 4 hours with 7-AAD, C) Melanoma cell 0.8mg ancotil and viability at 4 hours with annexin, D) Retinal cells 0.8 mg ancotil and viability at 4 hours with annexin.
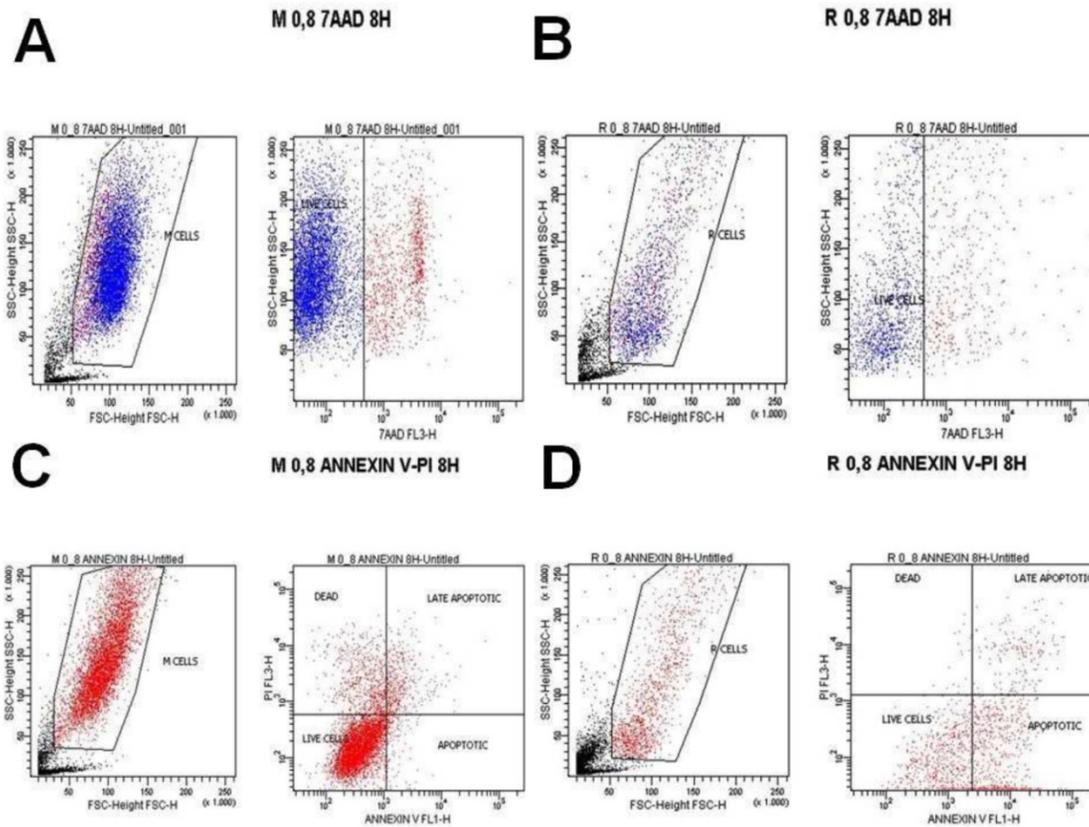
Figure 4. A) Melanoma cells 1.2 mg ancotil and viability at 4 hours with 7-AAD, B) Retinal cells 1.2 mg ancotil and viability at 4 hours with 7-AAD, C) Melanoma cell 1.2mg ancotil and viability at 4 hours with annexin, D) Retinal cells 1.2 mg ancotil and viability at 4 hours with annexin.
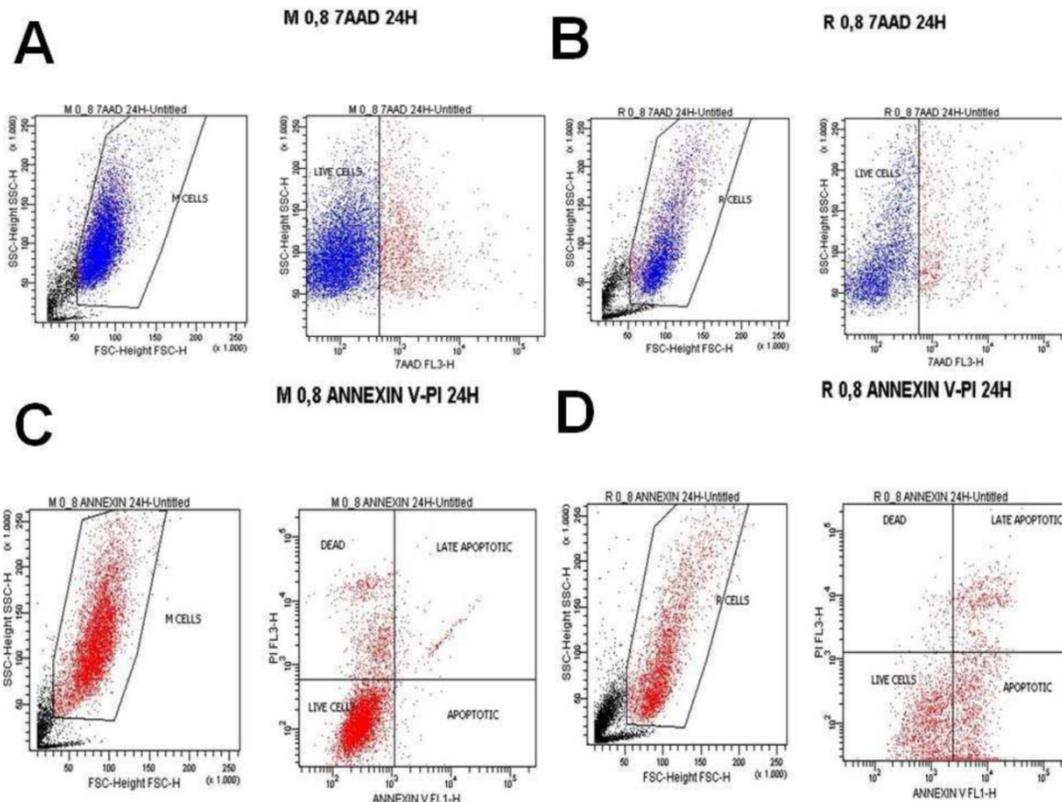

Figure 5. A) Melanoma cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 8 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 8 hours with annexin.

Figure 6. A) Melanoma cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 8 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 8 hours with annexin.

Figure 7. A) Melanoma cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 8 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 8 hours with annexin.
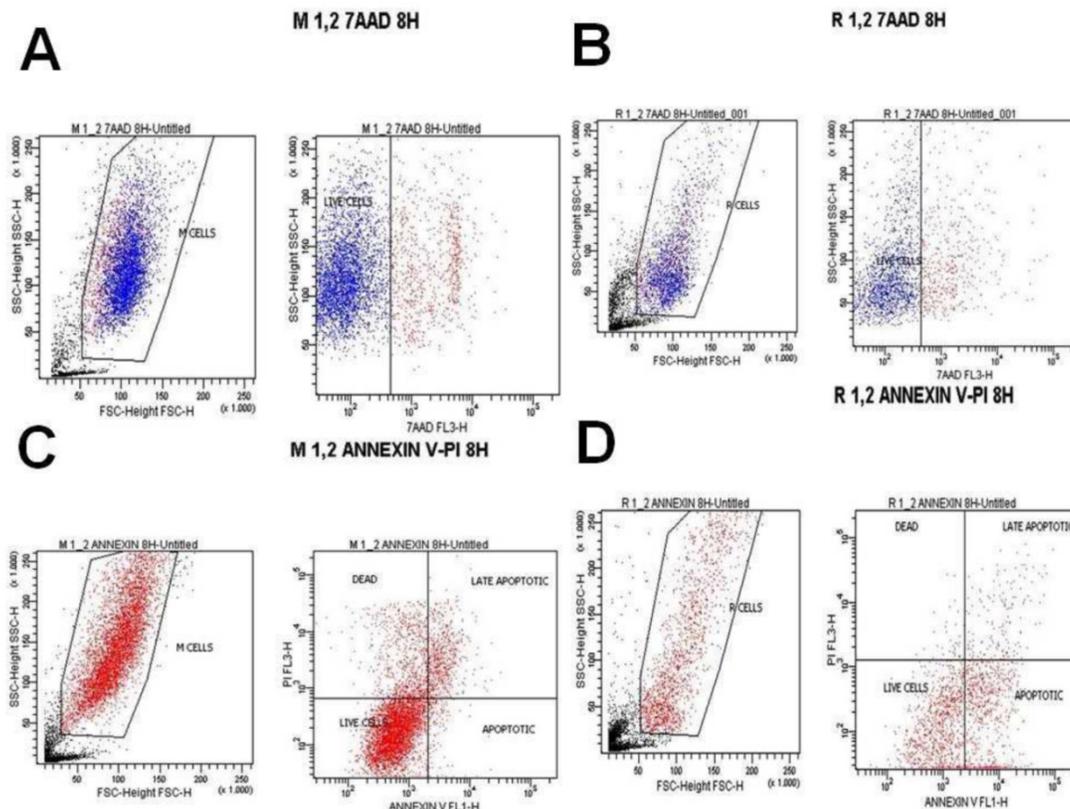
Figure 8. A) Melanoma cells 0.2 mg ancotil and viability at 24 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 24 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 24 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 24 hours with annexin.
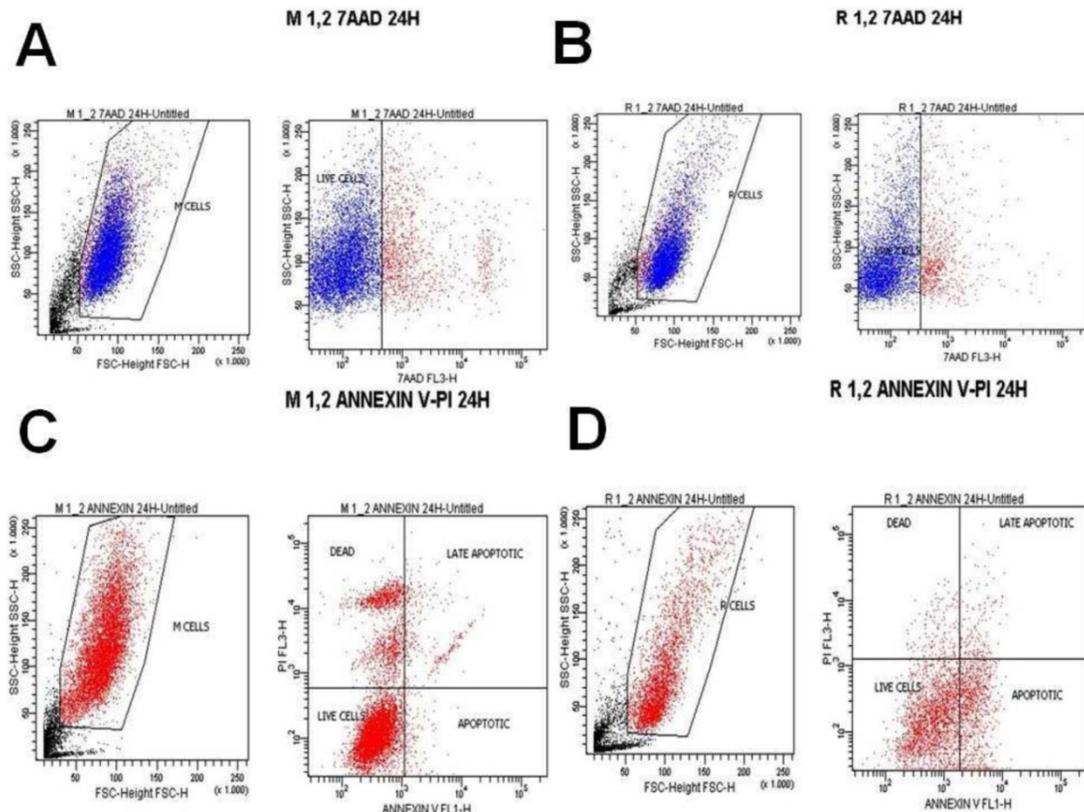

Figure 9. A) Melanoma cells 0.2 mg ancotil and viability at 24 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 24 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 24 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 24 hours with annexin.

Figure 10. A) Melanoma cells 0.2 mg ancotil and viability at 24 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 24 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 24 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 24 hours with annexin.

Acknowledgments

Conflict of Interest

None to declare.

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