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Development of gene therapy to deliver the receptor-specific rhTRAIL DHER variant to induce apoptosis in activated Hepatic Stellate Cells for the treatment of Liver Fibrosis

Ana Raquel Pato Santa Maria

Dissertation

Integrated Master in Biological Engineering

Dissertation directed by:

External Supervisor: Prof. Dr. Hidde Haisma

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Declaração da Autoria do Trabalho

Eu, _____declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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First of all I want to thank to Professor Hidde Haisma, my supervisor at the University of Groningen for accepting me to do my internship in the PGM laboratory. I appreciate all the support, understanding, teaching, criticism and for having encouraged me to want to know more.

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“Do the best you can until you know better.

Then when you know better do better. “

Maya Angel

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List of Abbreviations

Acetyl-CoA	Acetyl Coenzyme A
Ad5	Human Adenovirus
APS	Ammonium PerSulfate
BSA	Bovine Serum Albumin
CIP	Calf Intestinal Alkaline Phosphatase
CLD	Chronic liver diseases
DcR 1 or 2	Decoy Receptor 1 or 2
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's minimum essential medium
DRs	Death receptors
EASL	European Association for the Study of the Liver
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
ET-1	Endothelin-1
FADD	Fas-Associated Death Domain
FBS	Fetal Bovine Serum
HA	Human Influenza Hemagglutinin
HAT	Histone AcetylTransferase
HDACS	Histones Deacetylases
HMG	High Mobility Group
HSC	Hepatic Stellate Cells
IAP	Inhibitor of Apoptosis

IGK	Immunoglobulin K light chain
LB	Luria- Bertani broth medium
MFs	Myofibroblast
NAD	Nicotinamide Adenine Dinucleotide
NF-KB	Nuclear Factor Kappa B
OPG	Osteoprotegerin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PMS	Phenazine Methyl Sulfate
RER	Rough Endoplasmic Reticulum
RhTRAIL 4C7	Recombinant human TRAIL Variant 4C7
RhTRAIL DHER	Recombinant human TRAIL Variant DHER
RhTRAIL WT	Recombinant humam TRAIL Wild Type
RT	Room Temperature
SER	Smooth Endoplasmic Reticulum
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
TGF-β	Transforming Growth Factor Beta
TNF	Tumor Necrosis Factor
TRAIL	Tumor Related Apoptosis Inducing Ligand
VEGF	Vascular Endothelial Growth Factor
α-SMA	Smooth Muscle Actin

Abstract

Liver fibrosis is caused by excessive accumulation of extra-cellular matrix, produced by the hepatic stellate cells (HSC), when they evolve from the quiescent state to an activated state. These cells express on their surface receptors for Tumor Necrosis Factor- Related Apoptosis Inducing Ligand (TRAIL), a member of the TNF family. Five TRAIL receptors have been identified to date: the pro-apoptotic DR4 and DR5, and the decoy receptors DcR1, DcR2 and OPG. It is known that activated hepatic stellate cells overexpress the DR5 receptor. Soluble human recombinant TRAIL (rhTRAIL) is an interesting protein for the treatment of liver fibrosis, due to the efficacy of death induction. In this project, we constructed a vector capable of producing the DR5-specific variant rhTRAIL DHER in mammalian cells, for specific binding to the TRAIL DR5 receptor, and to efficiently induce apoptosis in activated stellate cells. We verified that the protein is functional and active when added to a cell line (SW948) sensitive for the rhTRAIL proteins. Moreover, we evaluated the effects of the protein rhTRAIL DHER, produced in *E.Coli* bacteria, in LX2 cells and HepG2 cells. Our results demonstrate that the rhTRAIL DHER protein induces apoptosis in LX2 cells, without producing any effect in the HepG2 cells. However, to develop a therapy capable to induce a continuous production and a more efficient apoptosis in activated HSC, we used an adenoviral vector (pAdEasy-1) to recombine with our constructed vector, in other to produce an adenovirus. However, the recombination of both plasmid were not achieved, due to the complexity of the vectors used to recombine and create a unique vector.

As previously demonstrated, the normal cells in the liver express the TRAIL DR5 receptor, to a lesser extent than the activated cells. For this reason, it was necessary to create a treatment that promotes apoptosis specifically in the target cells, but does not affect the other cells. We aimed to investigate the effects of combinations between HDAC (SAHA and Entinostat) and HAT (C646 and MG149) inhibitors with rhTRAIL variants. Our results confirm the synergistic effect of combinations of SAHA and rhTRAIL variants in H460, HUH-7 and HepG2, while the effect of Entinostat with rhTRAIL

variants did not promote a combination effect. For the HAT inhibitors we demonstrated a protective effect in MG149 with rhTRAIL variants, which was even more visible in HUH-7 and HepG2 cells. For C646 the combination effect was low in HepG2, HUH-7 and H460 cells.

In conclusion, we constructed a vector capable of producing the DR5-receptor specific protein rhTRAIL DHER in mammalian cells, and we have shown the synergistic effect of SAHA with rhTRAIL variants and a possible HAT inhibitor that may protect the normal cells in a fibrotic liver.

Keywords: TRAIL, Hepatic Stellate Cells, Histone acetylasetransferase, Histone deacetylase, Liver Fibrosis, Cancer.

Resumo

O fígado é um dos maiores órgãos do corpo humano e é responsável por funções vitais para o organismo, compreendendo o suporte e o apoio a outros órgãos. Este órgão é responsável pela síntese de componentes sanguíneos, regulação dos níveis de glucose, desintoxicação, produção de bílis e síntese de componentes essenciais à função de outros órgãos. O fígado é constituído por quatro lobos com diferentes tamanhos e formas, e por dois principais vasos sanguíneos, a artéria hepática e as veias-porta. A artéria hepática transporta o sangue da artéria aorta, enquanto as veias-porta transportam o sangue rico em nutrientes da digestão e também do pâncreas e baço. Os vasos sanguíneos subdividem-se em capilares, que se ligam a um lóbulo, a unidade funcional do fígado. Cada lóbulo contém milhões de células hepáticas (~80% de todo o fígado), as responsáveis pela função metabólica. As restantes células que constituem o fígado são células endoteliais, estreladas, linfócitos, células de Kupffer e células biliares epiteliais.

As doenças hepáticas crónicas são caracterizadas por uma repetida lesão nas células hepáticas, devido a infeção por vírus (hepatite B e C), doenças autoimunes, excesso de álcool e drogas, doenças inflamatórias, doenças genéticas ou fatores esporádicos. Contudo, a constante lesão destas células leva a acumulação de ferimentos no fígado induzindo este órgão a um estado fibrótico, podendo evoluir para cirrose ou cancro. Como resposta aos ferimentos causados pelas mais diversas lesões, inicia-se um processo de acumulação de matriz extracelular. Este processo deve-se sobretudo às células estreladas do fígado, que evoluem para um estado ativo, devido ao aumento da sua resposta fenotípica, sintetizando em excesso diversos fatores de crescimento que são responsáveis não só pela fibrogénese como também por respostas inflamatórias crónicas.

A ativação das células estreladas do fígado é realizada em três fases: a iniciação ou fase pré-inflamatória, onde se inicia a alteração do fenótipo e a expressão dos genes, devido sobretudo à estimulação pancreática graças ao excesso de produção de fatores de crescimento; a fase de perpetuação é devida a numerosas reações distintas, tais como, a proliferação, a contractilidade, a fibrogénese, a degradação da matriz, a perda de

retinóides e de células inflamatórias; Por fim, a fase de reversão ou apoptose, é a fase em que nas células estreladas é desencadeada a apoptose, e, é nesta fase que podem interferir determinados fatores que levam à reversão da lesão das células e, por conseguinte, a reversão da fibrose.

O Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) é um dos fatores que está associado à promoção da apoptose das células estreladas ativadas, pois estas células expressam em excesso o recetor para este fator. Sendo um membro da família Tumor Necrosis Factor (TNF) é uma proteína transmembranar tipo 2 e é expressa nos mais variados tecidos. O TRAIL interage com os recetores na superfície membranar das células e induz a apoptose. Até ao momento foram descritos cinco recetores: TRAIL DR4, DR5, DcR1, DcR2 e osteoprotegerin (OPG). O recetor TRAIL DR4 e DR5 têm um domínio e sinais de apoptose. Apesar de os DcR1 e DcR2 terem homologia com domínios de apoptose, o DcR1 tem um domínio de morte citoplasmática truncada e não funcional, enquanto o DcR2 falta uma região citosólica e está ancorado na membrana plasmática.

O TRAIL recombinante solúvel é interessante para terapias do cancro e fibrose no fígado, uma vez que é mais específico na indução da apoptose, e a produção de um TRAIL solúvel recombinante específico para os recetores DR5 e DR4 é permitirá o desenvolvimento de terapias mais específicas. Um dos objetivos do nosso trabalho é a construção de um vector que contenha o gene para o TRAIL recombinante variante D269H e E195R, promovendo uma ligação mais específica ao recetor TRAIL DR5, logo uma apoptose mais rápida e eficaz.

Contudo, a administração de proteínas diretamente ao paciente não é tão eficaz como quando a proteína é produzida diretamente nas células alvo. Por esta razão, nós pretendemos construir um vector adenoviral, que contenha o gene de interesse para a produção da proteína rhTRAIL DHER.

Os nossos resultados demonstraram que o vector construído, pADTRACK-CMV-IGK-HÁ-rhTRAIL DHER, tem capacidade de expressar aquando transfectado em células, HEK-293. Além disso, a proteína produzida tem atividade biológica e exerce as funções esperadas, quando o mesmo é adicionado a uma linha celular bastante sensível

para o TRAIL recombinante solúvel, onde promove a apoptose das células, como esperado. A utilização de vectores adenovirais é de grande importância em terapia genética, devido à rápida infeção do vírus nas células alvo, à baixa patogenicidade em seres humanos e ao facto de o genoma do vírus não sofrer rearranjo nas células hospedeiras. Porém, a construção do vector adenoviral não correu como esperado, possivelmente pela baixa possibilidade de recombinação de um vector digerido com outro não digerido por enzimas de restrição.

No entanto, sendo um dos objetivos a promoção da apoptose em células estreladas ativadas, a proteína rhTRAIL DHER foi adicionada a uma linha celular fibrótica do fígado (LX2) e também a uma linha celular hepática não fibrótica (HepG2). A proteína, rhTRAIL DHER, de origem bacteriana, foi utilizada em vez da proteína produzida em células humanas devido à quantidade disponível. Os nossos resultados demonstraram um efeito promotor de apoptose nas células LX2 e a ausência de efeito nas HepG2. Este efeito, embora reduzido, permitiu concluir que a indução de apoptose foi específica para as células que expressam o recetor TRAIL DR5 (LX2). Terapeuticamente, este resultado é importante porque as células saudáveis expressam o recetor TRAIL DR5 em quantidades reduzidas, comparando com células fibróticas. Como descrito na literatura, a administração conjunta de inibidores HDACs com as proteínas rhTRAIL DHER, 4C7 ou WT poderá promover maior indução de apoptose e ao mesmo tempo aumentar a especificidade celular.

Assim, o último objetivo do nosso trabalho consistiu em confirmar uma maior indução de apoptose aquando da administração conjunta da proteína rhTRAIL DHER,4C7 ou WT com inibidores HDAC (histonas deacetilases) como, SAHA e Entinostat. Simultaneamente, pretendemos verificar se inibidores HAT (histona acetiltransferase), MG149 e C646 exercem um efeito de proteção aquando da conjugação com as proteínas rhTRAIL DHER, 4C7 ou WT. Os inibidores HAT, histona acetiltransferase, transferem um grupo acetil da acetil co-enzima A para o grupo ε-amino num resíduo específico de lisina existente nas caudas amino-terminal das histonas, resultando de uma neutralização de carga positiva. Porém a reação inversa é catalisada por as histonas deacetilases. A nossa hipótese é que os HAT, combinados com as

proteínas rhTRAIL DHER, 4C7 e WT poderiam ter um efeito protetor nas células cancerígenas. Os nossos resultados mostram que, MG149 não exerce qualquer efeito em combinação com as proteínas.

Os nossos resultados comprovaram o efeito sinergético da combinação de SAHA com as proteínas rhTRAIL DHER, 4C7 e WT, quando adicionado em diferentes linhas celulares, HepG2, HUH-7 e H460. Contudo, ao contrário do que já foi demonstrado por outros autores, a combinação do Entinostat com as proteínas rhTRAIL DHER,4C7 e WT demonstrou efeito sinérgico reduzido, o que poderá ser considerado um efeito de proteção, pois não existe promoção de apoptose das células. Relativamente, aos inibidores HAT, C646 e MG149, os resultados demonstram que a combinação do MG149 com a proteína rhTRAIL DHER não exerce qualquer efeito de apoptose, o que pode indicar um efeito de proteção. O mesmo se verificou para o inibidor C646, apesar de o efeito de proteção ser menor, pois promove uma pequena percentagem de apoptose nas células.

Palavras-chave: Apoptose, fibrose no fígado, TRAIL, HDAC, HAT, proteína rhTRAIL DHER, 4C7 e WT.

-Chapter 1-

Introduction

1.1. Healthy and fibrotic Liver

The liver is the largest organ in the human body, providing essential metabolic, exocrine and endocrine functions [1, 2]. These functions include metabolism of dietary compounds, regulation of glucose levels, detoxification, production of bile and blood homeostasis [3]. The liver is a brown organ with four lobes of different shape and size. This organ is connected with two large blood vessels, the hepatic artery and the portal vein [1, 2]. The blood vessels subdivide into capillaries, which lead to a lobule (the functional units of the liver). Each lobule contains millions of hepatic cells which are the basic metabolic cells [2] (see Figure 1.1).

The hepatic cells are the largest part of the liver (~80% of the mass) in the adult organ. These cells are responsible for the secretion of essential serum proteins and for the synthesis of different proteins. These important cells have the capacity for detoxify, metabolize, and inactivate drugs toxins and endogenous compounds [3]. The other 20% of cells consist of endothelial cells, stellate cells, lymphocytes, Kupffer cells, the hepatic sinusoids and biliary epithelial cells [2, 3].

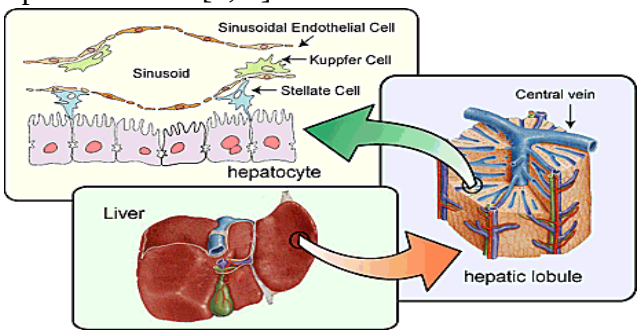

Figure 1.1. Components of a healthy liver. The image shows a healthy liver (in the center), the composition of a hepatic lobule (in the right), and the constituent cells of the liver (in the top). Adapted from [4].

The combination of all the cells that forms liver tissue, and the combination of functions gives a complexity to the organ becoming vulnerable to many diseases. Chronic liver diseases (CLD) are characterised by repetitive injury in the hepatocyte cells, induced by chronic viral hepatitis (hepatitis B and C viruses), autoimmune injury (neonatal liver disease, Wilson´s disease), alcohol abuse, drugs toxicity, chronic inflammatory conditions and vascular derangements, either congenital or acquire [5, 6]. When the liver is constantly suffering injuries the evolution of these damage can induce this organ to fibrosis, cirrhosis or cancer, depending on the damage this organ suffered.

In response to the injury, an extracellular matrix or scar is produced, and its accumulation in the liver is called hepatic fibrosis. An end stage of fibrosis is denominated cirrhosis, resulting in nodule formation that can outcome in alteration of hepatic functions and blood flow [6]. The primary effector of liver fibrosis are hepatic stellate cells (HSC). Due to the injuries the cells became activated into a myofibroblast (MFs) phenotype [7] and contributing to CLD progression because of their highly phenotypic responses, such as: an excess deposition of extracellular matrix (ECM), and a synthesis and delivery of several critical growth factor capable of supporting not only fibrogenesis but also chronic inflammatory responses and neo-angiogenesis [5].

According to the European association for the study of the liver (EASL) , until 2013, 59% of patients that needs a liver transplants in Europe suffer from cirrhosis which in majority is caused by virus infection and alcohol misuse [8].

1.1.1 Hepatic Stellate Cells

The extracellular matrix normally produced by the liver is essential for maintaining the different functions of resident liver cells, like hepatocytes, stellate cells and sinusoidal endothelium [6]. This interstitial ECM is limited to the capsule around large vessels, and in the portal areas. When the liver becomes fibrotic, the collagen and non-collagenous constituents growths three- to fivefold followed by a change in the subendothelial space from a low-density basement to an interstitial type matrix [6].

Following this change in the matrix, the loss of hepatocyte microvilli and the disappearance of endothelial fenestration occur when the liver becomes fibrotic (Figure 1.2.) [6].

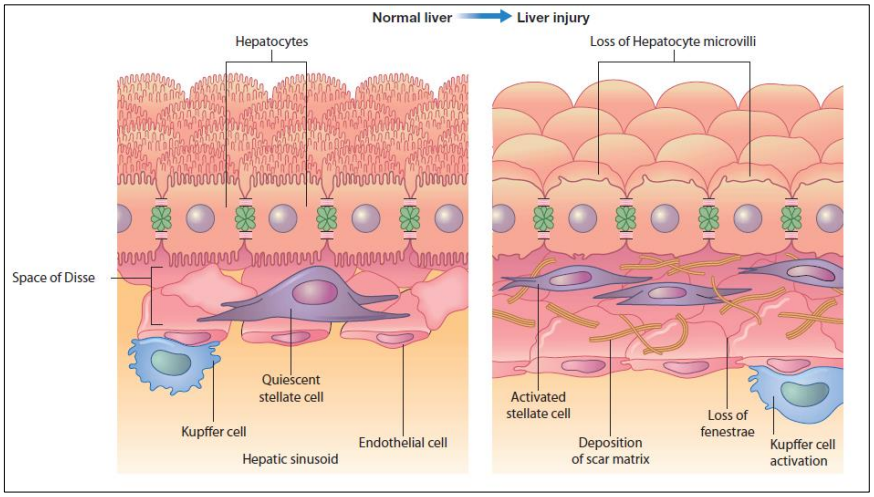

Figure 1.2. Hepatic liver cells in a normal and injured liver. On the left is shown the normal liver cells, the stellate cells are situated in the subendothelial space of Disse; on the right is shown the liver cells after injury, and the effects are visible: the stellate cells become activated, the hepatocytes lose the microvilli and the activated HSC produce a deposition of an extra cellular matrix. Adapted from [6].

When the liver becomes fibrotic this is due to the activation of stellate cells in which follows in three phases (Figure 1.3.): the initiation phase, the perpetuation phase and the resolution phase [9].

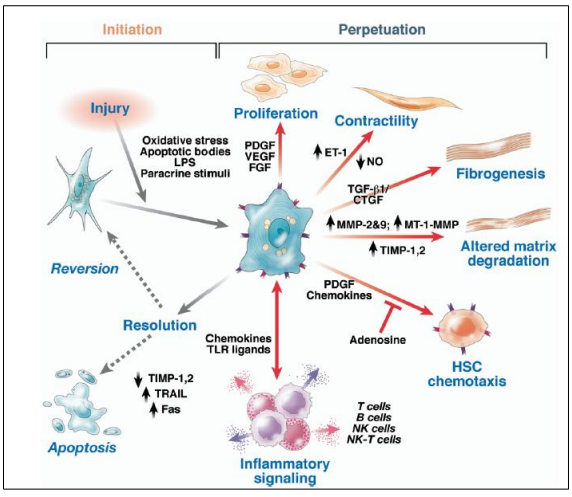

Figure 1.3. Pathways of activation of hepatic stellate cells. The different phases can be distinguished in the Figure, namely the initiation, perpetuation and resolution or reversion phases. The initiation is provoked by oxidant stress, apoptotic bodies and paracrine stimuli from the other cell types; the perpetuation is characterized by specific changes; and in the resolution or reversion phase, the cells can induce the apoptosis or revert the injury. Adapted from [10].

I. Initiation Phase:

The response of the quiescent hepatic stellate cells to paracrine stimulation by all neighboring cell types, like sinusoidal endothelium, Kupffer cells, hepatocytes and platelets, leads the HSC to activation [9]. The Kupffer cells stimulate matrix synthesis, cell proliferation, and release of retinoid by stellate cells through the actions of cytokines (TGF-β) and reactive oxygen intermediates/lipid peroxides [10, 12]. In case of injury, the hepatocytes also promote HSC initiation with a process mediated by FAS, and can involve the Tumor Necrosis Factor (TNF)-related apoptosis inducing ligand (TRAIL) [10, 13, 14]. Apoptotic fragments liberated from hepatocytes are fibrogenic and may contribute to the activation of HSC [6, 15]. Platelets are the most important paracrine stimuli, generating different important mediators, like platelet-derived growth factor (PDGF), TGF-β1 and epidermal growth factor (EGF) [6].

II. Perpetuation Phase:

To accumulate the ECM, the hepatic stellate cells undergo a series of phenotypic changes, including proliferation, contractility, fibrogenesis, chemotaxis, matrix degradation, pro-inflammatory responses and cytokine release [6]. In proliferation, a lot of mitogenic factors, but also cognate tyrosine kinases receptors, are unregulated. PDGF is one of the principal mitogenic factors that increases their expression. Other mitogens factors are involved in the proliferation of the hepatic stellate cells [6]. The contraction by activated hepatic stellate cells obstructs hepatic blood flow by constricting individual sinusoids and by contracting the cirrhotic liver [6]. A principal contractile stimulus to HSC is ET-1, and due to the activation of stellate cells there are increase of expression of smooth muscle actin (α-SMA) [6]. The fibrogenesis is the key of the stellate cells to contribute for liver fibrosis. TGF- β1 is an important fibrogenic factor, because several mechanisms surrounding this factor contribute for the increase of activation of ESC, another important is TNF factor [6, 16]. Sinusoidal endothelial cells and hepatic cells are inflammatory effectors, they can rapidly lose their fenestrations up on injury and express pro-inflammatory molecules, ICAM-1, VEGF and adhesion molecules [6, 18, 19].

III. Resolution/Reversion Phase:

The reversion of stellate cell activation, or selective removal of activated HSC by apoptosis, can explain the loss of activated cells in the resolving phase in liver injury [6]. The apoptosis can occur due to soluble factors and matrix components that are present during injury. Moreover, cell death ligands, such as TRAIL and FAS, are expressed in liver injury, and activated HSC are more vulnerable to TRAIL mediated apoptosis [6, 14, 20, 21]. The resolution/reversion phase in the activation of the ESC is one important phase that can be used to promote the apoptosis of HSC cells through_tumor-specific TRAIL receptor binding. The HSC have a central role in the liver fibrosis, when they are activated overexpress the TRAIL receptor in their surface becoming an ideal targets for TRAIL agonists [20].

1.2. Tumor Necrosis Factor–related apoptosis inducing ligand

Tumor necrosis factor (TNF) related apoptosis inducing ligand (TRAIL) belongs to a family of cytokines that function as protuberant mediators of immune regulation and inflammatory responses [21]. TRAIL can bind to two pro-apoptotic receptors, DR4 and DR5 [22, 23]. With HSC activation there is an increase in DR4 and DR5 expression levels, while that of DR5 remains 103 times higher than that of DR4 [19]. At the same time the expression of anti-apoptotic receptors DcR1, DcR2 and osteoprotegerin (OPG) also increases (Figure 1.4) [23, 27–30]. TRAIL protein is expressed in a wide range of tissues and interacts with TRAIL receptors to induce the apoptosis of the cells [23,25,26].

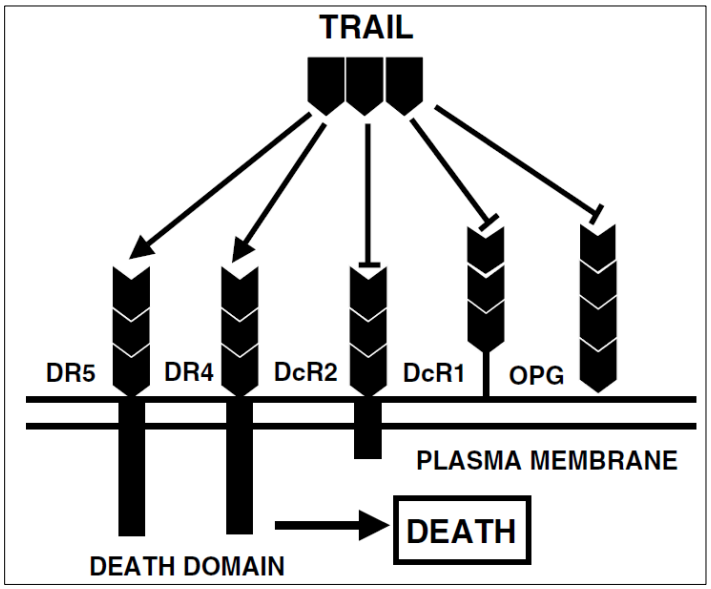

Figure 1.4. The receptors expressed on the surface of the cells that can bind to TRAIL protein. Receptor DR4 and DR5 have a death domain that can bind to TRAIL protein and induce apoptosis. The other hand, DcR1, DcR2 and OPG compete with receptor DR4 and DR5 for biding to the TRAIL protein, and these receptors do not promote the apoptosis. Adapted from [22].

The death receptors TRAIL DR4 and DR5 have an intracellular death domain motif via which the apoptotic signal is initiated. In contrast, DcR2 has a truncated and nonfunctional cytoplasmic death domain, whereas DcR1 lacks a cytosolic region and it is anchored to the plasma membrane, and OPG is a soluble decoy receptor [22].

When TRAIL protein binds to TRAIL receptor the extrinsic pathways are activated inducing apoptosis of mammalian cells [29]. The binding of TRAIL to its receptors results in trimerization of the receptor and clustering of the receptors intracellular death domain, allowing formation of the death-inducing signaling complex (DISC) [22] via binding of the adaptor molecule, Fas-associated death domain (FADD). This leads to binding and activation of caspase-8 and caspase-10. When caspase-8 or caspase-10 is activated, this cleaves caspase-3, leading to apoptosis (Figure 1.5.) [22].

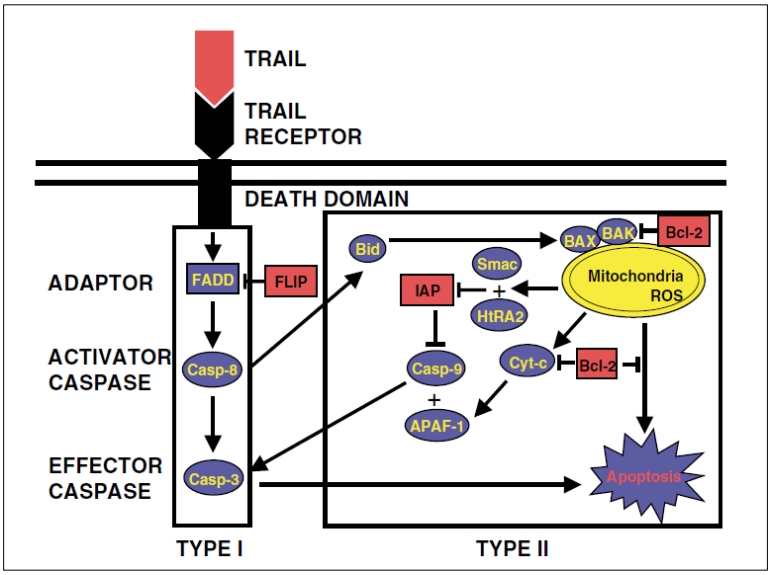

Figure 1.5. Pathways of induced apoptosis by TRAIL. The TRAIL binds to the receptor, leading to a recruitment of FADD and this molecule activates the caspase-8. Type I pathway is sufficient to induce apoptosis. However in other cell types, the type II pathway, through mitochondria, is necessary to induce the apoptosis. Adapted from [22].

In response to DNA damage, cell cycle checkpoint defects, loss of survival factors and other stressed factors, induce the intrinsic pathway of apoptosis in the cells. In this pathway, the activation of proapoptotic Bcl-2 gene induces the mitochondria to release apoptotic factors, such as cytochrome-c and SMAC/DIABLO, to the cytoplasm [23, 32–34]. The cytochrome-c connects with the APAF-1 adaptor, creating an apoptosome, activating the protease caspase-9. On the other hand, SMAC/DIABLO induces apoptosis binding to inhibitor of apoptosis (IAP), and preventing the caspase activation [23, 35, 36]. The intrinsic and extrinsic pathways can works together, and the capase-8 cleaves the proapoptotic Bcl-2 family member Bid. The cleaved product is translocated to the mitochondria to promote cytochrome-c release due to the interaction with Bax and Bak [23, 33].

TRAIL protein can bind to the five TRAIL receptors, however a soluble recombinant TRAIL can be an interesting treatment for cancer therapies and liver fibrosis. The soluble recombinant human TRAIL (rhTRAIL) is specific in inducing apoptosis in cancer cells and does not affect healthy cells. However, the capability of wild-type rhTRAIL to bind to all five receptors can reduce the levels of apoptosis [23, 37]. In order

to overcome this, receptor-specific variants of rhTRAIL were constructed: DHER and 4C7 that can bind specifically to DR5 and to DR4 receptors, respectively. The rhTRAIL DHER variant comprises mutations D269H and E195R, and the rhTRAIL 4C7 variant carries mutations G131R, R149I, S159R, N199R, K201H and S215D, using the rhTRAIL denominated WT (Wild Type) [37, 38]. Both variants, rhTRAIL DHER and 4C7, have a higher affinity to the receptor DR5 and DR4, respectively. Especially mutant rhTRAIL DHER can be used to develop new therapies to treat liver fibrosis, due to the higher affinity to DR5 receptor and the induced expression of DR5 receptor in HSC.

1.3. Gene therapy using adenovirus

Genes are the units of heredity and their specific sequences encode different proteins. When genes suffer alterations, the production of proteins cannot occur, resulting in genetic disorders. Gene therapy is fundamental to replace defective genes, which are responsible for diseases [39–41]. This therapy can replaced a mutated gene that causes disease by a healthy copy, it can inactivate the mutated gene or it can introduce new gene to fight the disease. One of the most difficult problems to achieve this goal is to replace the gene into the patient's target cells. For this reason it can be used a vector to replace the defective gene [39]. To deliver the vectors there are two techniques, *ex vivo* and *in vivo* [41, 42]. With the *in vivo* therapy the vector is directly injected into the patient´s blood to bind to the target cells [39]. For gene therapy, the vectors used include: viral vector or non-viral methods. The viral method uses retrovirus, adenovirus, adeno-associated viruses and herpes simplex virus [39].

The characteristics of the adenovirus, they are best suited for gene therapy: the ubiquitous of the vector, because of the isolation from the different species, the most common used in this therapy is the serotypes 2 and 5; the rapid infection of the adenovirus and the high levels of gene transfer in a range of human cells; the low pathogenicity of the vector in humans; the capacity of the vector to accept large sequences of DNA and transduce these transgenes; the genome of the adenovirus do not suffer rearrangement

and the DNA of the insert is maintained without alterations; lastly, the adenovirus are easily to manipulate using recombinant techniques [41].

The adenovirus has a double stranded DNA. When the infection occurs the DNA enters the nucleus, and the genes from the early region 1 (E1a and E1b) are transcribed. During the infection, the region from E1 to E4 is expressed, and this region is the principal for the process of viral gene expression [41] . The adenovirus infects the host cells, introducing the genome inside of the cells. The DNA of the adenovirus is not introduced into the genome of the cells, but is transcribed as the other genetic material of the cells (Figure 1.6.)[39].

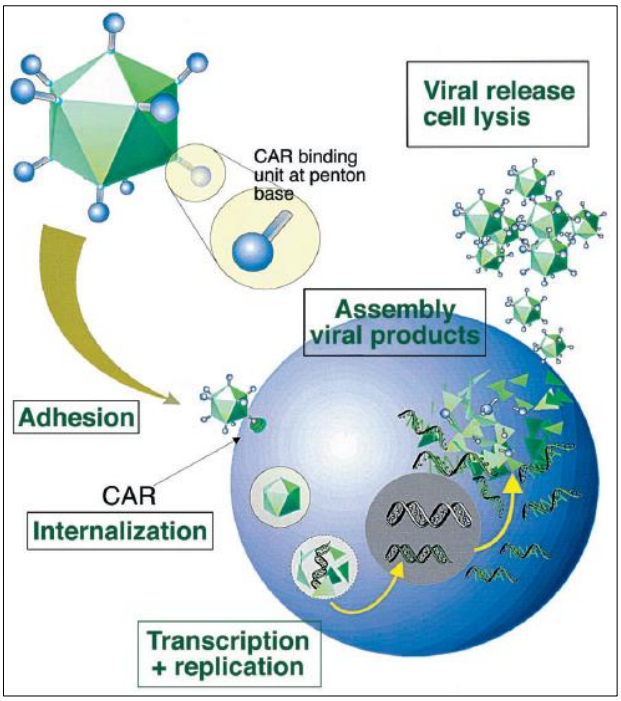

Figure 1.6. The replication cycle of an adenovirus vector. The image depicts the replication of an adenovirus vector in the cells, encoding the desired proteins. Adapted from [41].

The recently constructed adenoviral vectors have deletions in E1, E2 and E4 genes, due to the capability of these sequences to produce viral proteins, responsible to induce the immune response in the host and decrease the toxicity *in vivo* [41]. However, with the use of the adenovirus it is difficult to obtain efficient gene transfer after a second administration, due to the neutralizing antibodies, and the viral DNA disappears over time, so that in chronic diseases it is necessary repeat the infection of the adenovirus from

time to time [41]. The use of adenovirus are more advantageous than the use of protein added directly to the patients, due to the capability of the adenovirus to produce the protein in the target cells and maintaining constant levels of protein over a long period, so the efficacy will be larger. For this reason, the use of a adenovirus vector carrying the gene of rhTRAIL DHER for delivery in the liver is an interesting therapy for the treatment of liver fibrosis.

In a preclinical perspective, it was demonstrated that adenoviral DNA is expressed in liver, skeletal muscle, heart, brain, lung, pancreas and tumor tissue, once the vector is administrated intravenously. However, most of the virus accumulates in the liver [43, 44]. Gene therapy is a relatively new procedure, and the scientists do not yet understand all the associated to this therapy. Potential problems include: short lived nature of gene therapy, immune response, problems with viral vectors, multigenic disorders and insertional mutagenesis [39].

In an ethical perspective, the risk of the gene therapy may not be greater than the potential benefit [41, 45]. However, ethical committees have some concerns about this therapy, such as, the distinction between what is normal and what is disability, whether some diseases should be cured, whether the somatic therapy is more or less ethical than germ line therapy, etc. The primary treatments of gene therapy were conducted in patients whose first treatment failed, so the associated risk is small. Moreover, the viruses used for gene therapy should be extensively studied, to avoid greater risk for the patients [39]. Gene therapy can be an interesting therapy for liver fibrosis, moreover the use of this therapy combined with other agents/drugs (like HDACs and HAT inhibitors) can increase the efficacy of the therapies so the treatment it will be more powerful.

1.4. Histone deacetylation and histone acetyltransferase inhibitors

Liver fibrosis is caused by the activation of the HSC, the combination of the rhTRAIL mutants with the HDACs and HAT inhibitors can be an interesting therapy to treat liver fibrosis. Cells are constituted by DNA and the chromatin preserves the DNA-protein complex in the eukaryotic genome, where the predominant protein components are the histones [44]. The nucleosome is the principal element of chromatin, where 146 bp of DNA is wrapped around a core histone octamer, which contains two molecules of histones [46, 47]. The complexity of DNA in chromatin, at the level of histones, has an important role in the transcription, recombination, repair and replication of DNA [44].

Acetylation is a dynamic energy intense phenomenon: the stable state balance is mediated by the opposing activities of histone acetyltransferase (HATs) and deacetylase enzymes (HDACs). During acetylation an acetyl group from the acetyl coenzyme A (acetyl-CoA) is transferred to the ε-amino group in a specific lysine residue existent in the amino-terminal tails in the histones or the α-amino group of the amino-terminal residue [48–51], resulting in the neutralization of a single positive charge [46, 52]. Histone are acetylated by the histone acetyltransferases (HATs) [51, 53, 54], whereas the reverse reaction is catalyzed by the histones deacetylases (HDACs) [53]. HATs can also acetylate non histone proteins, like HMG proteins, transcription factors, nuclear receptors and α-tubulin [49]. Similarly, HDACs can also deacetylate non-histone proteins [49].

Histone acetyltransferases are characterized into Type A and B, located in the nucleus or cytoplasm, respectively [49]. The type A is based in acetylating nucleosome histones and is related to transcription. However, the type B acetylates nascent histones in the cytoplasm during the chromatin assembly process [49]. The HAT inhibitors can be grouped in to four different families, according to the sequence conservation: Gcng/PCAF (includes yeast Gcn5 and PCAF), MYST (contains MOZ, Ybf2/Sas3, Sas2 and Tip60), p300/CBP (p300 and CBP), and Rtt109 [49].

Histone deacetylases can be subdivided into 4 classes: classes I (comprisisng HDACs 1, 2, 3 and 8), classes II (HDACs 4, 5, 6, 7, 9 and 10) and Classes IV (HDAC 11) that are zinc-dependent enzymes, whereas the classes III are zinc-independent but nicotinamide adenine dinucleotide-NAD-dependent [56,57]. In the HDAC inhibitors, there are determinant features to lethality: the down-regulation of anti-apoptotic proteins such as caspase inhibitors (e.g. X-linked inhibitor of apoptosis (XIAP), surviving and cellular FLICE-like inhibitory protein (c-FLIP)), up-regulation of pro-apoptotic proteins such as Bim, Bmf and Noxa (through acetylation of p53), activation of the death receptor pathway, induction of Bid cleavage and activation, due to linking the intrinsic and extrinsic pathways of apoptosis, induction of the endogenous cycling dependent kinase (CDK) inhibitor p21 and disruption of chaperone protein [55].

The HDAC inhibitor SAHA inhibits cell proliferation blocking progression in G1 or G2/M phases in the mitotic cycle of the cell, supresses angiogenesis, and induces cellular differentiation and apoptosis [56]. The inhibitory activity improves the acetylation of histones, which persuades chromatin relaxation [56]. The HDAC inhibitor SAHA combined with protein TRAIL has already been tested. These combinations induce synergistically the apoptosis by mitochondrial pathway in the cells [56]. However the mechanisms behind the synergy are not complete understood. One possibility for this synergy includes the activation of one of the death receptors resulting in trimerization of the receptor and formation of DISC, activating the caspase-8, and directly inducing the pro-caspase-3 [57]. The activation of these caspase, through extrinsic pathway, takes in turn to the ultimate initiation of apoptosis [56]. Another possibility when DISC- activated caspase-8 is the intrinsic pathway (mitochondrial pathway). The truncated BID (tBID) translocate to mitochondria, reducing the mitochondrial membrane, and releasing the cytochrome-c [56]. The formed apoptosome activates the pro-caspase-9. Proteolytic cleavage of pro-caspase-3 by caspase-9 induces the programmed cell death and amplifies the caspase-8 and caspase-9 initiation signals [58, 60].

The HAT inhibitor, MG149, belongs to the family of MYST (tip 60), although the inhibitor C646 belongs to the group p300/CBP (p300). These two inhibitors was discovered very recently, and the mechanisms that occur in the cells are not complete

understood [60, 61]. Figure 1.7 demonstrates the interaction occurring with HAT and HDAC.

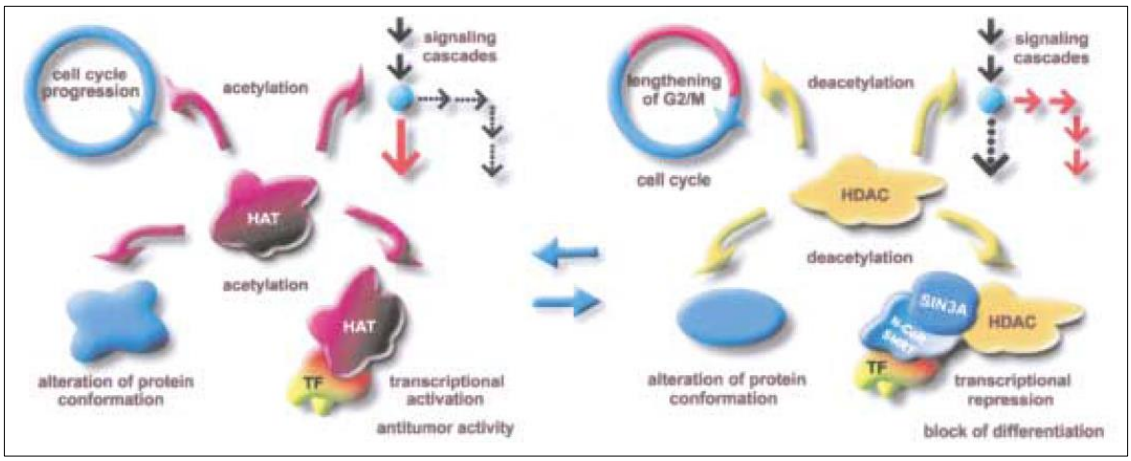

Figure 1.7. The dynamic equilibrium of HATs and HDACs in the cells results from histone acetylation. The change in histone levels has effect in transcriptional regulation, signal transduction cascades, cell survival, differentiation, and the activities of target proteins. HATs are connected to the cell-cycle progression, whereas HDAC are connected with extension of cell cycle G1 and G2. Adapted from [61].

1.5. Objectives

Researchers have found that the use of the DR5-specific TRAIL variants D269H/E195R could significantly reduce binding to decoy receptors and improve DR5 specific TRAIL receptor binding, resulting in a lower administrated dose with possibly fewer side effects [35].

The aim of our project is to use the gene therapy for the delivery of rhTRAIL DHER, which can selectively induce apoptosis in activated HSC, and hopefully, cure fibrotic livers. We will investigate the specificity and efficacy of the vector expressed proteins containing the rhTRAIL DHER. To this end, we will use the rhTRAIL mutants to determine their specificity in the context of different hepatic cell types. A specific research line will be the use of rhTRAIL mutants, designed to reduce binding to decoy receptors and to improve DR5 TRAIL receptor binding. Moreover, we will use the

rhTRAIL variants (DHER and 4C7) and the wild type rhTRAIL protein, to combine with HDACs (SAHA and Entinostat) and HATs (MG149 and C646) inhibitors, and evaluate the effects in different cancer cell lines (SW948 and H460) and transformed hepatic cells lines (HepG2 and HUH-7).We hope to find a combination that is highly efficient towards cancer cell line, but can protect the normal liver cells.

-Chapter 2-

Materials and Methods

2.1. Cell culture and cell lines

The human embryonic kidney cell line (HEK-293 ATCC® CRL-1573™), the human colorectal adenocarcinoma cell line (SW948 ATCC® CCL-237™) and the human hepatic cellular carcinoma cell line (HUH-7 JCRB0403™) were maintained in complete Dulbecco's minimum essential medium, with high glucose, GlutaMax™ and pyruvate (DMEM; Gibco®, Life Technologies™, Carlsbad, CA, USA) with 10% of FBS (Gibco®, Life Technologies™, Carlsbad, CA, USA)) and penicillin (100 IU/mL)/streptomycin (100 µg/mL) (1% P/S). The large lung carcinoma cell line (H460 ATCC® HTB-177™) and hepatocellular carcinoma cell line (HepG2 ATCC® HB-8065™) was maintained in complete DMEM with 10% of FBS and penicillin (100 IU/mL)/streptomycin (100 µg/mL). Immortalized human hepatic stellate cell line (LX-2), a kind gift provided by Prof. Scott Friedman (Mount Sinai Hospital, New York), was maintained as described previously [20]. All of the cells lines were maintained in a T-75flask at 37ºC with a 5% of CO_2 in a humidified atmosphere, and routinely cultured when they were at 90% of confluence. After the experiments with the cells, a T-75 flask was incubated again with the cell line, in order to have the cell line available for other assays.

2.2. Antibodies and rhTRAIL proteins

The Mouse IgG1 Anti-HA Tag antibody was from InvivoGen (13L18-MM, San Diego, CA, USA). Rabbit polyclonal Anti-TRAIL antibody was from AbCam® (ab2435,

Cambridge, England, UK). Polyclonal Swine Anti-Rabbit Immunoglobulins/HRP was from Dako© (P 0217, Agilent Technologies, Santa Clara, CA, USA). Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP was from Dako© (Z0259, Agilent Technologies, Santa Clara, CA, USA). The proteins rhTRAIL DHER, 4C7 and WT were a kind gift of Dr. R.H. Cool and the proteins were produced in *Escherichia coli* BL21 (DE3) bacteria using pET15b expression plasmids.

2.3. Materials

The agar and agarose solution were from Invitrogen™ (Life Technologies™, Carlsbad,CA, USA) and the electrophoresis in agarose gel was performed in a Sub-Cell® GT Cell, a horizontal electrophoresis system from Biorad®(Hercules, California, USA). The solution of Tris-HCl (Tris-HCl, Molecular Biology Grade (Tris-Hydrochloride) from Promega® (#H5123, Fitchburg, Wisconsin, USA). The SDS, and bromophenol blue Loading solutions were from Promega® (Fitchburg, Wisconsin, USA) and the electrophoresis of the 12,5% SDS-polyacrylamide gels and blotting of the gels were performed in Mini-Protean® Electrophoresis System and Mini Trans-Blot® system from Biorad®(Hercules, California, USA), respectively. The T-Flask 75 cm^2 and trypsin with 0,5% of EDTA were from Life Technologies™ (Carlsbad,CA, USA). The EDTA, Glycine, PBS (Phosphate buffered saline) were from Sigma Aldrich® (St. Louis, MO, USA). The methanol was from VWR® (Radnor, PA, USA). The 30% Acrylamide/Bis solution, Temed and β-mercaptethanol were from Biorad® (Hercules, California, USA). The Tris-acetate-EDTA (TAE) solution was done using Tris Base, Acetic Acid, EDTA and water until one liter. The primers used to the cloning process were created in a Clone Manager® Professional version 9 and purchase in Sigma Aldrich® (St. Louis, MO, USA).

2.4. Construction and production of the vector pADTRACK-CMV-IGK-HA- rhTRAIL DHER

2.4.1. Cloning process to create the vector

One of our goals was to construct a vector containing the rhTRAIL DHER protein. To this end, the gene was amplified from the pET15b-rhTRAIL DHER (kindly provided by Dr. R.H. Cool [35]) and cloned in a shuttle vector, pADTRACK-CMV, for the expression of specific transgenes [62]. The vector pADTRACK-CMV-IGK-HA-PDGF-sTRAIL (kindly provided by M. Arabpour, University of Groningen, The Netherlands) was used to construct the new plasmid pADTRACK-CMV-IGK-HA- rhTRAIL DHER. To construct the new vector, a specific region in the pET15b- rhTRAIL DHER was amplified by PCR with Phusion enzyme (Promega®, Fitchburg, Wisconsin, USA) using a forward primer 5´ GGGCCCAGCCGGCCGTGAGAGAAAGAGGTCCTCAGAG 3´ and a reverse primer 3´GGATATCGGATCCCTATTAGCCAACTAAAAAGGCCCC 5´. The restriction site for SfiI (New England Biolabs®, Ipswich, Massachusetts, USA) was added in the forward primer, and a restriction site for BamHI (New England Biolabs®, Ipswich, Massachusetts, USA) and EcoRV (Promega®, Fitchburg, Wisconsin, USA) was added in the reverse primer. The conditions of the amplification were 98ºC for 30 sec in the initial denaturation, 30 cycles at 98ºC for 10 sec, 60ºC for 15 sec and 72ºC for 1min 45sec and a final extension at 72ºC for 5 min. The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up system (Promega®, Fitchburg, Wisconsin, USA) according with the manufacturer's instructions.

The amplified product and the vector pADTRACK-CMV-IGK-HA-PDGF-sTRAIL was digested by the restriction endonuclease SfiI and EcoRV, and in the digested vector was added CIP (New England Biolabs®, Ipswich, Massachusetts, USA) to remove the dephosphorylate ends of the DNA, to prevent the reconnection of the plasmid. The products resulting from digestion were cleaned with the Wizard® SV Gel and PCR Clean-Up system (Promega®, Fitchburg, Wisconsin, USA) according with the manufacturer's

instructions. The digested vector and the digested sequence of the rhTRAIL DHER was ligated using the DNA ligase T4 (Promega®, Fitchburg, Wisconsin, USA) kept overnight at 4ºC, and a new plasmid was created (Figure 2.1). The pADTRACK-CMV-IGK-HA-rhTRAIL DHER vector was transformed into *Escherichia coli* XL1-Blue Bacteria. The transformation was done using 10µL of the ligation with 100µL of bacteria, incubated on ice 10 minutes, 45seconds at 42ºC, and 2 minutes on ice again. After thermal shock LB medium was added without antibiotic and incubated at 37ºC to allow the bacteria to recover from the shock. The bacteria containing the cloned vector were added in LB plates and selected using kanamycin.

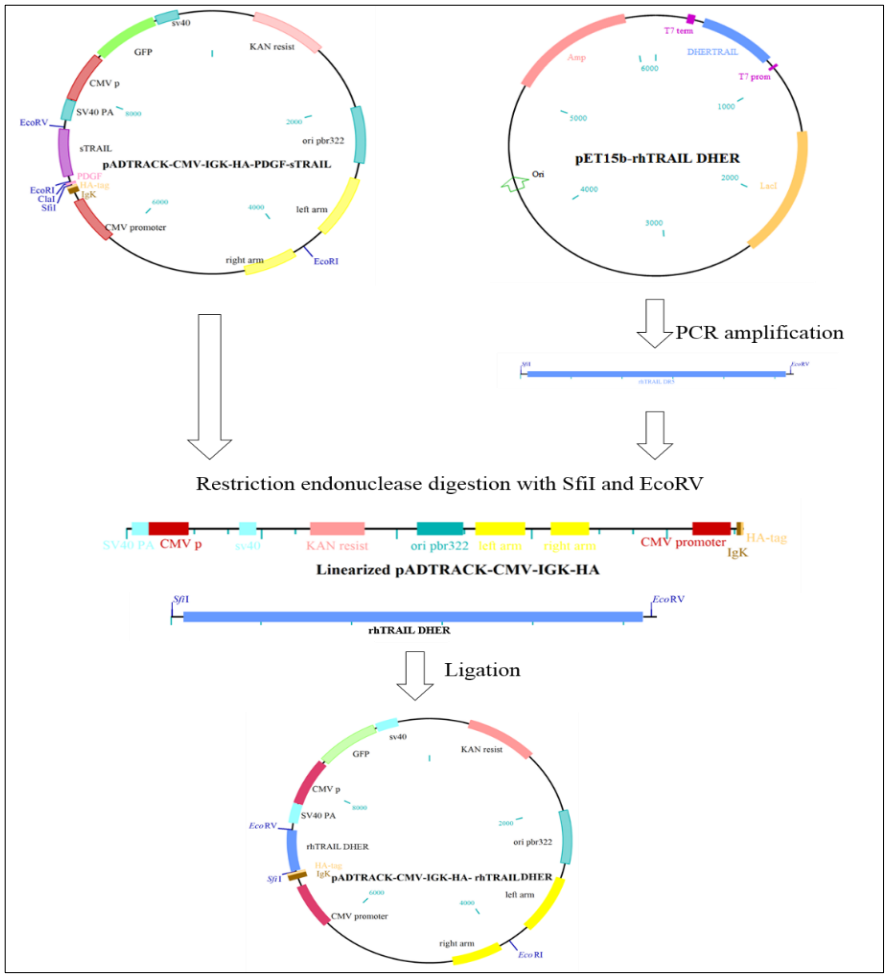

Figure 2.1 Schematic diagram of the cloning process to create the pADTRACK-CMV-IgK-HA-rhTRAIL DHER vector. A PCR amplification in the pET15b-rhTRAIL DHER occurs to amplify the rhTRAIL DHER gene. Then, the restriction endonuclease digestion with SfiI and EcoRV was done to create cohesive ends. Lastly, the ligation of the products was done to create the new vector.

2.4.2. Analysis of the cloned vector

In order to analyze whether the ligation of the amplified gene with the shuttle vector occur, two analyzes were performed. The selection in kanamycin produced several colonies, but only fifteen colonies were used to continue with the analysis. After the extraction of the DNA using a PureYield™ Plasmid Miniprep System (Promega®, Fitchburg, Wisconsin, USA) according with the manufacturer's instructions, the vector was analyzed through PCR reaction and restriction endonuclease digestions. The gene coding for the rhTRAIL DHER was amplified by PCR with Phusion™ enzyme (Promega®, Fitchburg, Wisconsin, USA) using the same primers used for the cloning process. The restriction endonuclease digestions were prepared in three different reactions: the first with ClaI restriction enzyme (Life Technologies™, Carlsbad,CA, USA); the second with ClaI and EcoRI (New England Biolabs®, Ipswich, Massachusetts, USA) restriction enzymes; and the third with EcoRI restriction enzyme, using as a control non digested DNA. The digestions were held for 1h30min at 37ºC. The bands sizes of the digestions were analysed using the electrophoresis technique in a 1% agarose gel (50 mL of Tris-acetate-EDTA (TAE) 1%, 0.5g agarose) and using the 1Kb Plus DNA Ladder marker from Invitrogen™ (Life Technologies™, Carlsbad, CA, USA). After both analysis two clones were chosen and sent for sequencing in the BaseClear® Company (Leiden, The Netherlands). The sequencing results are in Appendix A.1.

2.4.3. Expression of the protein in HEK-293 cells

After validation and confirmation of the presence of the gene of interest in the vector, it was necessary verified that the vector was expressing the protein of interested, to performed this analysis was used only the clones that was sent to sequence. The vectors pADTRACK-CMV-IGK-HA-rhTRAIL DHER were transfected into HEK-293 cells using the FuGENE® HD Transfection reagent (Promega®, Fitchburg, Wisconsin, USA) according with the instructions of the manufacture. As positive control, the vector

pADTRACK-CMV-IGK-HA- rhTRAILWT (kindly provided by M. Arabpour, University of Groningen, The Netherlands) was used, and, as negative, the transfection without DNA.The HEK-293 cells was cultured in a 24-well plate (Nunclon Delta Surface, Thermo Fisher Scientific® Waltham, Massachusetts, USA) with an 80% confluence (around $15x10^5$ cell/well), and allowed to adhere for 24 hours. The cells were cultured in 1mL of complete DMEM, containing 10%FBS and 1% penicillin (100 IU/mL)/streptomycin (100 µg/mL), then incubated at 37ºC in a humidified atmosphere with 5% of CO_2.

The efficiency of the transfection was observed in fluorescence microscopy (Axiovert 25 from Zeiss) by the GFP expression in the cells, because the vector contains the gene for GFP.Three days after the transfection, the culture medium (supernatant) was saved and the cells were harvested adding 50 µL of Trypsin containing 0.5% EDTA (Life Technologies™, Carlsbad, CA, USA), then incubated for 20 min at 37ºC in a humidified atmosphere with 5% of CO_2. The trypsinized cells were neutralized using 200 µL of the medium collect before. Afterwards, the neutralized cells were centrifuged at 1000g during 5 min (Beckman Coulter®, Brea, California, USA), and the supernatant was collected and saved. The pellet was ressuspended in 25 µL of 100mM Tris-HCl pH 7.8 and underwent 5 cycles of freeze-thawing in liquid nitrogen to promote the disruption of the cells. The final volume for the supernatant was 2mL and, for the pellet, 25 µL. Both the cells and the supernatant were stored at -20ºC.

2.5. Analysis of the expression profile of the rhTRAIL DHER protein in HEK-293 cells

2.5.1. Analysis of the expression of the protein through western blotting analysis

After confirming the presence of the gene in the vector, it was necessary to verify that the cloned protein expressed by the vector is working, which was done by western blotting. The supernatant and the lysates cells, obtained in the transfection in HEK-293 cells, were used to search for the specific protein rhTRAIL DHER expressed by the plasmid pAdTRACK-CMV-IGK-HA-rhTRAIL DHER. In 10 µL of the supernatant and 1 µL of the pellet were added 4 µL sodium dodecyl sulfate (SDS) buffer (1M Tris-HCl, pH 6.8, 0.8% SDS, 40% Glycine (w/v), 10% 0.5M EDTA, 0,01% bromophenol blue) and 1 µL of β-mercaptethanol, then warmed at 110ºC during 5 min, and 6µL of marker (Page Ruler Plus Prestained Protein Ladder, Thermo Fisher Scientific® Waltham, Massachusetts, USA) was added to allow the identification of the size. Allsamples were placed in a 12.5% sodium dodecyl sulphate polyacrylamide gel (SDS Gel) (1.5 M Tris-HCl, pH 8.8, 0.5M Tris-HCl, pH 6.8, 30% acrylamide/bisAA, 20% SDS, 1X Tris-glycine-SDS Buffer (TEMED), 10% Ammonium PerSulfate (APS)), and resolved by electrophoresis on a SDS running buffer (25mM Tris-HCl pH 8.3, 192mM Glycine, 0,1% SDS) during 30min at 70V, then the voltage was increased to 150V during 1hour 20min. After the SDS gel was transferred onto an activated polyvinylidene difluoride (PVDF) membrane (#162-0177 Biorad®, Hercules, California, USA), this membrane was activated for 2 min with 100% methanol, and blotted with electrophoretic transfer for 2 hours at 250mA in 4ºC, with blotting buffer (25mM Tris-HCl pH8.3, 192mM Glycine, 10% methanol). The membrane was blocked overnight at 4ºC in PBS (Gibco®, Life Technologies™, Carlsbad, CA, USA) with 0.1% Tween®20 (Promega®, Fitchburg, Wisconsin, USA) and 5% powder milk, after blocking was incubated with the primary antibody. The Mouse IgG1 Anti-HA Tag antibody (1:1000 dilution) and Rabbit

polyclonal Anti-TRAIL antibody (1:1000 dilution) were incubated in 10 mL of PBS with 0.1% Tween®20 and 5% of bovine serum albumin (BSA) (A9418 Sigma Aldrich®, St. Louis, MO, USA) during 1hour30min moderately shaking at RT. Then, the membrane was washed with 10 mL of PBS+0.1% Tween®20 (Promega®, Fitchburg, Wisconsin, USA), three times at RT, during 10min shaking smoothly. After washing, the peroxidase conjugated secondary antibodies, as polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP antibody (1:1000 dilution) and polyclonal Swine Anti-Rabbit Immunoglobulins/HRP (1:1000 dilution) antibody were incubated in 10 mL PBS with 0.1% Tween®20 and 5% BSA over 1hour at RT, with moderate shaking. Finally the membrane was washed as mention previously and the signal was revealed with enhanced chemiluminescence substrate (Western Lightning® Plus-ECL, PerkinElmer, Waltham, MA, USA), according to the manufacturer's instructions. The picture of the membrane was done in a gel imaging for fluorescence and visible applications (GBOX, Syngene, England, UK).

2.5.2. Analysis of the function of protein through survival assay in SW-948 cells

As described in the previously section to determine whether the express protein is working, first a western blotting analysis was done to detect the protein, and, second, a survival assay was done adding the express proteins to the cells SW948.

The cells SW-948, cultured in flasks T-75cm^2, were washed two times with 1xPBS buffer, trypsinized with 2mL during 30min at 37ºC, in a 5% CO_2 incubator. Then 2 mL of complete DMEM were added, to neutralize the trypsin. The suspension of cells was counted and seeded in 96-well plate (Costar™, Thermo Fisher Scientific, Waltham, MA, USA) with 12 x 10^3 cells/well at a final volume of 100 µL of complete DMEM and allowed to adhere overnight. After 24 hours of plating, different concentrations of the supernatant from transfection (3.1µL, 6.25µL, 10µL, 12.5µL, 25µL, 50µL, and 100µL) were added and 1 µL of pellet from transfection was added to the cells. The positive

control and the negative control from the transfection in HEK-293 cells were used. To reach the 200 μL as a final volume complete DMEM was added. After treatment for 72hours was removed carefully the culture medium from the wells, and washed slightly with PBS buffer concentrate 1x (warmed at RT). The PBS buffer was removed gently and 50 μL of the crystal violet solution (70% ethanol and 30% crystal violet solution) were added and incubate for 10min at RT. After incubation, the plates were washed twice by immersion in a large beaker with tap water. Then the plates were drained upside down on filter paper, and 100 μL of 1% SDS (1%SDS, 100mL sterile water) was added in each well, and put on shaker during 2 hours (until the color was uniform). As a final step the absorbance of each well was measured at 570nm, in a synergy H1 hybrid Multi-Mode Microplate reader (Biotek®,Winooski, VT, USA). The crystal violet (Figure 2.2), a triarylmethane dye, is a simple assay, useful for obtaining information about the adherent cells to the surface of the dishes. This assay stains the DNA. When soluble the amount of dye taken up by the monolayer cells can be quantified in a plate reader [63].

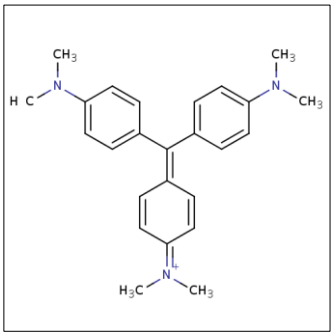

Figure 2.2 . The crystal violet structure. The crystal violet is a triarylmethane dye (methylrosanilide, tris[4-(dimethylamino)phenyl methanol) useful for cell adhesion studies. Adapted from [64].

The stained cells where an indication that the cells were alive and they adhered to the plate. To calculate the survival of the cells, after the measurement in the plate reader, the equation (1) was used and all the calculations was done in the Microsoft® Excel program.

$$\text{Survival}(\%) = \frac{\text{Avrg(Abs values of the sample)}}{\text{Avrg(Abs values of the control only with cells)}} \text{x}100 \qquad \text{equation (1)}$$

Avrg= Average; **Abs**=absorbance

2.6. Generation of adenovirus plasmid by homologous recombination in bacterial cells

2.6.1. Preparation of electrocompetent cells

In order to construct an adenovirus plasmid to produce the protein rhTRAIL DHER and to be able to use in humans, the *Escherichia Coli* strain BJ5183 bacteria was used, to obtain the homologous recombination of the constructed vector (pAdTRACK-CMV-IGK-HA-rhTRAIL DHER) with an adenoviral backbone vector (pAdEasy-1 adenoviral plasmid). The bacteria BJ5183 was transformed with the backbone vector pAdEasy-1 (kindly provided by M. Arabpour, University of Groningen, The Netherlands). The transformation procedure was the same as described in section 2.4.1. Then the transformed bacteria grew overnight in plates of LB medium (LB medium, 1% agar, 50 µg/mL ampicillin) at 37ºC in an incubator. One colony was selected and grew overnight in 6 mL of LB medium (with 50 µg/mL ampicillin) at 37ºC, in a Thermoshake-incubator shaker (Gerhardt® analytical systems). Subsequently 5mL of the cultured bacteria were added in 250mL of medium LB (with 50 µg/mL ampicillin), a sample was taken and the absorbance at 600 nm (A_{600}) was measured in a Eppendorf photospectrometer (Eppendorf®, Hamburg, Germany).The bacteria were then incubated for 4 hours until the A_{600}=0.75-0.90. The cells were collected in a 250mL flask and incubated on ice for 30 min, then centrifuged (Beckman Coulter®, Brea, California, USA) 4000g at 4ºC during 10 min. The pellet was washed with 250 ml of prechilled washing buffer (10% glycerol in sterile milli Q water), and the step of centrifuge was repeated again but for 30 min. The washing and centrifuge steps were repeated two more times, but decreased the volume of washing buffer, for 125mL and 75mL. The supernatant was discarded leaving only 15mL that were transferred to a 50 mL tube, and washed with 15 mL of washing buffer. Then the result was centrifuged at 2500g at 4ºC during 10 min, and the supernatant was aspirated, leaving only 1 mL. The remaining solution was ressuspended in 2mL of washing buffer, and aliquot 20 µL in cooled down tubes at -80ºC and stored.

2.6.2. Generation of recombinant adenoviral plasmid in BJ5183

With the goal of creating an adenovirus containing the gene of interest, the shuttle vector created was recombined with the plasmid pAdEasy-1, would be able to reproduce in the human body. For that reason, the prepared electrocompetent bacteria BJ5183 was taken from the -80ºC and thawed on ice. The plasmid pAdTRACK-CMV-IGK-HA-rhTRAIL DHER contained in XL1-Blue bacteria was grown overnight in medium 2mL of LB with 50 µg/mL of kanamycin and purified by phenol/chloroform extraction and ethanol precipitation using the kit PureYield™ Plasmid Miniprep System (Promega®, Fitchburg, Wisconsin, USA) according with the manufacturer's instructions. After purification 0.5-1.0µg of plasmid was linearized by restriction endonuclease with PmeI (New England Biolabs®, Ipswich, Massachusetts, USA) restriction enzyme (reaction of the digestion in Appendix 7.2), and incubated for 2 hours at 37ºC. After the incubation, the digested plasmid was purified using the kit Wizard® SV Gel and PCR Clean-Up system (Promega®, Fitchburg, Wisconsin, USA), following the rules of the manufacturer. Then, 1 µL of purified DNA was added to the electrocompetent bacteria BJ5183 (containing the pAdEasy-1), and incubated on ice for 30 seconds. The bacteria were transferred to a prechilled electroporation cuvette (Micropulser electroporation cuvettes, BioRad®, Hercules, California, USA), and the electroporation was performed at 2500V in the electroporation apparatus (MicroPulser, BioRad®, Hercules, California, USA). After electroporation 500µL of medium LB (without antibiotic) was added to the cuvette. The bacterial suspension was transferred to a micro tube and incubated at 37ºC for 40 min in a Thermoshake-incubator shaker (Gerhardt® analytical systems), to allow bacteria to recover and express the antibiotic resistance. To finish the procedure, 50µL of the bacterial suspension were added in a plate of LB medium (1% agar, 50 µg/mL of kanamycin), and then the bacterial suspension was centrifuged at 5000g during 1 min. The supernatant was removed, and 50 µL were left to ressuspend the pellet and add to other LB plate (with kanamycin) (see Figure Appendix A.3). In other to see the competence of the electrocompetent bacteria and the efficiency of the recombination, 50

µL of the bacteria suspension were added in a LB plate with 50 µg/mL of ampicillin (see Figure Appendix A.3). All the plates were incubated at 37ºC overnight.

2.6.3. Analysis of the recombinant adenoviral plasmid

In order to determine whether the recombination was achieved, it was necessary to evaluate the formed clones. The recombinants were selected by kanamycin and several colonies were generated. Usually, small colonies (10-25) are generated after recombination, because the genetic material inside the bacteria is large, and the replication is slow. Only four colonies were selected to continue with the analysis. The colonies grew overnight at 37ºC in 2mL of medium LB containing 50µg/ml of kanamycin, in a Thermoshake-incubator shaker (Gerhardt® analytical systems). The recombinant plasmids were purified using a PureYield™ Plasmid Miniprep System kit (Promega®, Fitchburg, Wisconsin, USA), and performed as the manufactures instructions. Those clones were further tested by restriction endonucleases.

First, 0.6µg of the DNA was digested with PacI restriction enzyme (New England Biolabs®, Ipswich, Massachusetts, USA), as described before [65], the reaction of the digestion is provided in Appendix A.2. Subsequently, to confirm that the clones contain the recombination of both plasmids (shuttle vector with adenoviral vector), the restriction endonuclease digestions were repeated (0.6-1.0µg DNA) in three endonuclease groups: the first with restriction enzyme PacI (New England Biolabs®, Ipswich, Massachusetts, USA), the second with restriction enzyme SfiI, and the third with restriction enzyme ClaI (Life Technologies™, Carlsbad,CA, USA) and SfiI (New England Biolabs®, Ipswich, Massachusetts, USA), the reactions of the digestions is in Appendix A.2. The digestions were compared with the restriction endonuclease of the controls, pAdEasy-1 and pAdTRACK-CMV-IGK-HA-rhTRAIL DHER. These controls were digested with the same reactions for the clones, but a restriction digestion only with ClaI (Life Technologies™, Carlsbad, CA, USA) was added to confirm the activity of this enzyme.

Due to the size of the plasmid (~43114bp) a marker was used containing a larger size in the bands. The marker used was Lambda DNA/EcoRI+HindIII Marker 3 (Thermo Fisher Scientific® Waltham, Massachusetts, USA).

2.7. Analysis and evaluation of the activity of receptor rhTRAIL mutants in LX2 cells and HepG2 cells.

2.7.1. Activity of specific proteins rhTRAIL DHER, 4C7 and WT in LX2 cells

We constructed the vector pADTRACK-CMV-IGK-HA-rhTRAIL DHER to be able to produce the rhTRAIL DHER protein, although the quantity produced for this type of vectors is not in high quantities. For this reason was used the protein rhTRAIL DHER , produced in the pET15b-rhTRAIL DHER vector, the same vector used to amplify the gene of the protein in the cloning process. This protein was added in LX2 (hepatic stellate cells), and the specific protein rhTRAIL 4C7 and WT were used as controls, and as produced in the vectors pET15b-rhTRAIL 4C7 and pET15b-rhTRAIL WT, respectively. These controls were used to evaluate the difference between the different proteins, and to observe the activity of the rhTRAIL DHER. The cells needed to be activated, so a treat plate was used. They would adhere and became activated after 6 days of incubation [20]. The LX2 cells, cultured in T-75 flask, were washed 2 times with 1xPBS, trypsinized with 2mL and incubated 20 min at 37ºC. To neutralize the trypsin 2mL of complete DMEM were added and the cells were counted. Then, 1000 cell/well were seeded in a 96 well-plate (Coated Plate, Gibco®, Life Technologies™, Carlsbad, CA, USA) with 100 µL of complete DMEM, and allowed to adhere overnight in the incubator at 37ºC with 5% CO_2. After three days, the medium was changed and 100 µL of complete DMEM were added and incubated for more 72 hours. Then, the specific proteins rhTRAIL DHER, 4C7 and WT were added to the cells in different concentrations (7.5 ng/mL, 15ng/mL, 35ng/mL,

70.5ng/mL, 153ng/mL, 306ng/mL,) using as a positive control cells without treatment (only complete DMEM) for a final volume of 200µL .The cells were incubated in 37ºC with 5% of CO2, during 72 hours. The tetrazolium assay (MTS- (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium)) was used to determine the cytotoxicity. In the cells 20µL of the solution MTS/PMS were added, which had been previously done according to the protocol that is attached in Appendix A.4.3, and incubated for 1 hour at 37ºC. The plates were read at 490nm in a synergy H1 hybrid Multi-Mode Microplate reader (Biotek®,Winooski, VT, USA). The survival of the cells was defined as the growth of the treated cells compared with untreated cells, and to determine this parameter the equation 1 was used, described in section 2.5.2. In this assay, the tetrazolium reagent is reduced by viable cells to generate formazan products that are straight soluble in the medium of cell culture [66]. The formazan products are negatively charged, contributing to the solubility in the medium, and limiting the cellular permeability to the tetrazolium. This set of reagents are used in combination with an intermediate electron acceptor such as phenazine methyl sulfate (PMS), being reduced in the cytoplasm and left the cells, where they can convert the tetrazolium to the soluble formazan product [66].

2.7.2. Activity of specific proteins rhTRAIL DHER, 4C7 and WT in HepG2 cells

In order to see how the specific protein rhTRAIL DHER (the same used for the previous assay) interacts with an hepatic cell line, a non-fibrotic cell, was combined the protein rhTRAIL DHER with the cells, using the rhTRAIL 4C7 and WT as a control, to see the sensibility of this type of cells to the proteins, and to see the difference between the proteins. The HepG2 cells, cultured in T-75 flasks, were washed with 1xPBS, and trypsinized with 2mL during 40 min at 37ºC. The trypsin was neutralized with 2mL of complete DMEM, and counted. Then 10000 cells/well were added in a 96 well-plate (Costar™, Thermo Fisher Scientific, Waltham, MA, USA) with 100µL of complete DMEM, and allowed to adhere overnight at 37ºC with 5% of CO_2. After 24 hours the

proteins rhTRAIL DHER, 4C7 and WT was added in each well with 100 µL of complete DMEM in different concentrations (100ng/mL, 500ng/mL, 5000ng/mL, 10000ng/mL and 50000ng/mL) and incubated for 72hours at 37ºC with 5% of CO_2. After incubation, 20 µL of MTS/PMS solution were added in the cells to perform the tetrazolium (MTS) assay as described in section 2.7.1.

2.8. Evaluation of the effects of the combination of rhTRAIL DHER, 4C7 and WT with HDACs and HATs inhibitors

Another goal of this project was to use the rhTRAIL proteins (DHER, 4C7 and WT) combined with HDACs and HATs inhibitors to determine the synergy or protection in different cancer (SW948 and H460) and transformed (HUH-7 and HepG2) cell lines. The inhibitors, SAHA, Entinostat, MG149, and C646 were kindly provided by Frank Dekker (University of Groningen, the Netherlands).

All the cells lines, SW948, H460, HUH-7 and HepG2 were processed in the same way for the assays. The cells, cultured in a T-75flask, were washed with PBS concentrate one time, trypsinized with 2mL and left at 37ºC with 5% CO_2 for different times: H460 and HUH-7 for 35min, HepG2 for 45min and SW948 for 20 min. After trypsinization, 2mL of complete DMEM were added to neutralize. Cells were counted and added 12000 cells/well with 100 µL of complete DMEM (DMEM, 10% FBS, 1% P/S) in a 96 well-plate (Costar™, Thermo Fisher Scientific, Waltham, MA, USA) and allowed to adhere overnight at 37ºC in a 5% CO_2. Then, for each cell line, the same combination of proteins inhibitors were added. For 100 ng/mL and 10ng/mL of rhTRAIL DHER, 4C7 and WT were combined with 1µM, 5µM of SAHA; 0.01µM, 0.05µM of Entinostat; 10 µM, 50 µM of MG149 and 10µM, 50µM C646. As control the cells without any drug and the cells only with one drug were used, to see the effect of combination. In all wells the final volume is 200 µL (100 µL there is already in culture, and more 100 µL that is added with

the drugs). Then, cells were incubated for 72 hours, at 37ºC with 5% CO_2. After three days, a crystal violet assay was used to determine the survival of the cells, and the procedure was the same as described in section 2.5.2. The same assay was repeated, but the incubation time for the rhTRAIL DHER, 4C7 and WT was 48 hours, i.e. the HDACS and HAT inhibitors were added to the cells (with the same concentrations), and after 24 hours the rhTRAIL DHER, 4C7 and WT were added (alone and for the combination) and incubated for 48 hours.

2.9. Statistical Analysis

All results represent independent experiments and are expressed as mean ± Standard error (SE) of the mean. The statistical significance was done using the program Prism5©.Tests were performed with one-way ANOVA test and a *Bonferroni* post-test for multiple comparisons.

-Chapter 3-

Results

3.1. Construction and production of the vector pDTRACK-CMV-IGK-HA- rhTRAIL DHER

The overall approach established for the cloning process is diagrammed in Figure 2.1 and involve several steps. Firstly, the gene of interested (rhTRAIL DHER) was amplified from the vector pET15b-rhTRAIL DHER by a PCR reaction and screened in a 1% agarose gel to see the amplification of the gene (Figure 3.1). Considering the size of the insert as 524bp, validation of this size was done with a simulation using the program Clone Manager© , described by other authors [35], and confirmed after a PCR amplification.

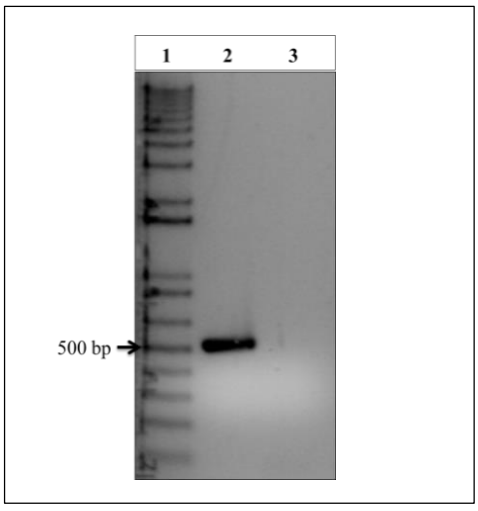

Figure 3.1 Amplification of the rhTRAIL DHER gene by a PCR reaction. The rhTRAIL DHER gene was amplified from the pET15b-rhTRAIL DHER vector using a PCR reaction. The reaction was done using the primers described in section 4.4.1. Lane 1- molecular marker (1Kb Plus DNA Ladder); Lane 2- Amplification of the gene rhTRAIL DHER; Lane 3- PCR reaction without DNA, to use as negative control.

Secondly, the amplified gene and the vector pADTRACK-CMV-IGK-HA-PDGF-sTRAIL were cleaved with restriction endonucleases (SfiI and EcoRV) to create the cohesive ends to allow the ligation of the gene into the vector of interest. Thirdly, the digested products were ligated overnight, transformed in XL1-Blue bacteria and cultured in a plate containing kanamycin to select the resistant. The bacteria that contained the new plasmid have the gene that confers resistance, to allow the bacteria grew in medium with this antibiotic (Figure 3.2).

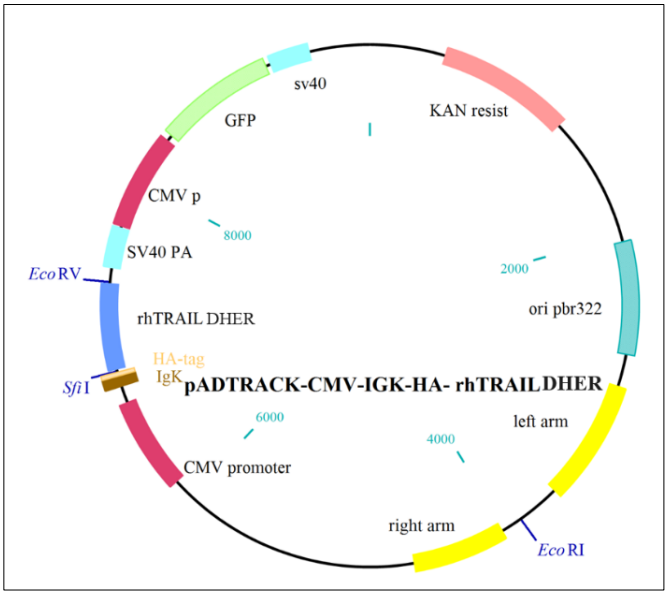

Figure 3.2. The constructed vector pADTRACK-CMV-IGK-HA-rhTRAIL DHER. This vector contains the gene that confers resistance to kanamycin. This vector results from ligation of the rhTRAIL DHER gene into the pADTRACK-CMV-IGK-HA linearized vector.

Indicating an efficient transformation, several colonies were generated and fifteen were selected. To further confirm the presence of the gene rhTRAIL DHER in the new vector, the clones were analysed by a PCR reaction and cleaved with a restriction endonuclease (ClaI and EcoRI). As shown in Figure 3.3, all the colonies express the gene for the rhTRAIL DHER, comparing with the control (lane 17), to which DNA was not added. We can observe that all of the selected clones have the gene for the rhTRAIL DHER and, based on the migration rate of the bands, the size was the expected (524bp).

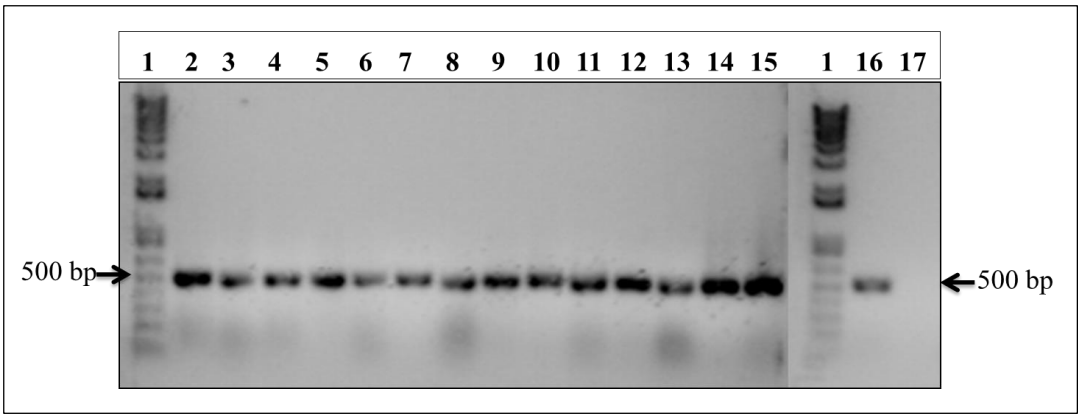

Figure 3.3 PCR reaction for the analysis of the new constructed vector. The DNA was analysed by electrophoresis through a 1% agarose gel and SYBR® Safe DNA gel staining. Lane 1, molecular marker (1Kb Plus DNA Ladder); Lane 2-16, pAdTRACK-CMV-IGK-HA-rhTRAIL DHER clones; Lane 17, PCR reaction without DNA. Based on the migration rates, all the clones were positive for the presence of the gene.

Candidate clones were digested with several restrictions endonucleases to verify the presence of the gene (the reactions used for the digestions are described in Appendix A.2). As indicate in Figure 3.4, for the pADTRACK-CMV-IGK-HA-PDGF-sTRAIL the endonuclease ClaI cleaves in the sequence for the PDGF gene, and in the new plasmid this endonuclease cannot cleave. Consequently, this enzyme is used as a control for the presence of PDGF sequence. The endonuclease EcoRI cleaves in old plasmid after the sequence of the PDGF gene and between the left and right arm, whereas in the new plasmid it only cuts once between the left and right arm, so that a control reaction was done without the addition of endonucleases.

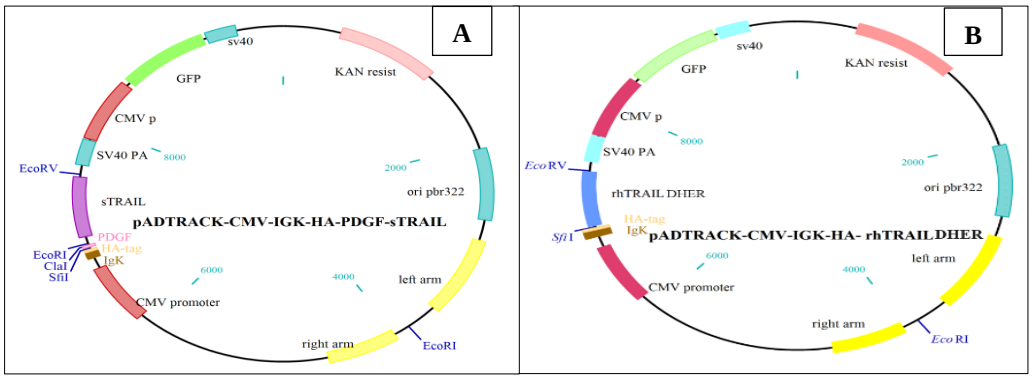

Figure 3.4 The plasmid constructed and the original shuttle plasmid. (A) Vector pADTRACK-CMV-IGK-HA-rhTRAIL DHER showing the restriction endonucleases cuts; (B) Vector pADTRACK-CMV-IGK-HA-PDGF-sTRAIL showing the restriction endonucleases cuts.

As shown in Figure 3.5.A, the expected restriction fragments were created in all the pADTRACK-CMV-IGK-HA-rhTRAIL DHER clones. In all the analyzed colonies: ClaI did not produce a cleaved fragment (Figure 3.5A, lane 2); the produced fraction is compared to the control where there is no cleaved DNA (Figure 3.5A,lane 5), in addition to the 9836bp fragment generated from pADTRACK-CMV-IGK-HA-PDGF-sTRAIL (Figure 3.5B, lane 2); the digestion of the ClaI and EcoRI endonucleases generated a 9664bp fragment in the new plasmid (Figure 3.5A, lane 3), while in the old plasmid were generated a 3005bp and a 6814bp fragments; the EcoRI endonuclease produced a 9664bp fragment in the new plasmid, while in the old plasmid a 3002bp and 6814bp fragments were generated. As revealed in Figure 3.5, the clones generated the expected fragments, even though the sizes of the fragments are not exactly the size projected in the simulation program (Clone Manager©), which may be due to the supercoiled form of DNA. In this form, the DNA is more condensed, consequently, the migration during electrophoresis will be larger. Furthermore, two colonies were sent to sequencing and proved the presence of the rhTRAIL DHER gene (Appendix A.1). These colonies will be mentioned in the following experiments as rhTRAIL DHER-A and rhTRAIL DHER-B.

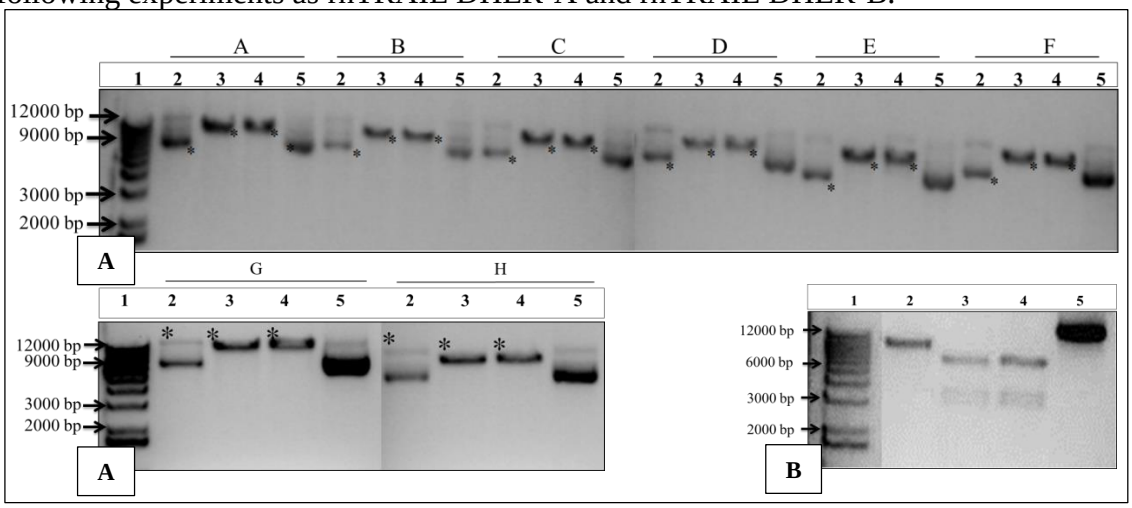

Figure 3.5 Restriction endonuclease digestions for the generated clones of pADTRACK-CMV-IGK-HA-rhTRAIL DHER. The clones isolated from the plate containing kanamycin were digested with endonucleases (ClaI and EcoRI), to confirm the presence of the gene for the rhTRAIL DHER. The clones were digested, and analysed by electrophoresis through a 1% agarose gel and SYBR® Safe DNA gel staining. A- Digestion of the clones (A-H); B- Digestion of the control pADTRACK-CMV-IGK-HA-PDGF-sTRAIL; Lane 1, molecular marker (1Kb Plus DNA Ladder); Lane 2, endonuclease restriction digestion in the constructed pADTRACK-CMV-IGK-HA-rhTRAIL DHER with ClaI; Lane 3, endonuclease restriction digestion in the constructed pADTRACK-CMV-IGK-HA-rhTRAIL DHER with ClaI and EcoRI; Lane 4, endonuclease restriction digestion in the constructed pADTRACK-CMV-IGK-HA-rhTRAIL DHER with EcoRI; Lane 5, pADTRACK-CMV-IGK-HA-rhTRAIL DHER without endonuclease restriction digestion. The signal * indicates the expected fragments for a positive evaluation of the constructed plasmid.

3.2. Expression and analysis of the protein in HEK293 cells

The constructed plasmid pADTRACK-CMV-IGK-HA-rhTRAIL DHER was confirmed, by sequencing, that contains the gene of interested. To verify that the plasmid is working properly and it is capable to expresses the protein, the DNA was transfected into HEK-293 cells, and used as positive control the functional vector pADTRACK-CMV-IGK-HA-rhTRAILWT, which is capable to express the protein rhTRAIL WT. The cells were prepared 24 hours before the transfection, and the same number of cells was added in each wells. The cells were transfected with 1µg of the pADTRACK-CMV-IGK-HA-rhTRAIL DHER, 1 µg of the DNA from the positive control and without DNA for the negative control, using FuGENE® HD Transfection reagent and incubated for 72 hours. After the transfection the cells were monitored by GFP expression. As shown in Figure 3.6. the transfected cells were visualized by fluorescence microscopy after 72 hours of incubation, all the plates were full of cells (lanes A, C, E, G), and the efficiency of the transfection was high when observed in fluorescent microscopy (lane B, D, F), except for the negative control, where there was no cell transfected, as expected. Both for the positive control (lanes E and F) or the constructed plasmid (A, B, C, D) the efficiency of the transfection was 80-90%; and comparing the positive control with the new plasmids, the number of transfected cells was similar. Evidently, numerous cells express the presence of the plasmid, which is confirmed by the manifestation of GFP. However, to confirm the production of the protein, it was necessary to screen by western blotting the protein rhTRAIL DHER (19KDa) and by application of the protein in a very sensitive cell line (SW948) [67].

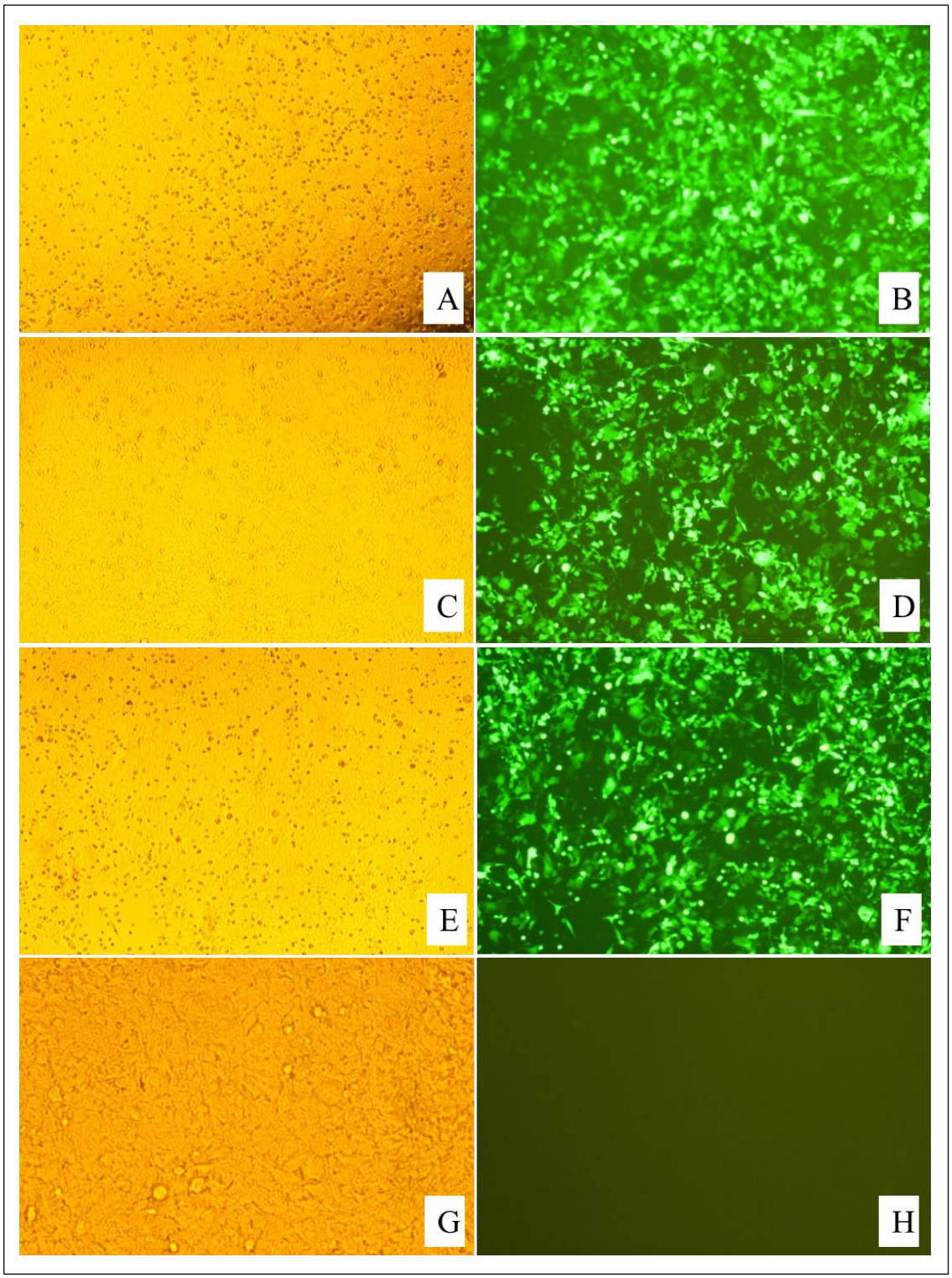

Figure 3.6 Transfected HEK-293 cells after 72 hours of incubation. The HEK cells were transfected with the pADTRACK-CMV-IGK-HA-rhTRAIL DHER, and incubated for 72 hours. After the incubation period, the cells were motorized through GFP expression. The amplification of the images was 12,5X. (A), (B): The plasmid pADTRACK-CMV-IGK-HA-rhTRAIL DHERA; (C),(D):The plasmid pADTRACK-CMV-IGK-HA-rhTRAIL DHERB; (E),(F): the positive control plasmid pADTRACK-CMV-IGK-HA-rhTRAIL WT; (G),(H): the negative control, cells without DNA.

The pADTRACK-CMV-IGK-HA-rhTRAIL DHER has a sequence for the immunoglobulin K light chain (IgK), a specific sequence responsible for the secretion of heterologous proteins [68], like rhTRAIL DHER. A sequence for the human influenza hemagglutinin (HA), specific for the detection and isolation of the protein, which does not interfere with the bioactivity of the protein [69]. Accordingly, the protein rhTRAIL DHER was screened with a western blotting, using as primary antibodies the Mouse IgG1 Anti-HA Tag antibody and Rabbit polyclonal Anti-TRAIL antibody. As shown in Figure 3.7, the presence of the protein rhTRAIL DHER was detected with the Mouse IgG1 Anti-HA antibody. The HA sequence is linked to the sequence of the protein, being possible to confirm the presence of the rhTRAIL DHER protein and it is the expected size (19kDa) comparing with the control (the protein rhTRAIL WT). Observing the Figure 3.6 the expression of GFP is similar either in the positive control or in the clones, but when observed the Figure 3.7 the size of the band for the positive control is what we expected (21kDa), but the intensity of the band for the supernatant in the Anti-HA antibody it is bigger, it means that the cells excreted more the rhTRAIL WT protein. Comparing the two clones of the rhTRAIL DHER, the second clone as higher production of protein that the first one, either in the cellular lysates or the supernatant.

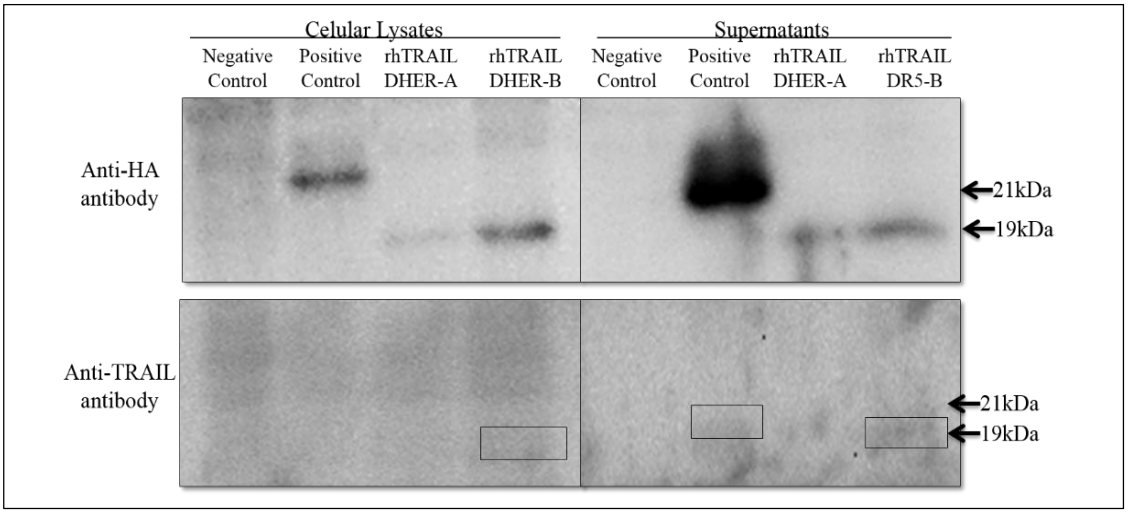

Figure 3.7 Screening of the rhTRAIL DHER protein through western blotting analysis. The supernatant and pellet collected from the transfection of HEK-293 cells with pADTRACK-CMV-IGK-HA-rhTRAIL DHER were added in a 12.5% SDS gel, and screened by western blotting using as primary antibodies the Mouse IgG1 Anti-HA Tag antibody (in a 1:1000 dilution) and Rabbit polyclonal Anti-TRAIL antibody (in a 1:1000 diluiton), and then incubated for 1 hour and 30min at RT.

The Rabbit polyclonal Anti-TRAIL antibody has a very low capacity detection of the protein, as indicate in Figure 3.7. The detection of the rhTRAIL DHER is visible in the second clone, both in the supernatant and in the cellular lysates, however the presence of the rhTRAIL WT (positive control) is only detected in the supernatant. A higher expression of the proteins in the supernatants was expected, since the plasmid has the sequence of IGK to secrete the protein outside the cytoplasm. Considering the positive control and the produced proteins, it can be concluded that the vector is capable to produce and express the proteins rhTRAIL DHER.

In order to confirm the activity of the protein the supernatant and pellet from the transfection was added in SW948 cells. The produced rhTRAIL WT was used as positive control, and as a negative control only cells were used, but the supernatant and the pellet of the negative control from transfection were added, since these are the control, to see the effect of the protein. As presented in Figure 3.8, the protein rhTRAIL DHER is active, the cell line SW948 is a very sensitive one for the rhTRAIL DHER protein, as described previously [67].It is clearly shown in Figure 3.8A a decrease of survival of SW948 cells for the rhTRAIL DHER-A and B, respectively, for 100µL (91.88% ± 8.43%; 102.23% ± 18.60%), 12.5µL (109.42% ± 2.14%; 115.30% ± 7.97%) and 6.25 µL (94.69% ± 10.85%; 95.40% ± 14.40%) of supernatant, relating with the survival of the negative control where no addition of protein (131.63% ± 7.44%).

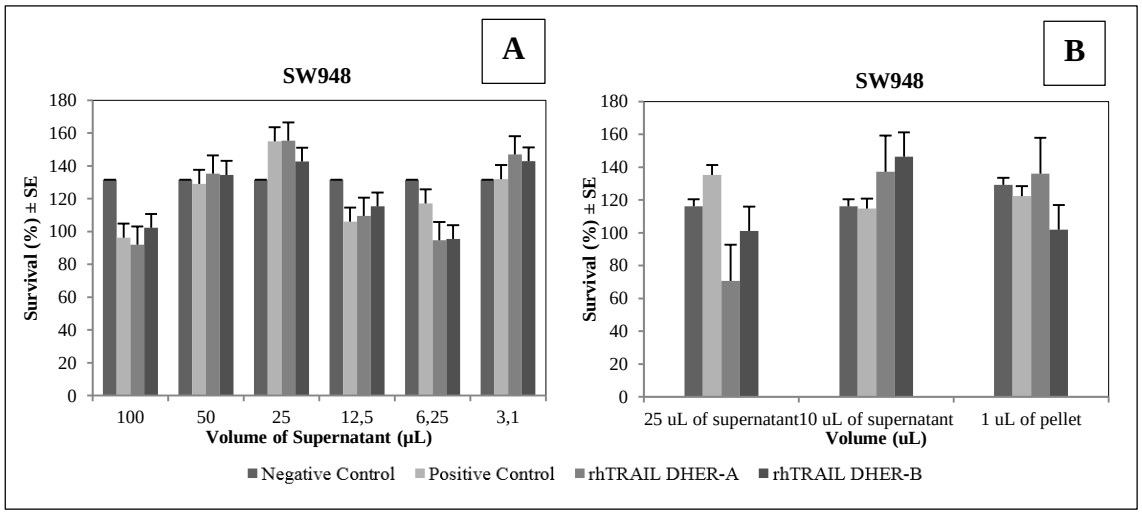

Figure 3.8 Analysis of the function of the rhTRAIL DHER in SW948. (A) Quantification of the supernatant (from the clones, the positive control and negative control) from the transfection; (B) Different amounts of supernatant and pellet (from the clones, positive control and negative control). The protein was produced in HEK-293 cells from the vector pADTRACK-CMV-IGK-HA-rhTRAIL DHER. The supernatant and the pellet from transfection were removed and added to the SW948 cells, a sensitive cell line in the presence of rhTRAIL DHER protein. The negative control contains cells with the supernatant and pellet from the transfection. The survival of the cells was measured 72 hours after the addition of the protein, using crystal violet technique, and the percentage of the survival of the cells was calculate using the equation (1), described in material and methods. The graphs showed the average of independent experiments and the error bars indicate the standard error of the mean. The statistical significance tests were performed with one-way ANOVA test and a Bonferroni post test was conducted for multiple comparisons; in this case there is no statistical significance between groups.

As demonstrated in Figure 3.8B, a decrease in the survival of the cells occurs with 25µL of supernatant for the rhTRAIL DHER-A and B (70.78% ± 6.8%; 101.11% ± 25.29%), related with the 116.16% ± 35.04% of the control established with cells only (negative control). However, for the 10 µL of supernatant the rhTRAIL DHER-A and B, did not promote the apoptosis of the cells (137.25% ± 8.47%; 146.36% ± 13.49%). It is evident that there is a decrease in the survival of the cells with 1µL of the protein rhTRAIL DHER-B from the pellet (101.99% ± 44.54%). However, for rhTRAIL DHER-A protein there is no evidence for the function of the protein for 1 µL of pellet.

It can be concluded that the protein rhTRAIL DHER (in both clones) are functional and that the cells are more sensitive for the rhTRAIL DHER than the rhTRAIL WT (positive control), as described before [67].

3.3. Generation of adenovirus plasmid by homologous recombination in bacterial cells

The overall scheme developed to create the recombination is diagrammed in Figure 3.9. The bacteria *E.Coli* BJ5183 transformed with pAdEasy-1 were used to mediate the recombination with the plasmid pADTRACK-CMV-IGK-HA-rhTRAIL DHER. After transformation, the bacteria were prepared to be electrocompetent.

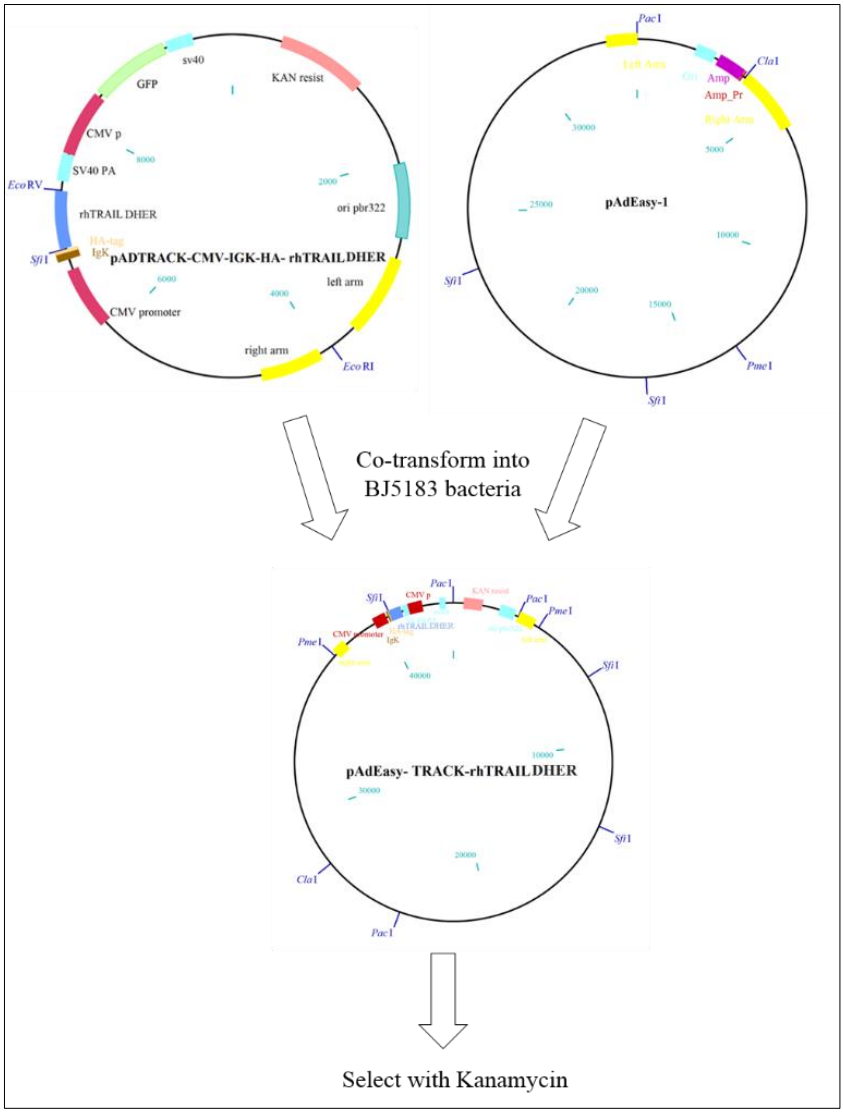

Figure 3.9 Recombination of pADTRACK-CMV-IGK-HA-rhTRAIL DHER into pAdEasy-1. The gene of interest was cloned in the shuttle vector (pADTRACK-CMV) and linearized with restriction endonuclease PmeI. The linearized plasmid was cotransformed into BJ5183 containing the pAdEasy-1 vector and selected by kanamycin. The size of the recombinant plasmid is 43114bp.

The adenoviral backbone vectors (pAdEasy-1) hold the sequence for Ad5 (human adenovirus), except nucleotides 1-3533 (including the E1 genes) and nucleotides 28130-30820 (including the E3 genes) [62]. The pADTRACK-CMV vector contains a polylinker site for insertion of the gene rhTRAIL DHER using the GFP as a marker to see the expression of the plasmid when transfected. The polylinker site is enclosed by adenoviral sequences ("arms") that permit the homologous recombination with the adenoviral backbone vector. The left arm encloses the Ad5 nucleotides (34931-35935), which facilitate the recombination with the pAdEasy vector in BJ5183, the inverted terminal repeat (ITR) and packaging signal sequences (1-480 of Ad5), essential for viral production in mammalian cells. The right arm holds the Ad5 nucleotides 3534-5790, which facilitate the recombination in the pAdEasy-1 vector. Both arms have the restriction endonuclease site for PacI (Figure 3.9).

The constructed vectors pADTRACK-CMV-IGK-HA-rhTRAIL DHER-A and B were cleaved with a restriction endonuclease, PmeI, to linearize the vectors. This endonuclease cleaves the vector between the right and left arm (Figure 3.9) to allow the ligation with the right and left arm of the adenoviral vector, and to generate a 9664bp fragment. As shown in Figure 3.10, the vector was digested (lane 2, 4) comparing with the no digested DNA (lane 3, 5), because the migration of DNA is larger in the digested DNA, which is due to the supercoiled form of the vector. The linearized clones were purified and transfected to electrocompetent *E.Coli* BJ5183 bacteria containing the pAdEasy-1 vector. The recombinants were carefully chosen with kanamycin and the small colonies were picked. Due to the 43114bp of the recombinant plasmid, the colonies of bacteria are small, since they have higher concentrations of DNA to replicate. In order to see the competence of the bacteria, a small volume of the recombinant bacteria was added in a plate with ampicillin. The competence of the bacteria were proved, because in the plates with ampicillin there were a lot of small colonies, indicating the efficiency of the bacteria (see Appendix A.3). Afterwards, the recombinants were screened with restriction endonuclease digestion (PacI, SfiI, ClaI), using as control the vectors pADTRACK-CMV-IGK-HA-rhTRAIL DHER and pAdEasy-1, and analysed by electrophoresis through a 0.8% agarose gel.

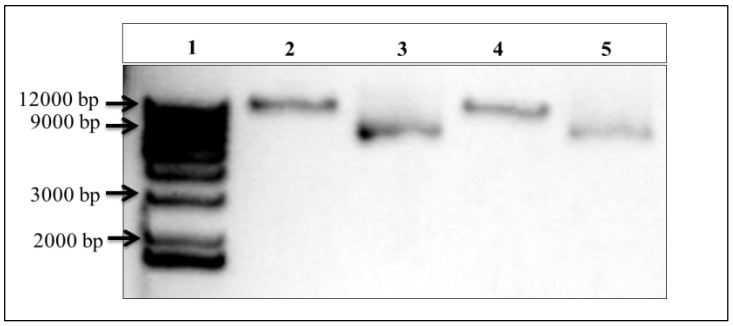

Figure 3.10 Linearization of shuttle vector with PmeI restriction endonuclease. The pADTRACK-CMV-IGK-HA-rhTRAIL DHER was linearized by a restriction endonuclease PmeI that cuts between the right arm and left arm of the shuttle vector. Then was purified and transfected into *E.Coli BJ5183* bacteria. The digestion was analyzed by electrophoresis through 1% agarose gel and SYBR® Safe DNA gel staining. Lane 1, molecular marker (1Kb Plus DNA Ladder); Lane 2, pADTRACK-CMV_IGK-HA-rhTRAIL DR-A linearized; Lane 3, pADTRACK-CMV_IGK-HA-rhTRAIL DR-A control (without endonuclease digestion); Lane 4, pADTRACK-CMV_IGK-HA-rhTRAIL DR-B linearized; Lane 5, pADTRACK-CMV_IGK-HA-rhTRAIL DR-B Control (without endonuclease digestion).

The restriction endonuclease PacI creates 2897bp, 19177bp and 21040 bp fragments in the recombinant, compared with controls that generate 2897bp and 6767bp fragments from the shuttle vector, and 33450bp fragment from the adenoviral vector. The restriction endonuclease SfiI generated three fragments (6710bp, 9721bp, 26683bp) in the recombinant, instead the 9664bp from the shuttle vector and two fragments (6710bp, 26740bp) from the pAdEasy-1 vector. The combination of the endonuclease PacI with ClaI produced four fragments in the recombinants (2897bp, 3675bp, 15502bp, 21040bp) comparing with only 2897bp and 6767bp from the shuttle vector (because the endonuclease ClaI does not cut in this vector), and two fragments (3675bp, 29775bp) from the adenoviral vector. As indicated in Figure 3.11, the expected fragments were not generated. For the digestion with PacI endonuclease (Figure 3.11C lane 1) were generated four fragments, instead of the expected three fragments, comparing with the Figure 3.11C lane 4 (DNA whitout endonuclease digestion) that appears two bands, due to the different forms acquired by DNA. It is possible to assume that two bands of recombinant clones may correspond to undigested DNA, and the other two bands to digested DNA (near to the 2027bp band and between the 21226bp-5148bp), so there is similarity with the expected bands for the pADTRACK-CMV-IGK-HA-rhTRAL DHER. For the digestion with SfiI endonuclease (Figure 3.11C lane 2) one fragment was generated instead of the

expected three fragments. Once more, it is possible to assume some similarity with the pADTRACK-CMV-IGK-HA-rhTRAL DHER, because it only expected one band. For the digestion with PacI and ClaI endonucleases (Figure 3.11C lane 3) two fragments were generated, instead of the expected four fragments. Once again, it is possible to compare them with the two expected bands for the shuttle vector. Considering the Figure 3.11 A and B, the pADTRACK-CMV-IGK-HA-rhTRAIL DHER and pAdEasy-1, respectively, the fragments obtained are what we expect. However, in the lane 2 and 4 of Figure 3.10A, instead of two fragments, there appear three, which is due to no undigested DNA, in contrast with the lane 5 (no endonuclease digestion).

This analysis of the recombinants through endonuclease digestions leads to the conclusion that no recombination occurred between the pADTRACK-CMV-IGK-HA-rhTRAIL DHER and pAdEasy-1 vector, which indicates that the vector present in the *E.Coli* BJ5183 can be the pADTRACK-CMV-IGK-HA-rhTRAIL DHER.

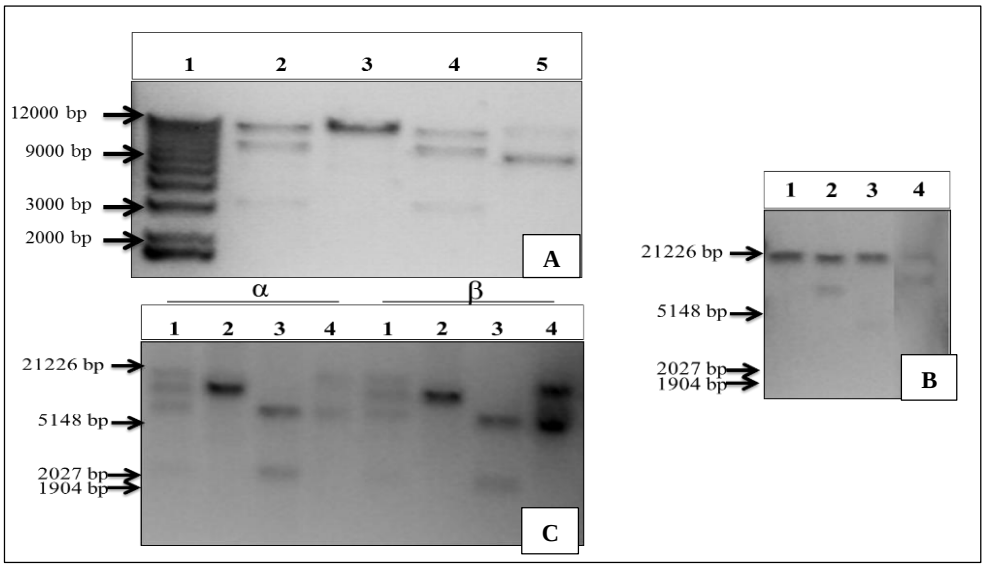

Figure 3.11 Endonuclease digestions of the recombinant plasmid and the controls. (A) DNA from the restriction endonuclease digestions in pADTRACK-CMV-IGK-HA-rhTRAIL DHER; Lane 1, molecular marker (1Kb Plus DNA Ladder); Lane 2, Endonuclease digestion with PacI; Lane 3, Endonuclease digestion with SfiI; Lane 4, Endonuclease digestion with PacI+ClaI; Lane 5, vector without a endonuclease digestion. (B) DNA from the restriction endonuclease digestions in pAdEasy-1, the molecular marker used was Lambda DNA/EcoRI+HindIII Marker 3; Lane 1, Endonuclease digestion with PacI; Lane 2, Endonuclease digestion with SfiI; Lane 3, Endonuclease digestion with PacI+ClaI; Lane 4, vector without a endonuclease digestion. (C) DNA from the restriction endonuclease digestions of the recombinant clones (α and β), the molecular marker used was Lambda DNA/EcoRI+HindIII Marker 3; Lane 1, Endonuclease digestion with PacI; Lane 2, Endonuclease digestion with SfiI; Lane 3, Endonuclease digestion with PacI+ClaI; Lane 4, vector without a endonuclease digestion. All the endonuclease digestions was analysed by electrophoresis through a 0.8% agarose gel and SYBR® Safe DNA gel staining.

3.4. Evaluation of the activity of rhTRAIL DHER in LX2 cells and HepG2 cells.

In order to verify that the protein has the desired function in fibrotic liver cells, different concentrations of rhTRAIL DHER protein were added in LX2 cells, using as control the rhTRAIL 4C7 and rhTRAIL WT, these protein was produced in *E.Coli* bacteria as described in material and methods. These controls were used to see the sensibility for the fibrotic cells to the rhTRAIL DHER, because fibrotic cells express almost 10 times more the receptor for the protein rhTRAIL DHER in their surface than the receptor for the protein rhTRAIL 4C7 [64,73]. The LX2 cells were plated in a 96 well/plate and allowed to grow during 72 hours. Afterwards, the cultured medium was changed and new medium was added, left for incubation for more 72 hours. This period of incubation (a total of 6 days) is very important for the activation of this type of cells. Even though the LX2 cells had some morphologic features of early activated stellate cells when plated, from day 6 of incubation the cells start expressing more morphologic features, like an increase in the production of α- SMA (Smooth muscle actin), a marker of the activation of hepatic stellate cells [70]. To further characterize the function of the protein rhTRAIL DHER in the activated hepatic stellate cells, different concentrations of the proteins were added, using as control the rhTRAIL 4C7 and WT. As shown in Figure 3.12A, the LX2 cells are sensitive to the different proteins of rhTRAIL. Interpreting the action of different concentrations of proteins in the LX2 cells, for 7.5ng/mL, 35ng/mL and 306ng/mL, the effect of the proteins is higher than for the other concentrations. For 100% of cells without the addition of the proteins, in the 7.5ng/mL the rhTRAIL WT as a higher effect (75.57% ± 1.15%) than the other proteins; in the 35ng/mL, the effect of rhTRAIL DHER (91.70% ± 3.15%) and 4C7 (86.32% ±8.75%) is higher than for the rhTRAIL WT; and in the 306 ng/mL all the proteins as effect in the cells (DHER: 86.07% ± 6.15%; 4C7: 94.69% ± 0.5%; WT: 87.36% ± 2.7%).

Taken together, these results show, for one hand, a low sensibility of the LX2 cells to the rhTRAIL DHER protein. On the other hand, they show a comparable sensibility

for all the rhTRAIL proteins. The insensibility of the cells to the rhTRAIL DHER may be due to a weak activation of the cells. Previous studies have shown that the LX2 cells begin to be more activated at the 6th day, but at the 14th day they will be much more activated and have more sensibility for the rhTRAIL DHER, due to a higher expression of the receptor TRAIL DR5 in the surface of cells and to associated factors of activation of HSC [70].

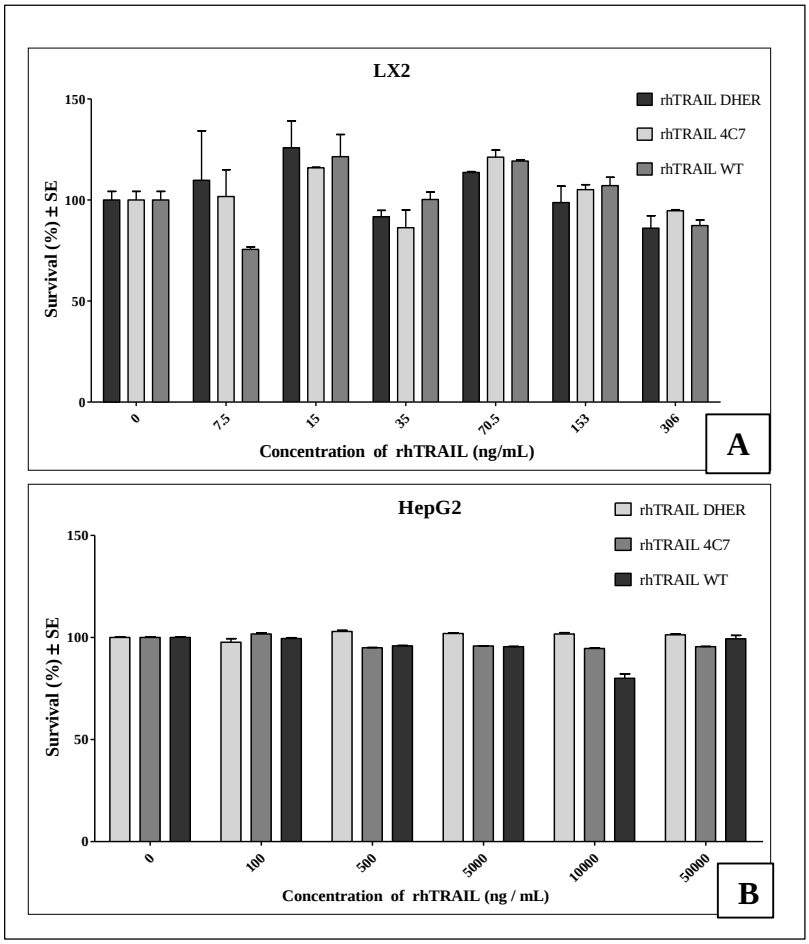

Figure 3.12 RhTRAIL DHER, 4C7 and WT combined in LX2 cells and HepG2 cells. (A)- The cells LX2 were plated in a 96well/plate (1000 cells/well), allowed to grow for 72 hours, afterwards the culture medium was changed, and more new complete DMEM was add, incubating more 72 hours. Then, the rhTRAIL DHER, 4C7 and WT were added in different concentrations, and a MTS assay performed to see the survival of cells. (B) The cells HepG2 was plated in a 96 well/plate (10000cells/well), and allowed to adhere overnight. Then different concentrations of rhTRAIL DHER, 4C7 and WT were added to the cells, and then incubated for 72 hours. After the incubation, a MTS assay was performed to assess the survival of the cells. The graphs showed the average of independent experiments and the error bars indicate the standard error of the mean. The statistical significance tests were performed with the one-way ANOVA test and a Bonferroni post-test for multiple comparisons; in this case there is no statistical significance between groups.

In relation to HepG2 cells this cell line was used to determine the effect of the rhTRAIL proteins in the survival of the cells. The effect of the proteins was expected to be the lowest possible or even not to promote any effect at all the cells. The HepG2 cells were cultured in a 96 well/plate and allowed to adhere overnight. After 24 hours, different concentrations of proteins were added, and the cells were left for incubation for 72 hours. After the incubation, a MTS assay was performed to assess the survival of the cells in those different concentrations. As shown in Figure 3.12B, the effect of the proteins is greatly reduced in cells, despite the concentrations of the rhTRAIL proteins being very high. These concentrations should have promoted some effect in the cells, but that did not occur. Comparing the effects of the rhTRAIL variants in LX2 and HepG2, it is visible the effect of rhTRAIL variants in the LX2 cells, although in the HepG2 cells was not visible, either with higher concentrations. As we expected the rhTRAIL DHER have some effect on the LX2 cells and a reduced effect in the HepG2, however the effect in fibrotic cells was very weak.

3.5. Evaluation of the effects with the combination of rhTRAIL variants with HDACs and HATs inhibitors

TRAIL induces apoptosis by binding to DRs receptors in the surface of the cells, which induces the activation of the caspase cascade [71]. HDAC inhibitors (like SAHA and Entinostat/MS-275) synergize with TRAIL in inducing apoptosis [71] and we hypothesize that HATs inhibitors (like C646 and MG149) promote the protection of the cells against the ligation of the rhTRAIL to the receptors, thus avoiding the apoptosis.

3.5.1. Combination of SAHA, Entinostat, MG149 and C646 with rhTRAIL (DHER, 4C7 and WT) induces apoptosis in SW948

We investigate the combination of SAHA/rhTRAIL (DHER, 4C7 and WT), Entinostat/rhTRAIL (DHER, 4C7 and WT), C646/rhTRAIL (DHER, 4C7 and WT) and MG149/rhTRAIL (DHER, 4C7 and WT) in SW948 cells, since this colon carcinoma cell line is very sensitive for the rhTRAIL (DHER, 4C7 and WT) [70, 75]. The SW948 cells were plated in a 96 well/plate and allowed to adhere overnight. Then, different concentrations of SAHA, Entinostat, MG149 and C646 combined with rhTRAIL DHER, 4C7 and WT were added to the cells, and the cells were incubated for 72 hours with 5% CO_2. In a second assay, the rhTRAIL DHER, 4C7 and WT were also added but only after 24 hours of HDACS and HATs incubation, and the incubation continued for another 48 hours. The drugs (SAHA, Entinostat, C646 and MG149) and the proteins (rhTRAIL DHER, 4C7 and WT) were added alone as controls, to assess the effect of combination, and to detect the survival of the cells a crystal violet assay was performed.

The colon carcinoma cell line (SW948) is very sensitive to the rhTRAIL (DHER, 4C7 and WT). However, the expression of the receptor rhTRAIL DR4is higher in the surface [70, 75]. As shown in Figures 3.13, 3.14 and 3.15 the cells are very sensitive for all rhTRAIL but for 100ng/mL of rhTRAIL 4C7 during 72 hours (Figure 3.14) the survival of the cells is lower (4.26% ± 1.50%) comparing with the cells only with medium (100%). However, for 48 hours, the rhTRAIL DHER (Figure 3.13) has a lower rate of survival (71.04% ± 1.56%) when compared with the rhTRAIL 4C7 (87.45% ± 6.49%) (Figure 3.14), using as control 100% of survival (0ng/mL of rhTRAIL). The combination of SAHA (1µM and 5 µM) and Entinostat (0.01µM and 0.05µM) with the rhTRAIL DHER (Figure 3.13A and B) demonstrated a combination effect either for 72 hours or 48 hours. The survival of the cells decreases when the combination occurs, for example, for 72 hours with 10ng/mL of rhTRAIL DHER combined with 1µM of SAHA, the survival

of the cells is 9.93% ± 4.17% against 21.80% ± 6.09% only 10ng/mL of rhTRAIL DHER and 56.96% ± 3.72% only 1µM of SAHA.

The combination of C646 with rhTRAIL DHER (Figure 3.13C) demonstrated a synergistic effect (for 72 hours and 48 hours) of rhTRAIL DHER, while the combination of MG149 shows a protection effect when the rhTRAIL DHER was added for only 48 hours (Figure 3.13D). However, for 72 hours of rhTRAIL a combination effect was revealed.

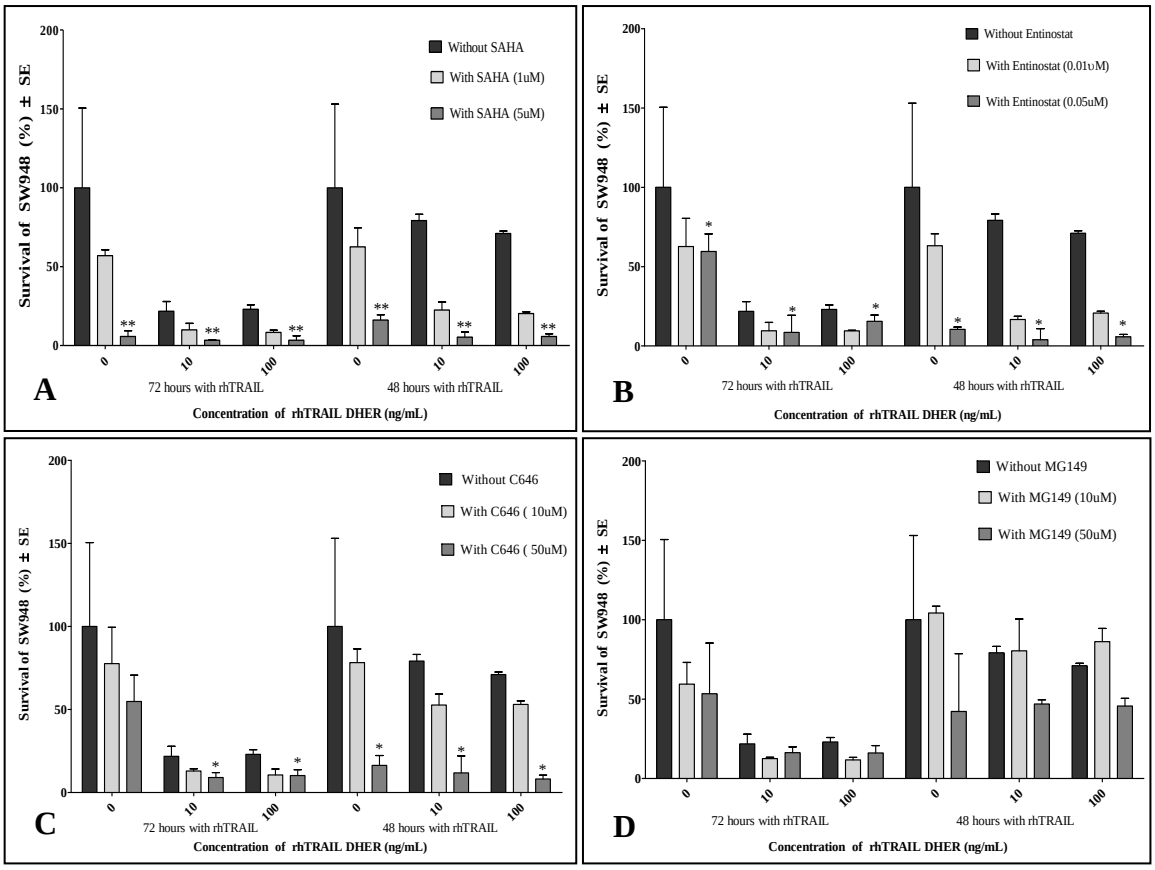

Figure 3.13 Combination of SAHA, Entinostat, MG149 and C646 with rhTRAIL DHER in SW948 cells induces apoptosis. The cells SW948 were incubated for 72 hours with rhTRAIL DHER, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL DHER, (left part of the graphics). On the right part of the graphics, the cells SW948 were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL DHER was added alone, and combined with the inhibitors. Accordingly, the incubation time of HDACs and HATs inhibitors in both parts of the graphics is 72 hours, but for rhTRAIL DHER the incubation period in the left part of the graphic is 72 hours, but in the right is 48hours. (A) SW948 cells with rhTRAIL DHER combined with SAHA; (B) SW948 cells with rhTRAIL DHER combined with Entinostat; (C) SW948 cells with rhTRAIL DHER combined with C646; (D) SW948 cells with rhTRAIL DHER combined with MG149. The graphs showed the average of independent experiments and the error bars indicate the standard error of the mean. *P<0.05, **P<0.01 versus control without treatment (0ng/mL, 10ng/mL and 100 ng/mL concentrations of rhTRAIL DHER).

Upon treatment with rhTRAIL 4C7 combined with SAHA and Entinostat (Figure 3.14 A and B), the synergistic effect was demonstrated. Furthermore, the effect of the combination C646/rhTRAIL 4C7 synergizes the induction of apoptosis. And MG149/rhTRAIL 4C7 shows an effect of combination in 72hours, and a low combination effect in 48hours.The effect of MG149 with 10ng/mL of rhTRAIL 4C7 in 48 hours is higher, the cells survive 89.47% ± 3.76%, against the 80.39% ± 20.03% of MG149 combined with rhTRAIL DHER in 48 hours (Figure 3.13D).

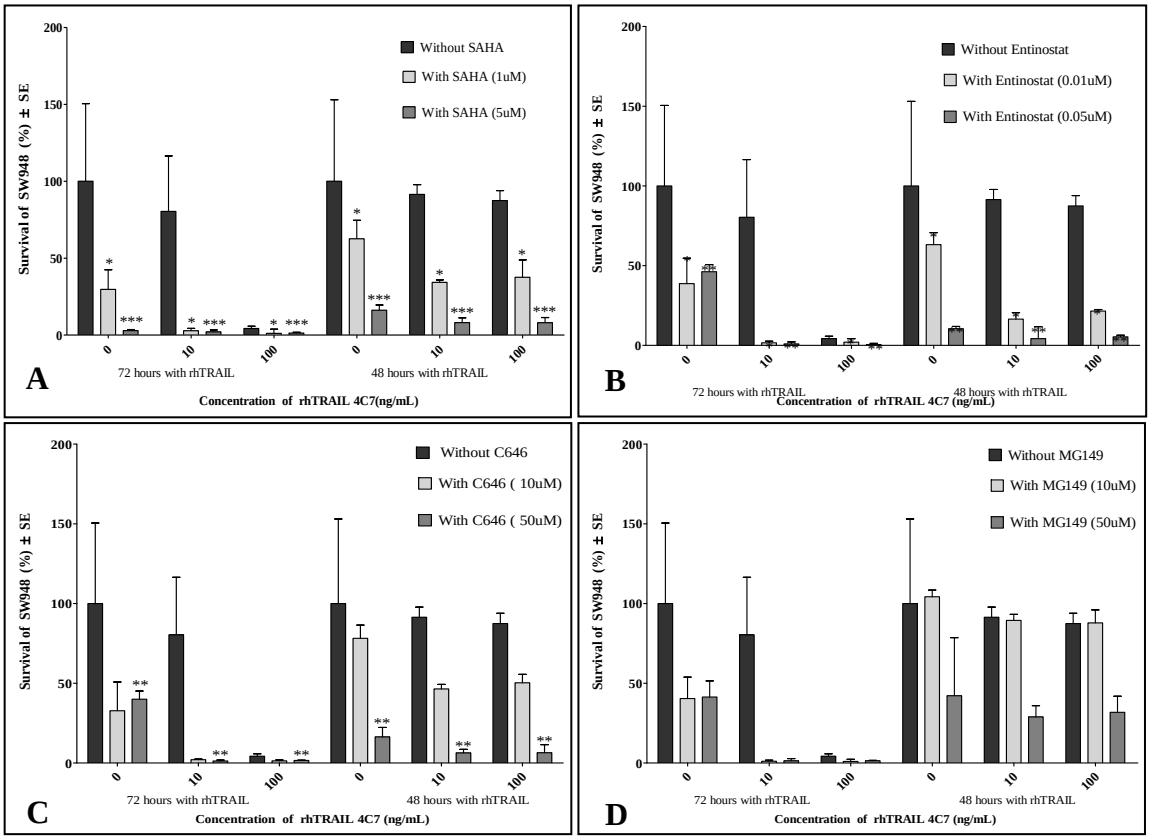

Figure 3.14 rhTRAIL 4C7 combined with SAHA, Entinostat, MG149 and C646 induces apoptosis in SW948. (A) SW948 cells with rhTRAIL 4C7 combined with SAHA; (B) SW948 cells with rhTRAIL 4C7 combined with Entinostat; (C) SW948 cells with rhTRAIL 4C7 combined with C646; (D) SW948 cells with rhTRAIL 4C7 combined with MG149. The graphs show the average of independent experiments and the error bars indicate the standard error of the mean. *P<0.05, **P<0.01, ***P<0.01 versus control without treatment (0ng/mL concentration of rhTRAIL 4C7). The cells were incubated for 72 hours with rhTRAIL 4C7, SAHA, Entinostat, MG149 and C646 alone, and all the inhibitors combined with rhTRAIL 4C7, (left part of the graphics); On the right part of the graphics, the SW948 cells were incubated with SAHA, Entinostat, MG149 and C646, and, after 24 hours, the rhTRAIL 4C7 was added alone and combined with the inhibitors. Accordingly, the incubation time of HDACs and HATs inhibitors in both parts of the graphics is 72 hours, but the rhTRAIL 4C7 the incubation period in the left part of the graphic is 72 hours, while in the right it is 48 hours. A crystal violet assay was done to detect the survival of the cells.

As demonstrated for rhTRAIL DHER and 4C7, the interaction of the combination SAHA/rhTRAIL WT, Entinostat/rhTRAIL WT, C646/rhTRAIL WT and MG149/rhTRAIL WT (Figure 3.15) is similar as described for the other rhTRAIL. The HDACS inhibitors promote higher a synergistic effect of apoptosis when combined with rhTRAIL WT. C646 also promotes synergetic apoptosis. However, the MG149 have a synergistic apoptosis with 72hours, but a not significant effect for 48 hours.

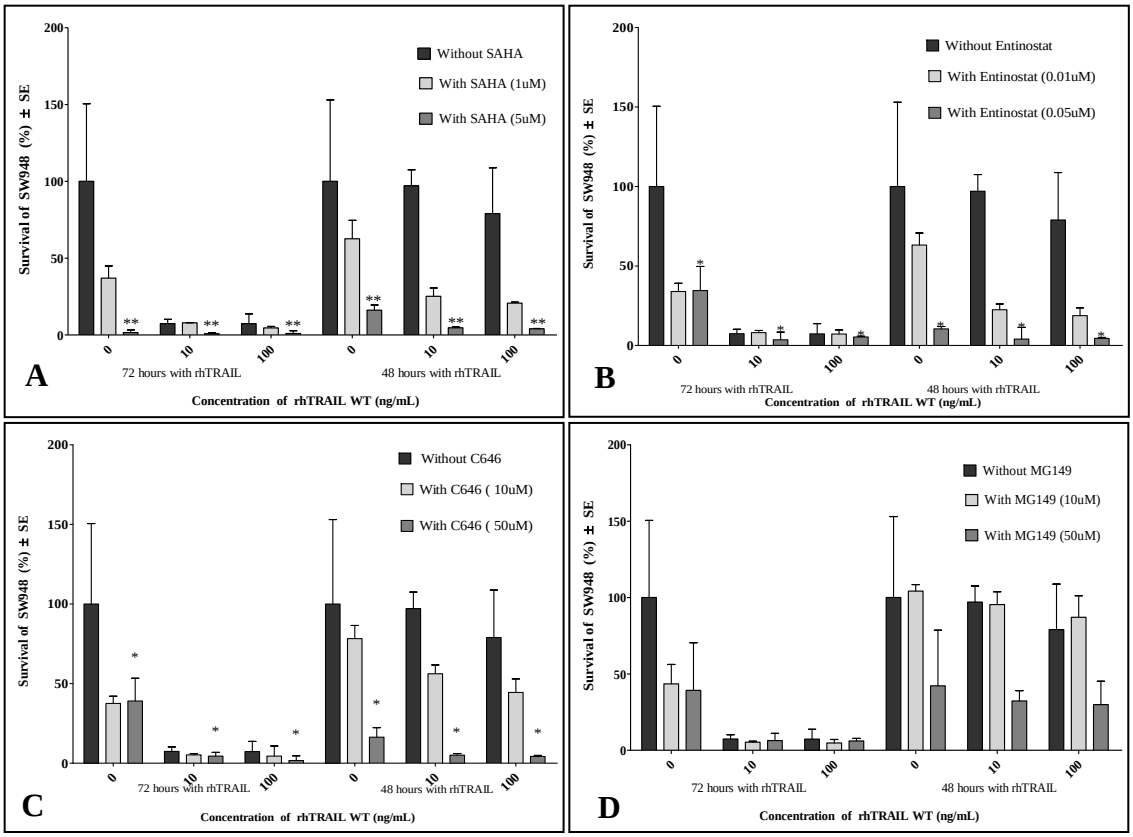

Figure 3.15 SAHA, Entinostat, MG149 and C646 combined with rhTRAIL WT induces in SW948. The SW948 cells were incubated for 72 hours with rhTRAIL WT, SAHA, Entinostat, MG149 and C646 alone, and all the inhibitors combined with rhTRAIL WT (left part of the graphics). On the right part of the graphics, the cells SW948 were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL WT was added alone, and combined with the inhibitors. A crystal violet assay was performed to determine the survival of the cells. (A) SW948 cells with rhTRAIL WT combined with SAHA; (B) SW948 cells with rhTRAIL WT combined with Entinostat; (C) SW948 cells with rhTRAIL WT combined with C646; (D) SW948 cells with rhTRAIL WT combined with MG149. The graphs show the average of independent experiments and the error bars indicate the standard error of the mean. *P<0.05, **P<0.01, versus control without treatment (0ng/mL concentration of rhTRAIL 4C7).

Moreover, these results demonstrate that the treatment for 72 hours with rhTRAIL (DHER, 4C7 and WT) promotes almost 80% apoptosis in the SW948 cells, and the concentration 100ng/mL of the rhTRAIL DHER, 4C7 and WT is more effective in apoptosis. The sensibility of the SW948 to the rhTRAIL 4C7 was confirmed as described before [70,75]. Due to this sensitivity, the combination with HDACs (SAHA and Entinostat) and the HATs (C646 and MG149) promotes a greater interaction, so the survival of the cells decreases. However, results demonstrated that the 10 µM MG149 do not promote any combination effect, so we suggest that is capable to protect the cells, when the addition of rhTRAIL (DHER, 4C7 and WT) is performed after 24 hours of HAT incubation. The results from all the experiments are described in Appendix A.5.

3.5.2. SAHA with rhTRAIL (DHER, 4C7 and WT) promote apoptosis while Entinostat, C646 and MG149 with rhTRAIL (DHER, 4C7 and WT) have no combination effect in H460 cells

We want to demonstrate that the HATs inhibitors combined with rhTRAIL (DHER, 4C7 or WT) can protect the cell against the binding of the protein to the receptor in the surface of the cells. The lung carcinoma cells (H460) can be used to see the effect of the rhTRAIL DHER, 4C7 and WT both alone, and when combined with the inhibitors (SAHA, Entinostat, C646 and MG149), allowing to prove the synergy effect of HDACs and the protective effect of HATs. The H460 cells were cultured in a 96 well/plate and allowed to adhere overnight. After 24 hours, different concentrations of SAHA, Entinostat, C646 and MG149 were combined with different concentrations of rhTRAIL (DHER, 4C7 and WT), and the drugs alone were used as control. The cells were incubated for 72 hours in a 5% of CO_2. The same assay was repeated, but the addition of the rhTRAIL (DHER, 4C7 and WT) was done after 24 hours of HDACs and HATs incubation, and the combination was incubated for another 48 hours. A crystal violet assay was performed to determine the survival of the cells. As shown in Figure 3.17, the

H460 are a very sensitive cell line for the rhTRAIL 4C7. For 72 hours with 10ng/mL of the rhTRAIL 4C7, the survival of the cells is 13.17% ± 1.63%, against 67.52% ± 1.10% of rhTRAIL DHER (Figure 3.16) and 61.16% ± 10.66% of rhTRAIL WT (Figure 3.18), comparing with 100% survival of cells without treatment. This sensibility is verified for 72 hours and 48 hours of rhTRAIL 4C7.

As indicate in Figure 3.16, the combination of SAHA/rhTRAIL DHER promotes apoptosis in a synergistic way. However, the combination of Entinostat/rhTRAIL DHER develops a very poor synergistic effect in apoptosis, and it can be considered as a protection of the Entinostat, against rhTRAIL DHER. For 10ng/mL of rhTRAIL DHER incubated during 72 hours, the survival of the cells is 67.52% ± 1.10% and the combination with 0.01µM of Entinostat the survival is 74.66% ± 2.68%. Although, for the rhTRAIL DHER incubated for 48hours, the effect of the combination is synergistic. The same reaction profile happened with the C646 inhibitor. In the combination of MG149 with rhTRAIL DHER (Figure 3.16 D), for 10ng/mL of rhTRAIL DHER incubated for 72 hours with 10µM of MG149, the survival of the cells is 77.79% ± 14.25%, against 67.52% ± 1.10% for only 10ng/mL rhTRAIL DHER, although, for 48 hours of rhTRAIL DHER, there is some protective effect but this is less than when the incubation carried out for 72 hours.

Concerning to the rhTRAIL 4C7 (Figure 3.17), the combination with SAHA promotes apoptosis in a synergistic way and the same effect occurs with the combination with C646. Though the combination Entinostat/rhTRAIL 4C7 has protection effect when the rhTRAIL 4C7 was added after 24 hours of Entinostat (Figure 3.17B), for 10ng/mL rhTRAIL 4C7 with 0.01µM of Entinostat, the survival of the cells is 16.66% ± 9.44%, against only 10ng/mL of rhTRAIL 4C7 it is 14.65% ± 6.65%. However, the protection effect is very slight, and for 72 hours the effect is the same. Regarding the combination MG149/rhTRAIL 4C7 (Figure 3.17D), the effect is similar to the one that happened with the combination of Entinostat.

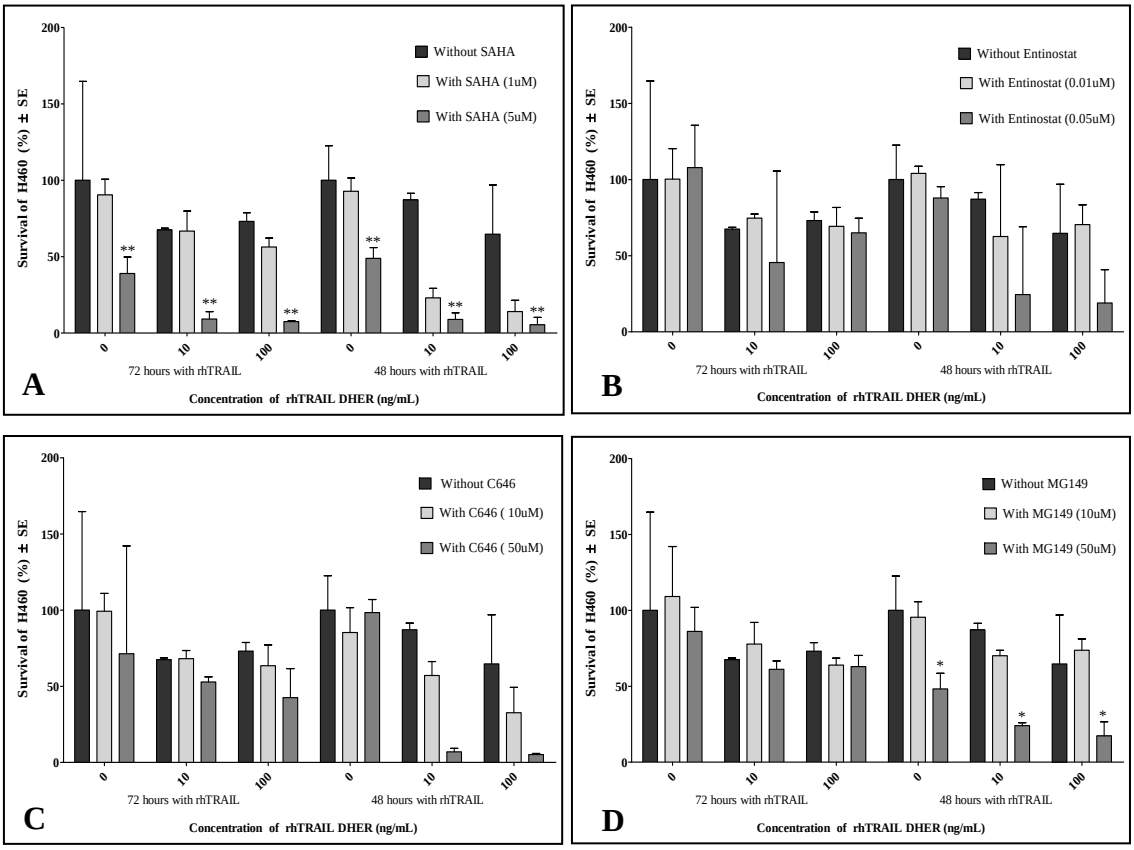

Figure 3.16 Combination of SAHA, Entinostat, C646 and MG149 and rhTRAIL DHER increase the apoptosis in H460. (A) H460 cells with rhTRAIL DHER combined with SAHA; (B) H460 cells with rhTRAIL DHER combined with Entinostat; (C) H460 cells with rhTRAIL DHER combined with C646; (D) SW948 cells with rhTRAIL DHER combined with MG149. The graphs show the average of independent experiments and the error bars indicate the standard error of the mean. *P<0.05, **P<0.01 versus control without treatment (0ng/mL concentration of rhTRAIL DHER). The H460 cells were incubated for 72hours with rhTRAIL DHER, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL DHER (left part of the graphics). On the right part of the graphics, the cells H460 were incubated with SAHA, Entinostat, MG149 and C646, and after 24hours the rhTRAIL DHER was added alone, and combined with the inhibitors. Accordingly, the incubation time of HDACs and HATs inhibitors in both parts of the graphics is 72 hours, but rhTRAIL DHER the incubation period in the left part of the graphic is 72 hours, while the in the right is 48 hours. To determine the survival of the cells, a crystal violet assay was done.

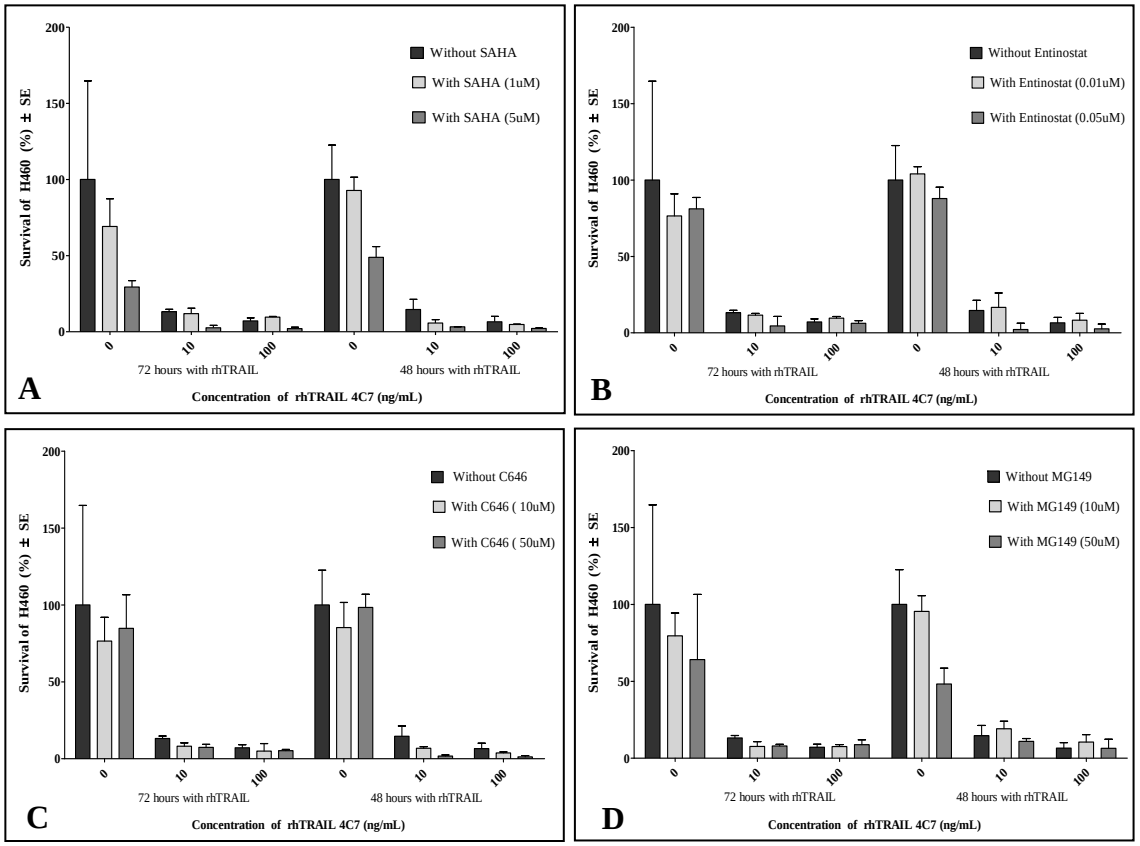

Figure 3.17 Combination of SAHA, Entinostat, C646 and MG149 with rhTRAIL 4C7 in H460 promote a higher apoptosis. The cells H60 were incubated for 72 hours with rhTRAIL 4C7, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL 4C7, (left part of the graphics). On the right part of the graphics, the cells H460 were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL 4C7 was added alone, and combined with the inhibitors. After incubation a crystal violet assay was done to determine the survival of the cells.(A) H460 cells with rhTRAIL 4C7 combined with SAHA; (B) H460 cells with rhTRAIL 4C7 combined with Entinostat; (C) H460 cells with rhTRAIL 4C7 combined with C646; (D) H460 cells with rhTRAIL 4C7 combined with MG149. The graphs showed the average of independent experiments and the error bars indicate the standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 versus control without treatment (0ng/mL concentration of rhTRAIL 4C7).

As regards to the rhTRAIL WT the H460 is not very sensitive for this TRAIL (Figure 3.18). However, the combination effect of SAHA/rhTRAIL WT decreases the survival of the cells to 40% in a synergistic manner. Still, looking for the effect of Entinostat and C646 combined with rhTRAIL WT (Figure 3.18B and C), do not occur a combination effect, suggesting a protection effect. Moreover, the MG149 combined with rhTRAIL WT provide protection to the cells (Figure 3.18D), because the cells were affected with the rhTRAIL WT, but when the combination occur did not happen an extra effect.

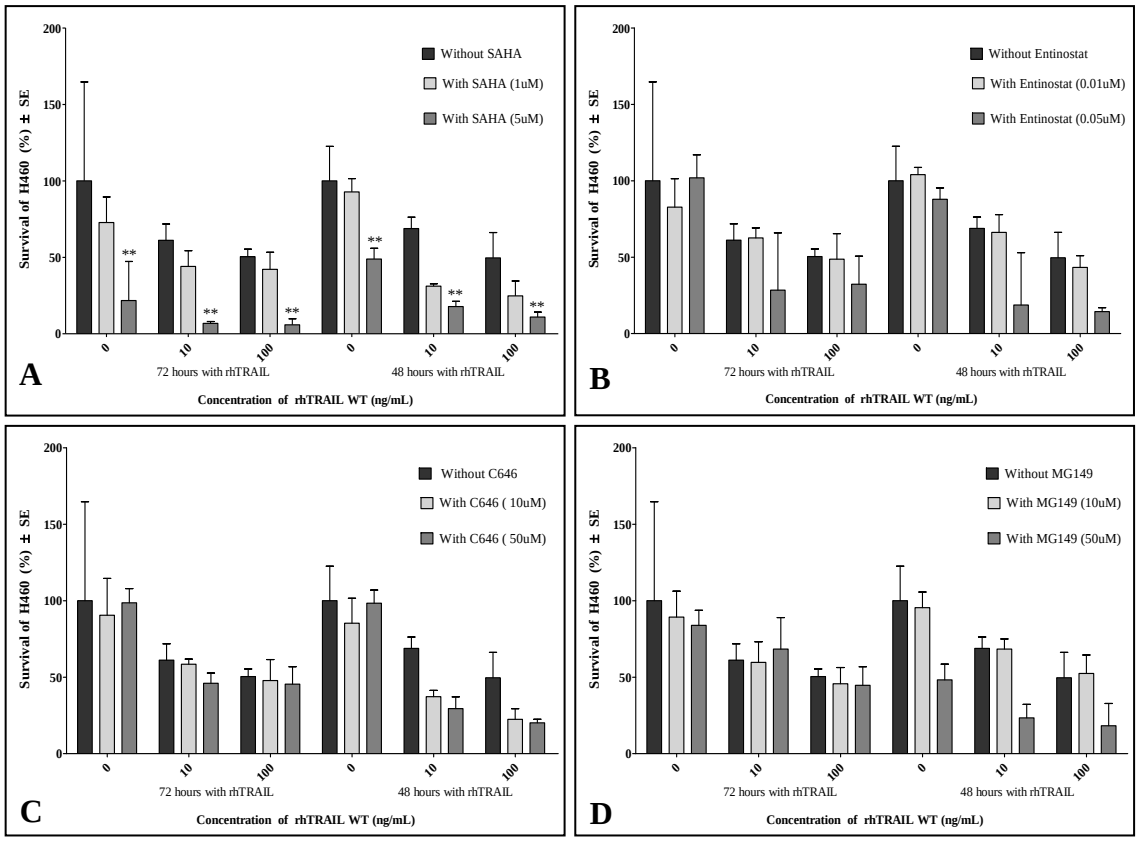

Figure 3.18 SAHA, Entinostat, C646, MG149 and rhTRAIL WT in H460 cells induces apoptosis. The cells H460 were incubated for 72 hours with rhTRAIL WT, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL WT, (left part of the graphics). On the right part of the graphics, the H460 cells were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL WT was added alone, and combined with the inhibitors. A survival assay (crystal violet) was performed. (A) H460 cells with rhTRAIL WT combined with SAHA; (B) H460 cells with rhTRAIL WT combined with Entinostat; (C) H460 cells with rhTRAIL WT combined with C646; (D) H460 cells with rhTRAIL WT combined with MG149. The graphs showed the average of independent experiments and the error bars indicate the standard error of the mean. **P<0.01, versus control without treatment (0ng/mL concentration of rhTRAIL WT).

The H460 showed a strong decrease in cell survival upon treatment with rhTRAIL 4C7 (Figure 3.17). Moreover, the synergistic effect was confirmed with the combination of SAHA/rhTRAIL (DHER, 4C7 and WT). An unexpected result was the combination of Entinostat with rhTRAIL (DHER, 4C7 and WT), indicating a very slight synergistic effect. But the combination of C646 and MG149 did not demonstrates a combination effect when compared with the addition of the rhTRAIL (DHER, 4C7 and WT). A very interesting result is the fact that the H460 cells, independently of the type of TRAIL tested, had the same profile of answer for the combination of HDACs and HAT with the

different rhTRAIL. This shows that, although the cells are more sensitive to one of the rhTRAIL, the response profile in combination with other drugs will be the same. The results from all the experiments are described in Appendix A.5.

3.5.3. SAHA combined with rhTRAIL (DHER, 4C7 and WT), promotes apoptosis more efficiently compared with the combination of Entinostat, C646 and MG149 in HUH-7 cells

Since the aim is to determine the effect of rhTRAIL DHER in liver cells, the hepatocellular carcinoma cells (HUH-7) were used as a liver cell model, to test the effect of rhTRAIL DHER protein, using as control the rhTRAIL 4C7 protein and WT so as to verify the sensitivity of the cells to each protein. The combination with HDACs and HATs inhibitors was done in order to determine whether the inhibitors promote a higher apoptosis in the cells, or whether they can protect the cells against the rhTRAIL. The HUH-7 was plated in a 96 well/plate and incubated overnight to allow adhering to the surface. Then, different concentrations of SAHA, Entinostat, C646, MG149, rhTRAIL (DHER, 4C7 and WT) were added, alone and in combination. The cells were incubated for 72 hours in a 5% CO_2. The same assay was repeated but the addition of rhTRAIL DHER, 4C7 and WT was done after 24 hours of incubation of HDACs and HAT inhibitors.

The results, shown in Figure 3.19, 3.20, 3.21, indicate that the HUH-7 is more sensitive for the rhTRAIL DHER, inducing a survival of the cells, with 10ng/mL of the protein for 72 hours, of 65.84% ± 1.23%, against survival of 65.22% ± 4.18% and 94.51% ± 0.99% for rhTRAIL 4C7 and WT, respectively. The results show the combination of MG149/rhTRAIL DHER (Figure 3.19D), either for 72 hours or 48hours, the rhTRAIL DHER has some effect in the cells (killing 10% of the cells), but the combination did not promote apoptosis in cells; instead, it cans an increasing rate of survival. Without the combination, for 10ng/mL of rhTRAIL DHER the survival is 65.84% ± 1.23%; but when

combined with 10µM of MG149 for 72 hours the survival is 110.77% ± 5.90%. The effect of 100ng/mL of rhTRAIL DHER for 72 hours is a survival rate of 53.75% ± 4.23%.Therefore, any affect happened with the combination, and the same profile was also seen for the combination of MG149 with 48 hours of rhTRAIL DHER. The response profile for SAHA, Entinostat and C646 is similar to that described in interaction with DHER rhTRAIL in H460 cells.

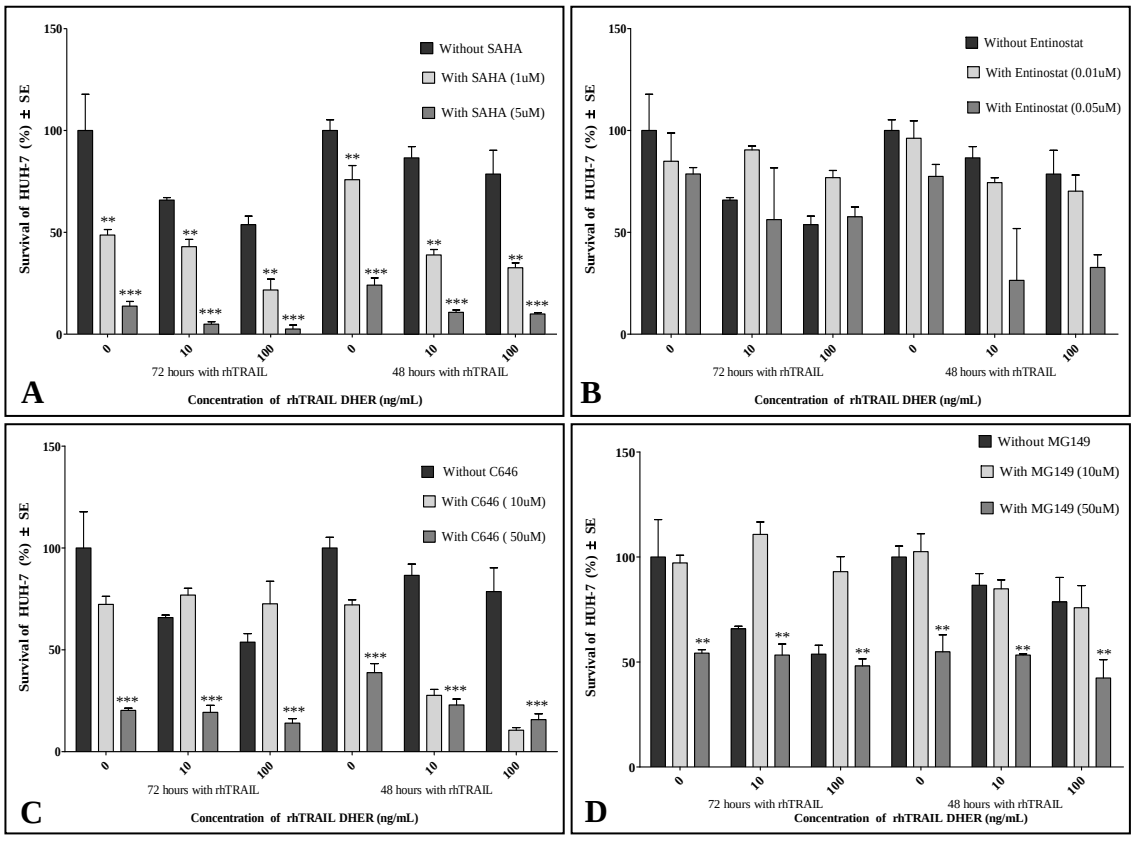

Figure 3.19 Combination of SAHA, Entinostat, C646, MG149 and rhTRAIL DHER in HUH-7. The cells HUH-7 were incubated for 72 hours with rhTRAIL DHER, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL DHER (left part of the graphics). On the right part of the graphics, the cells HUH-7 were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL DHER was added, alone and combined with the inhibitors. (A) HUH-7 cells with rhTRAIL DHER combined with SAHA; (B) HUH-7 cells with rhTRAIL DHER combined with Entinostat; (C) HUH-7 cells with rhTRAIL DHER combined with C646; (D) HUH-7-cells with rhTRAIL DHER combined with MG149. The graphs showed the average of three independent experiments and the error bars indicate the standard error of the mean. **P<0.01, ***P<0.001 versus control without treatment (0ng/mL concentration of rhTRAIL DHER).

Following rhTRAIL 4C7 treatment (Figure 3.20) in combination with SAHA a synergistic effect was observed, promoting a higher apoptosis in the cells. Looking for the effect of 10ng/mL during 72 hours of rhTRAIL 4C7, the survival of the cells is 65.22% ± 4.18%; when combined with 1µM of SAHA and 10µM of C646 the survival is 16.13% ± 1.36%and 41,62% ± 2,33%, respectively. For 24 hours they have the same profile of response. However, the combination of rhTRAIL 4C7 with Entinostat and MG149 shows a very weak combination effect in comparison with the 10ng/mL rhTRAIL 4C7 (65.22% ± 4.18%) for 72 hours with 0.01µM of Entinostat and 10µM of MG149, and the cells survival rate is 44.14% ± 0.84% and 54.19% ± 2.75%, respectively.

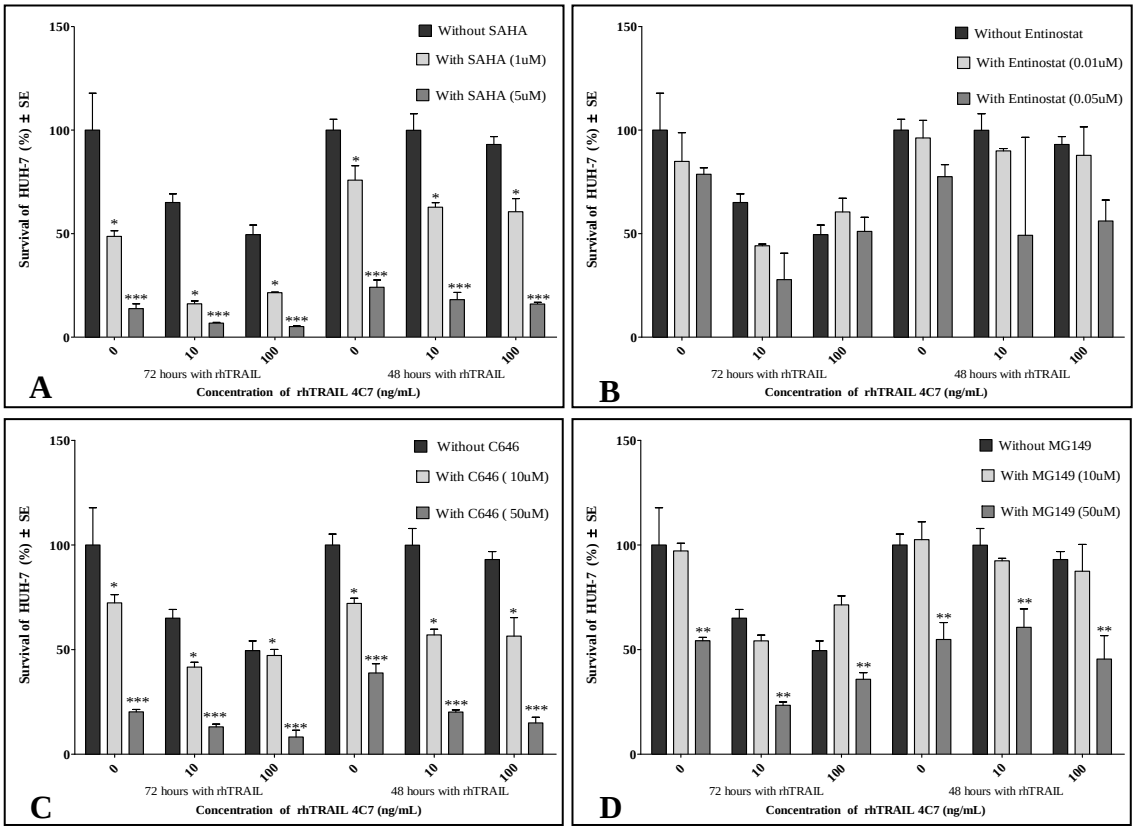

Figure 3.20 Combination of C646, MG149, Entinostat and SAHA with rhTRAIL 4C7 in HUH-7. The HUH-7 cells were incubated for 72 hours with rhTRAIL 4C7, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL 4C7 (left part of the graphics). On the right part of the graphics, the cells HUH-7 were incubated with SAHA, Entinostat, MG149 and C646, and after 24hours the rhTRAIL 4C7 was added, alone and combined with the inhibitors. (A) HUH-7 cells with rhTRAIL 4C7 combined with SAHA; (B) HUH-7 cells with rhTRAIL 4C7 combined with Entinostat; (C) HUH-7 cells with rhTRAIL 4C7 combined with C646; (D) HUH-7 cells with rhTRAIL 4C7 combined with MG149. The graphs show the average of three independent experiments and the error bars indicate the standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 versus control without treatment (0ng/mL concentration of rhTRAIL 4C7).

In respect to rhTRAIL WT (Figure 3.21), the cells are not sensitive for this protein. To 10ng/mL of rhTRAIL WT for 72 hours the survival is 94.51% ± 0.99%, but when combined with 1µM SAHA or 10µM C646, the survival decreases to 26.07% ± 4.30% and 63.28% ± 3.97%, respectively. With these results, can be conclude that there is a synergistic effect of SAHA and C646, promoting a higher apoptosis when combined with rhTRAIL WT. Still, for 0.01µM of Entinostat and 10µM of MG149, the combination effect is very small. With these interactions, the percentage of cells killed is 10%. Thus, it can be concluded that MG149 and Entinostat can protect the cells against rhTRAIL WT.

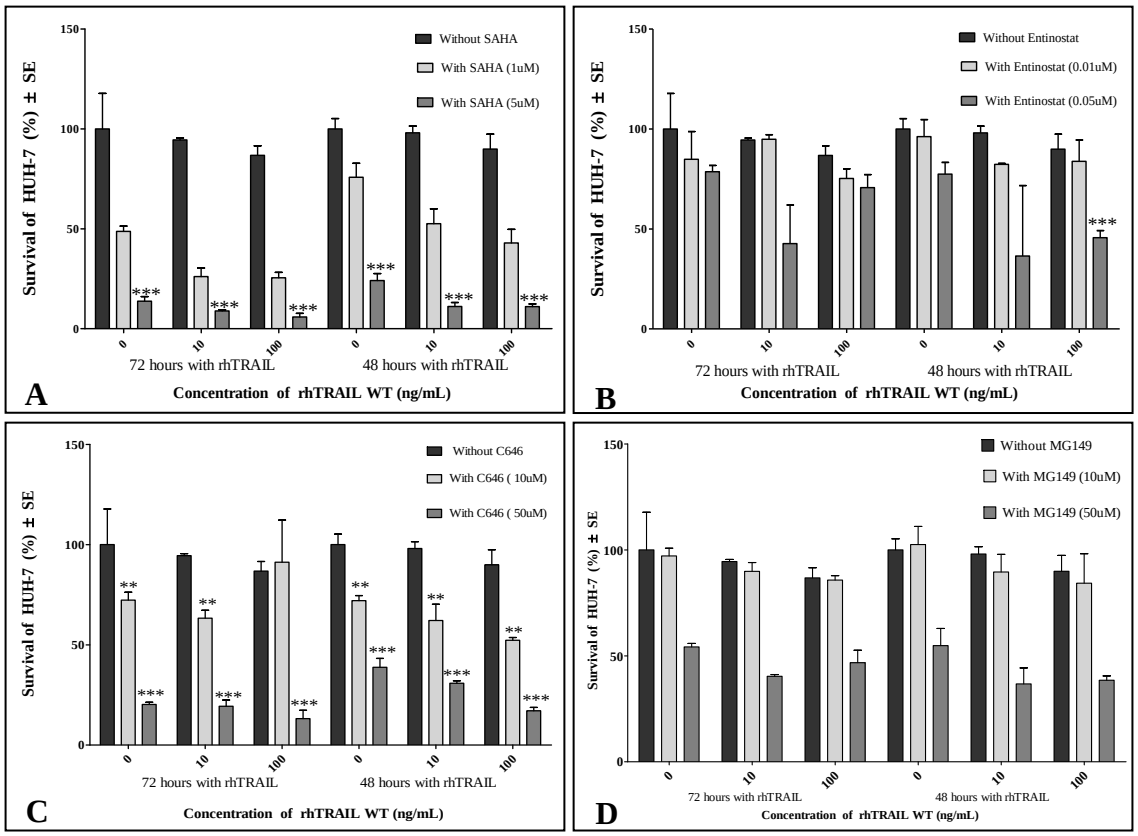

Figure 3.21 SAHA, Entinostat, C646 and MG49 with rhTRAIL WT in HUH-7 cells. (A) HUH-7 cells with rhTRAIL WT combined with SAHA; (B) HUH-7 cells with rhTRAIL WT combined with Entinostat; (C) HUH-7 cells with rhTRAIL WT combined with C646; (D) HUH-7 cells with rhTRAIL WT combined with MG149. The graphs show the average of independent experiments and the error bars indicate the standard error of the mean. **P<0.01,***P<0.001 versus control without treatment (0ng/mL concentration of rhTRAIL WT).The cells HUH-7 were incubated for 72 hours with rhTRAIL WT, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL WT (left part of the graphics). On the right part of the graphics, the cells HUH-7 were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL WT was added alone, and combined with the inhibitors. Accordingly, the incubation time of HDACs and HATs inhibitors in both parts of the graphics is 72 hours, but rhTRAIL WT the incubation period in the left part of the graphic is 72 hours, but in the right is 48 hours.

These results report the sensitivity of HUH-7 cells to rhTRAIL DHER, and a protection effect of MG149 against this protein, because of the very weak combination effect. The synergistic effect of SAHA in combination with rhTRAIL (DHER, 4C7 and WT) was confirmed, but the interaction of Entinostat and C646 with rhTRAIL (DHER, 4C7 and WT) is very weak. These results are very interesting because they have the same profile of answer as H460, and it can be suggested that instead of Entinostat (HDAC inhibitor) promoting apoptosis synergistically, it acts as a protector, such as C646, having a lower level of protection than the MG149. The results from all the experiments are described in Appendix A.5.

3.5.4. SAHA combined with rhTRAIL variants promotes apoptosis more efficiently compared with the combination of Entinostat, C646 and MG149 in HepG2 cells

As previously demonstrated, the HDACs (SAHA) inhibitors are active in inducing apoptosis in HepG2 cells [73]. We wanted to see the effect of another HDAC inhibitor (Entinostat) and the effect of HAT (C646 and MG149) inhibitors combined with rhTRAIL. As earlier showed, the HDACs inhibitors combined with rhTRAIL (DHER, 4C7 and WT) induce apoptosis in a synergistic manner [71]. The HepG2 cells is another hepatocellular carcinoma cells, and for the same reasons as for HUH-7, we wanted to use this cell line as a model of hepatic cells, to determine the effect of rhTRAIL (DHER, 4C7 and WT) alone, and to determine whether the combination with HDACs promotes a higher apoptosis, and whether HATs inhibitors protect the cells against the rhTRAIL (DHER, 4C7 and WT) as hypothesize. The HepG2 cells were cultured in a 96 well/plate, after 24 hours of plated, different concentrations of SAHA, Entinostat, MG149 and C646 were added, and combined with different concentrations of rhTRAIL (DHER, 4C7 and WT). Then, the cells were incubated for 72 hours at 5% CO_2. The same assay as repeated, but the addition of rhTRAIL (DHER, 4C7 and WT) was done after 24 hours of incubation of HDACS and HAT inhibitors. In Figures 3.22, 3.23 and 3.24 show the sensitive of this

cell line for rhTRAIL 4C7 with 10ng/mL, not for 72 hours but for 48 hours. This cell line is very sensitive for this rhTRAIL. As shown in Figure 3.22A there is a combination effect of SAHA/rhTRAIL DHER, both for 72 hours and 48 hours. However, for C646 and Entinostat the combination effect is inefficient (Figure 3.22B and C), and we can suggest a protection effect. A very interesting result has been produced for the MG149 inhibitor, both for 72 hours or 48 hours, of rhTRAIL DHER. This inhibitor can have a protection effect, because for 10ng/mL of rhTRAIL DHER, for 72 hours and 48 hours, the survival of the cells is 114.99% ± 12.21% and 65.83% ±8.37%, respectively, but when combined with 10µM of MG149, the survival of the cells is 140.91% ± 26.95% and 77.62% ± 6.66%. We can conclude that SAHA and MG149 have the results as we hypothesize, however, for Entinostat and C646, the efficiency of this inhibitors is smaller in comparison with the other two inhibitors.

Regarding rhTRAIL 4C7 (Figure 3.23) the sensitivity of HepG2 for this rhTRAIL was demonstrated. As expected SAHA had a synergistic effect in combination with rhTRAIL 4C7. However, for the MG149 a very slight protective effect was observed. This is shown for 10ng/mL of rhTRAIL 4C7 combined with 10µM and 50µM of MG149, during 72 hours, the survival of the cells is 134.65% ± 6.60% and 123.49% ± 12.22%, in comparison with 146.31% ± 21.45% of rhTRAIL alone. And for 100ng/mL of rhTRAIL 4C7 there is no difference. The same profile of interaction happened with Entinostat and C646, both for 72 hours and 48 hours of incubation.

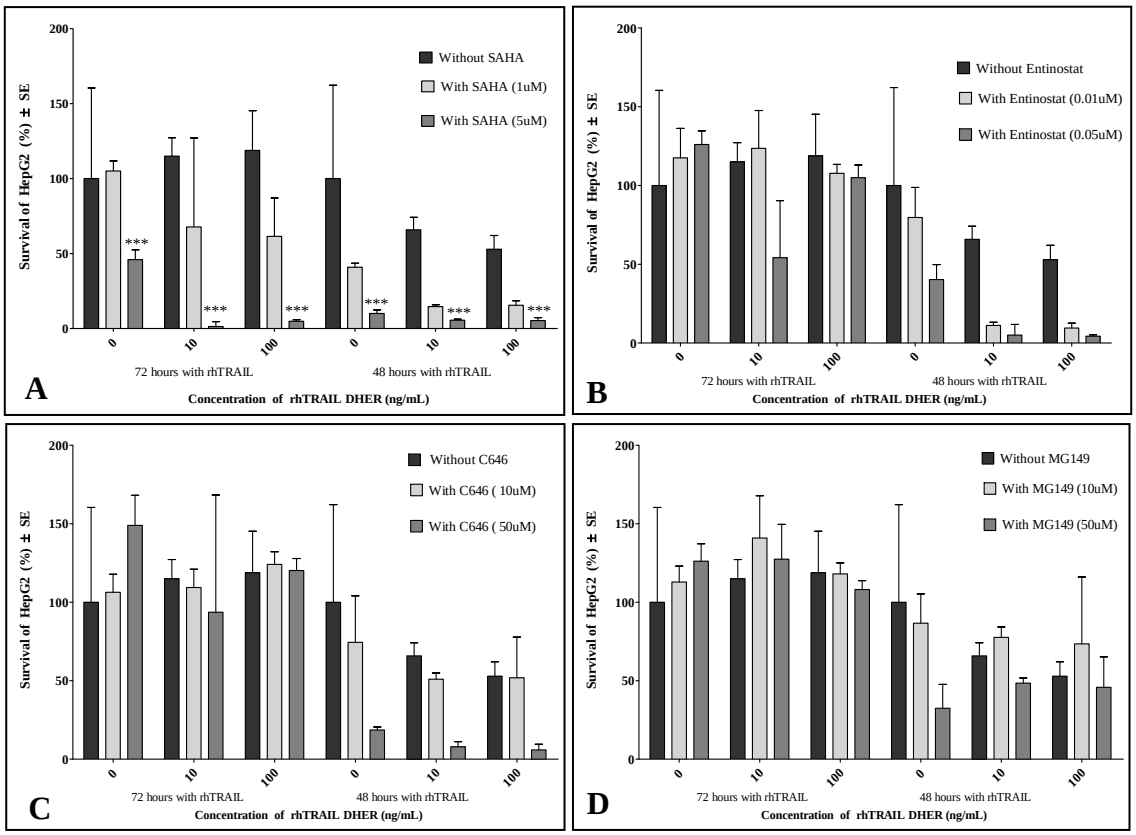

Figure 3.22. SAHA with rhTRAIL DHER in HepG2 cells induces apoptosis in a synergistic manner and the combination of MG149, C646 and Entinostat and C646 have no combination effect in the cells. The HepG2 cells were incubated for 72 hours with rhTRAIL DHER, SAHA, Entinostat, MG149 and C646, alone and all the inhibitors combined with rhTRAIL DHER (left part of the graphics). On the right part of the graphics, the cells HepG2 were incubated with SAHA, Entinostat, MG149 and C646, and after 24hours the rhTRAIL DHER was added alone, and combined with the inhibitors. (A) HepG2 cells with rhTRAIL DHER combined with SAHA; (B) HepG2 cells with rhTRAIL DHER combined with Entinostat; (C) HepG2 cells with rhTRAIL DHER combined with C646; (D) HepG2 cells with rhTRAIL DHER combined with MG149. The graphs showed the average of three independent experiments and the error bars indicate the standard error of the mean. ***P<0.001 versus control without treatment (0ng/mL concentration of rhTRAIL DHER).

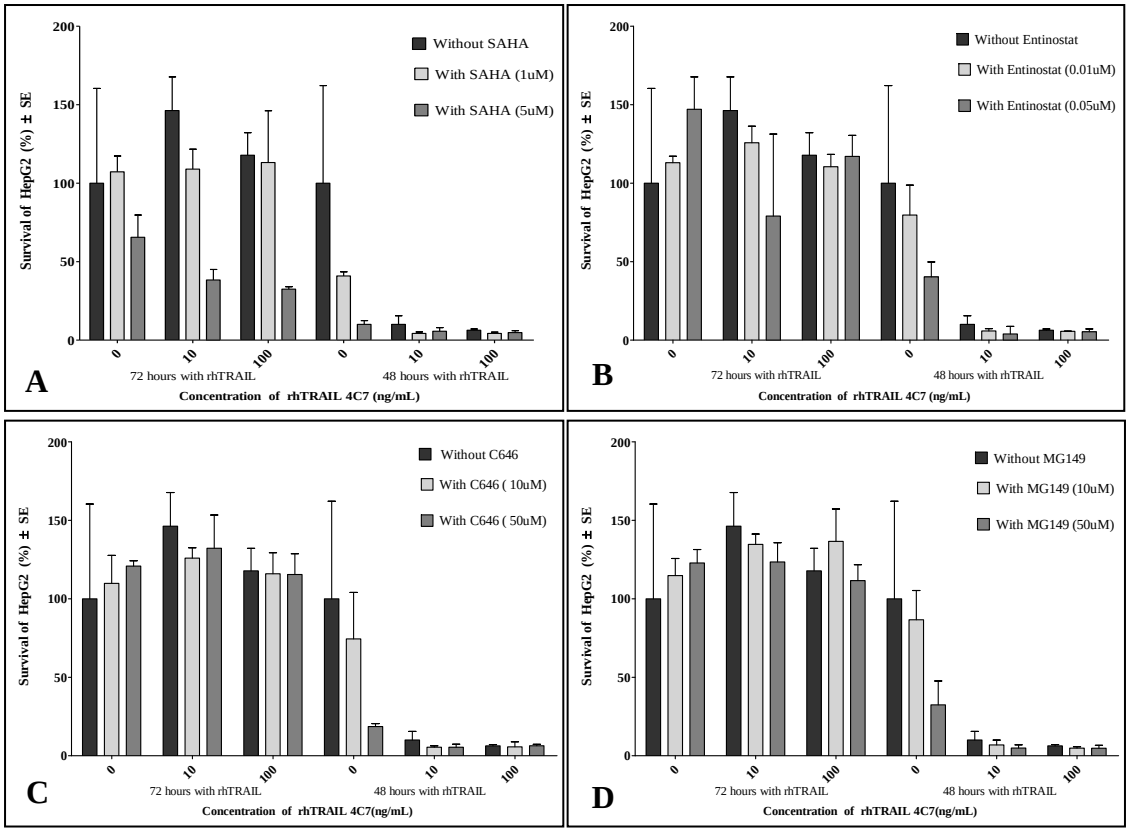

Figure 3.23. SAHA with rhTRAIL 4C7 in HepG2 cells induces apoptosis and MG149, C646 and Entinostat promote a slight apoptosis in the cells. (A) HepG2 cells with rhTRAIL 4C7 combined with SAHA; (B) HepG2 cells with rhTRAIL 4C7 combined with Entinostat; (C) HepG2 cells with rhTRAIL 4C7 combined with C646; (D) HepG2 cells with rhTRAIL 4C7 combined with MG149. The graphs show the average of three independent experiments and the error bars indicate the standard error of the mean. The statistical significance tests were done in three independent experiments, and there is no statistical significance between groups. The cells HepG2 were incubated for 72 hours with rhTRAIL 4C7, SAHA, Entinostat, MG149 and C646 alone and all the inhibitors combined with rhTRAIL 4C7 (left part of the graphics). On the right part of the graphics, the cells HepG2 were incubated with SAHA, Entinostat, MG149 and C646, and after 24 hours the rhTRAIL 4C7 was added, alone and combined with the inhibitors.

In respect to rhTRAIL WT (Figure 3.24) the combination with SAHA as a combination effect promoting a higher apoptosis. However, for Entinostat, MG149 and C646 have a synergistic effect, because with the combination of the inhibitors with the rhTRAIL WT, a decrease of the survival of the cells occurs. Still, this interaction is very slight and it can be suggested that interaction function as a protection of the cells. For 10ng/mL of rhTRAIL WT in 72 hours of incubation, the survival of the cells is 119.71% ± 8.36%, and for the combination with Entinostat, C646 and MG149, the survival is 87.96% ± 3.33%, 129.00% ± 24.80% and 113.81% ± 8.51%, respectively. Results suggest that, although there is some interaction when combined, this effect is very weak, and the combination may be used without causing major effects on cells.

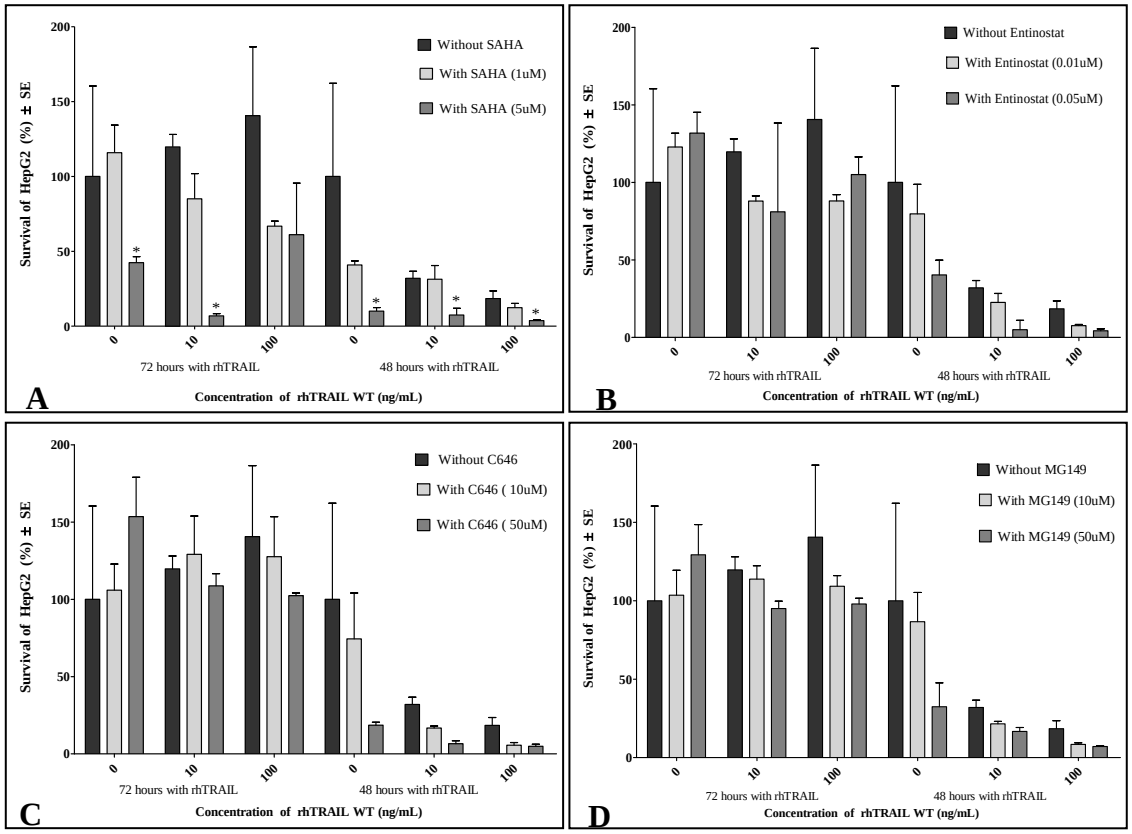

Figure 3.24. The effect of the combination of SAHA, Entinostat, MG149 and C646 with rhTRAIL WT in HepG2 cells. The cells HepG2 were incubated for 72 hours with rhTRAIL WT, SAHA, Entinostat, MG149 and C646, alone and all the inhibitors combined with rhTRAIL WT (left part of the graphics). On the right part of the graphics, the HepG2 cells were incubated with SAHA, Entinostat, MG149 and C646, and after 24hours the rhTRAIL WT was added, alone and combined with the inhibitors. (A) HepG2 cells with rhTRAIL WT combined with SAHA; (B) HepG2 cells with rhTRAIL WT combined with Entinostat; (C) HepG2 cells with rhTRAIL WT combined with C646; (D) HepG2 cells with rhTRAIL WT combined with MG149. The graphs showed the average of three independent experiments and the error bars indicate the standard error of the mean. **P<0.01 versus control without treatment (0ng/mL concentration of rhTRAIL WT).

Results indicate the sensitive of the HepG2 cells for the rhTRAIL 4C7, and the combination with SAHA promotes a synergistic apoptosis, as expected. Nevertheless, for MG149 a very interesting result shows that combined with rhTRAIL DHER this inhibitor, produces no combination effect, both for 72 hours and 48 hours, while for the other rhTRAIL (4C7 and WT) the same effect did not occur. For C646 and Entinostat, the profile of response to these inhibitors in the cells is similar, and the combination with rhTRAIL (DHER, 4C7 and WT) has a very weak combination effect. Moreover, the combination of rhTRAIL variants adding 24 hours after HDAC and HAT inhibitors, shows a higher toxicity than the rhTRAIL variants adding at the same time than HDAC and HAT. The results from all the experiments are described in Appendix A.5

-Chapter 4-

Discussion

Liver fibrosis is a reversible scaring response that occurs in almost all patients with chronic liver injury. This injury is caused due to metabolic, genetic and viral diseases, and represents a health problem in our society [77, 78]. According to the European Association for the Study of the Liver, until December 2009, 59% of transplantation in liver is due to cirrhosis [8]. Liver cirrhosis is an end stage consequence of fibrosis, and it is described by altered hepatic function and nodule formation [75].

Hepatic stellate cells (HSC) are responsible for this injury. These cells evolve from a quiescent state to an activated state, in response to liver damage. In the activation process, they became very proliferative and synthesize extra cellular matrix (ECM), rich in collagen type I [18]. The activated HSC have the capability to go through apoptosis either by specific targeting with apoptotic agents, or spontaneously [76]. The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), is one of the apoptotic agents that have a large apoptotic activity [77]. TRAIL, either in full-length or in a cleaved soluble form, binds to five transmembrane receptors: death receptors (DR) 4 and 5, decoy receptors (DcR) 1 and 2, and osteoprotegerin [29]. When TRAIL binds to the DRs receptors it induces apoptosis through caspase-8 into the death-inducing signaling complex (DISC) [81, 82]. As previously reported [64, 73], the receptor TRAIL DR5 is expressed 103 times more frequently in the surface of the activated HSC than the receptor TRAIL DR4. TRAIL appears to be the most promising factor of the TNF family to induce apoptosis in the activated HSC. Soluble recombinant human TRAIL protein (rhTRAIL) demonstrated, in other reports, low toxicity and significant antitumor activity in a large range of human cancer [31, 83–86]. However, the short half-life of only 30 minutes, in non-human primates, could be a limitation for the use of rhTRAIL in humans [82]. Another limitation is the delivery of rhTRAIL to the cells [77].Therefore gene therapy

could be an interesting approach. Moreover, the decoy receptors DcR1 or DcR2 do not contain a death domain and consequently could prevent apoptosis by sequestering accessible TRAIL or by interfering in the formation of a TRAIL 4C7 or DHER signaling complex [80, 83]. Researchers have found that the use of the rhTRAIL DHER variants D269H/E195R could significantly reduce the binding to decoy receptors and improve the connection of the rhTRAIL DHER protein to the TRAIL DR5 receptor, resulting in a lower administrated dose with possibly of lower effects [35]. This study uses gene therapy to deliver DHER specific TRAIL which can induce apoptosis in activated HSC.

As showed in Figure 2.1, a shuttle vector, pADTRACK-CMV-IGK-HA-rhTRAIL DHER, was construct capable to express the rhTRAIL DHER protein variant D269H/E195R. The constructed vector has three important sequences: the IGK specific for secretion of rhTRAIL DHER protein; the HA, specific for the detection and isolation of the protein; and the GFP to be able to visualize the expression of the vector. As indicate in Figure 3.6, the transfection in HEK-293 cells showed that the plasmid is working, because of the expression of GFP in the cells. Moreover, results show (Figure 3.7) the function of the plasmid through the expression of the interest protein, detectable across the antibody against HA, and the antibody against the rhTRAIL.

As demonstrated by Wang et al [22], once rhTRAIL protein binds to the DRs receptors, in this case, the rhTRAIL DHER connect to TRAIL DR5 receptor, the extrinsic pathway is initiated through activation of caspase-8 and caspase-10, which can directly cleave and activate caspase such as caspase -3, -6 and -7 [22]. Caspase-8 can also start the intrinsic apoptosis cascade by cleavage of the pro-apoptotic protein Bid (Bcl-2 interacting domain). Bid displaces to the mitochondria and activates Bax and Bak, which leads to dissipation of the mitochondrial membrane potential and adds the release of cytochrome c, formation of the apoptosome, activation of caspase-9 and finally cleavage of the apoptosis-executing caspases [83].

Furthermore, the function of the protein was confirmed by means of the addition of the expressed protein into the SW948 cells (Figure 3.8). Because of the sensibility of this cell line to rhTRAIL, the addition of the expressed protein (from the production in HEK-239 cells) into SW948 shows a decrease of survival of cells, indicating that the

protein is working. However, with 10 µL of supernatant (Figure 3.8A) did not promote apoptosis in the cells and this was unexpected once with 12.5µL and 6.25µL of supernatant (Figure 3.8A) the protein promotes the apoptosis, therefore 10µL should have promoted the apoptosis. This can be due to the inactivation of the protein during the 72 hours of incubation. During this period, the ligation of the protein rhTRAIL DHER to the receptor DR5 can be lower, and the pathways that would promote the apoptosis were not activated. Nevertheless, the apoptotic effect that the protein induces, in the SW948 cells is not very high. This can be due to the absence of purification after the transfection into HEK-293 cells. The purification is a very important step for the integrity and function of the protein, following by the quantification of the produced protein. The vector pADTRACK-CMV is a shuttle vector and has the capability to express transgenes under the monitoring of GFP expression. The site for the insertion of rhTRAIL DHER is bounded by adenoviral sequences ("arms") that permit homologous recombination with pAdEasy-1 [62]. Both arm contains Ad5 (adenovirus serotype 5, the serotype widely used for gene therapy [87–89]) nucleotides, which mediate homologous recombination with pAdEasy vectors in *E.Coli* and packaging signal sequences (nucleotides 1-480 of Ad5), a necessary feature for viral production in mammalian cells. A restriction endonuclease site for PacI was created, surrounding both arms. This vector also contains a gene for resistance to kanamycin and a polyadenylation site (SV40 poly A) [62]. The recombination of pADTRACK-CMV-IgK-HA-rhTRAIL DHER with pAdEasy-1 (Figure 3.11) did not occur, as proved in the restriction endonucleases digestions. However, the homologous recombination step is done by a restriction endonuclease that cleaves the shuttle vector and an intact supercoiled adenoviral vector. The capacity to use intact adenoviral plasmids, uncleaved by restriction endonucleases, has proofed to be critical to generate efficiently, the desired recombinants [62]. Another important step is the purification of DNA after the endonuclease digestions, before the transfection to BJ5183 bacteria, the quality of DNA can affect the efficiency of recombination [65, 68]. The strain BJ5183 was used because of its higher efficiency of transformation, but this efficiency depends of the competence of the cells [65, 90, 91].

Our data indicates that rhTRAIL (DHER, 4C7 and WT) protein induce apoptosis in activated hepatic stellate cells (LX2 cells) (Figure 3.12A), as expected. The proteins used is from other authors [35], due to the availability of this protein produced in pET15b vector, the same vector used for the extraction of gene to clone into our constructed plasmid. However, the protein of interest, rhTRAIL DHER, did not show a higher apoptosis rate, which could be due to the weak activation of LX2 cells. As demonstrated by other authors, LX2 cells increase the expression of TRAIL DR5 receptor during the activation after 14 days, the TRAIL DR5 mRNA and α-SMA expression also increase during activation [64, 73].

To confirm that the rhTRAIL DHER protein has no effect in hepatic cells, the HepG2 cells were treated with the same proteins as LX2 cells. Results (Figure 3.12B) demonstrated the lower efficacy of the rhTRAIL proteins (DHER, 4C7 and WT) into the HepG2 cells. As described by other authors, the rhTRAIL DHER,4C7 and WT has some effect in the activated HSC but they also show some toxicity in certain normal cells, including the hepatocytes [92, 93].

Results, demonstrated that 306 ng/mL of rhTRAIL DHER protein can induce apoptosis in activated hepatic stellate cells, without producing any effect in hepatic cells (Figure 3.12). However, the low rate of apoptosis that occurred with the addition of rhTRAIL DHER in the activated stellate cells demonstrated that the protein can induce the apoptosis but in a very inefficient way.

In the other hand, the protection of the quiescent stellate cells, and the other liver cells types is an aim for the treatment of liver fibrosis, defining more specific apoptotic therapies [70].In order to identify a treatment that induces an increase of apoptosis in target cells while protecting the normal cells, we suggest the combination of the rhTRAIL DHER (4C7 and WT as used as control) with HDACS and HATs inhibitors. HDAC inihibitors (SAHA and Entinostat) combined with TRAIL are extensibely studied as promotors to induce apoptosis in cancer cells lines [58, 61, 74, 76, 94–99]. Still, there is scarce information available about the HAT (C646 and MG149) inhibitors [61, 62, 100].

Histone acetyltransferase (HAT) is involved in chromatin structure modifications via acetylation, while Histone deacetylases (HDAC) are involved in chromatin structure modifications via deacetylation [97]. Recently, it has been shown that HAT and HDACs regulate the cell proliferation, differentiation, and apoptosis in various cancer cells lines [95, 101]. Since HDAC inhibitors synergize with rhTRAIL in inducing apoptosis [71], due to the overexpression of TRAIL DR5 receptor in the surface of the cells, we hypothesized that HAT inhibitors combined with rhTRAIL DHER (4C7 and WT as used as control) may interact with DcR and TRAIL DR5/DR4 receptors, avoiding the binding of rhTRAIL DHER, and preventing the apoptosis of the cells.

We confirmed that the interaction of SAHA with rhTRAIL DHER (4C7 and WT) promotes a higher apoptosis in H460, HUH-7 and HepG2 cells in a synergistic manner. Some reports [76, 96] have mentioned, that SAHA induces apoptosis by increasing TRAIL DR5 expression in the surface, downregulating c-Flip, and activating the caspase-8 as well as cleavage of NF-kB by caspase-3. Regardless of Entinostat, for all cell lines, the interaction of this HDAC inhibitor with rhTRAIL DHER (4C7 and WT) shows the effect of combination to be very low, contrary to what was expected. As results from this study conflicts with other studies, we proposed that for this HDAC/rhTRAIL not a synergistic interaction but an effect of protection occurs, though a very weak protection. Venza et al [93] evidenced that the combination of HDAC/rhTRAIL DHER induces apoptosis in a synergistic interaction, promoting the apoptosis, and upregulating a transcriptional repressor of C-Flip. C-flip is an important inhibitor of the extrinsic apoptotic pathway.

Regarding to HAT inhibitors, this study demonstrated no combination effect of this inhibitors in combination with rhTRAIL DHER, and it can be suggested a protection effect. Althought for C646, a weak combination effect occurs, suggesting that the protection effect is less than MG149. This effect is more evident in the HUH-7 and HepG2 cells in the combination of MG149/rhTRAIL DHER. Due to lack of information on this HAT inhibitor (MG149), the process involved in cells protection is understudied. We suggest that protection is due to the interaction of the inhibitor with DcR and TRAIL DR5 receptor, since this receptor is more active in cancer cells, as well liver fibrosis, as

shown by other authors [23, 97]. We suggest that the inhibitors block the apoptosis pathway in the cells and do not increase the expression of TRAIL DR5 receptor. Already proved by other authors[64,73], the hepatic stellate cells expression a lot of TRAIL DR5 receptor when activated, so the concentrations of this added inhibitor are important to not interfere with the binding of rhTRAIL DHER protein with TRAIL DR5 receptor. Another feature that we demonstrated was that the response profile of the interaction, either the HAT or HDACs inhibitors, with rhTRAIL DHER added at the same time or after 24 hours, does not affect the interaction of the inhibitors and protein in the cells.

Moreover, results show that the effect of 50μM of MG149 added alone or in combination with the rhTRAIL (DHER, 4C7 and WT) as the same effect,. For SW948 and HUH-7, this is observable for all rhTRAIL, although for H460 this is not observable and for HepG2 it is only observed in rhTRAIL DHER. Furthermore, the sensibility of cell line to the rhTRAIL 4C7 seems to have a interaction with the effect of combination. For MG149 inhibitor in SW948, the effect of combination is higherrelating with other rhTRAIL. Concerning, to HUH-7 and HepG2, the higher effect of the combination with MG149 is visible for rhTRAIL DHER, demonstrating a higher effect of protection against the other rhTRAIL.

The present study demonstrates that the sequencial treatments of SW948, H460, HUH-7 and HepG2 cells with SAHA followed by rhTRAIL DHER (4C7 and WT) are more effective in inducing apoptosis than when the drugs are added alone. As is was demonstrated by other authors, the interaction of HDAC inhibitors and TRAIL induces synergistic apoptosis due to upregulation of TRAIL DR5 and/or DR4 receptors, activations caspase -3, -9, and -8, induction of proapoptotic members from the Bcl-2 family, and inhibition of Bcl-2 and/or Bcl-X_L [71]. Futhermore, the protection effect of HAT inhibitors was demonstrated, as suggested. The MG149 inhibitor followed by rhTRAIL DHER (4C7 and WT) are effective in protecting the H460, HUH-7 and HepG2 cells,because any combination effect occurs. Moreover, it can be suggested that this inhibitor can prevente the activation of the apoptotic pathways of the cell.

In this research a vector was constructed capable to produce the rhTRAIL DHER in mammalian cells. The produced protein is working and active, and the vector is able

to recombine with an adenoviral vector, although the recombination was not succeeded in our study. Adenoviral vectors are attractive gene delivery vehicles for liver fibrosis, due to their capability of producing the interest protein, their high stability *in vivo*, high expression of the gene, and low pathogenicity in humans. The adenovirus can be administrated intravenously, targeting, infecting and expressing the gene responsible to produce the rhTRAIL DHER protein in the liver [99]. Furthermore, in this study the synergistic effect of HDACs inhibitors with rhTRAIL proteins was proved, and a possible inhibitor which may protect the normal cells in the liver was identified. The MG149 inhibitor may protect the normal cells in the liver when combined with rhTRAIL DHER protein. Thus, on one hand, the protein can have its function in the hepatic stellate cells, and, on the other hand, the MG149 inhibitor can protect the hepatocytes, since these cells have some expression of TRAIL DR5 receptor in its surface.

-Chapter 5-

Conclusion and Future Perspectives

In this study, we constructed a vector, pADTRACK-CMV-IgK-HA-rhTRAIL DHER, capable of producing the interest protein, rhTRAIL DHER. The study showed that the protein is working and active when added in SW948 cells. However, the recombination with an adenoviral vector was not succeessful. Once recombination is accomplished and verified, the adenoviral recombinant DNA can be simply transformed to a recA, endA strain of *E.Coli* (like DH5α) for better yields of DNA [62]. Moreover, the recombination with an uncleaved vector, the pAdEasy-1, is difficult to achieve. As described by other authors, the recombination can directly occur in mammalian cells [65,68]. The shuttle vector, pADTRACK-CMV-IgK-HA-rhTRAIL DHER, and the adenoviral vector, pAdEasy-1, can be digested with endonuclease restriction enzyme PmeI, and transformed in HEK-293 cells, allowing recombination to occur inside the cells. Furthermore, this packaging cell line is easy to transfect by lipid DNA complexes. The HEK-293 cells express the E1 gene products, essential for propagation of recombinant adenoviruses [62].

The treatment for patients with liver fibrosis would be rather lengthy using the rhTRAIL protein produced in a non-viral vector. For this reason, the production of the protein in an adenoviral vector and administrated in patients, targeting, infecting and expressing the gene, can reduce the activated hepatic stellate cells, thought rhTRAIL DHER protein binding to the TRAIL DR5 receptor. However, the normal cells in the liver express the TRAIL DR5 receptor [88, 93], although in smaller amount, this can be prejudicial to the treatment. To treat liver fibrosis, it is necessary to develop a therapy that can promote the apoptosis in the activated HSC, and have no effect in the other liver cells.

As demonstrated, the HDACs inhibitors induce synergistically the apoptosis in the cells, when combined with rhTRAIL proteins. However, it is necessary to prove what kind of interactions occur, like the overexpression of TRAIL DR5 or DR4 in the surface,

the activation of caspase cascade pathways, and what type of pathway is activated (the intrinsic or extrinsic).

In relation to the HATs inhibitors, it is important to study the effect of the interaction with rhTRAIL DHER. This study demonstrated the protection effect of this inhibitor in cancer cell lines, more evident in HepG2 and HUH-7 cells. The study suggests the interaction of this inhibitor with the TRAIL DR5 receptor and decoy receptors. As described by other authors [71], the HDACs inhibitors induce the apoptosis activating the intrinsic pathway and overexpressing the TRAIL DR5/DR4 receptor, so that the HAT inhibitor instead active the pathway to apoptosis and the overexpression of the receptors, this inhibitor MG149 could be able to block this pathways. To prove this hypothesis, it will be necessary to study the expression of these receptors in the surface of the cells during the treatment. Moreover, to determine whether they block some apoptotic pathway, it will be required to study the activation of the caspase cascade pathways and other pathways involved in cellular apoptosis. Concerning the C646 inhibitor, this can be used as a protection to cells, but this protection is not 100% efficient. This feature can be a positive effect of the interaction with rhTRAIL DHER/C646, because the protein can bind to the specific receptor. Though some of the normal cells may die, the combination of the inhibitor with the protein will block the apoptotic pathways of the cells. To confirm this hypothesis, it is necessary to study what pathways this interaction activates and the expression of death receptors and decoys receptors in the surface. Moreover, the concentration used, both for rhTRAIL proteins and for HAT or HDACs inhibitors, seems to be adequate for these drugs.

In a clinical perspective, the HDACs inhibitors combined with rhTRAIL protein are important to induce apoptosis in liver fibrosis, cancers and resistant cancer cells. Furthermore, the HAT inhibitors, like MG149 and C646, can be used as a protective enclosure of the cells that do not overexpress the death receptors. In addition, the use of adenoviral vectors to produce the protein in target cells will increase the efficiency of this type of treatment for liver fibrosis, because the production of the protein it will be constant during the time of the treatment, inducing a more efficacy apoptosis.

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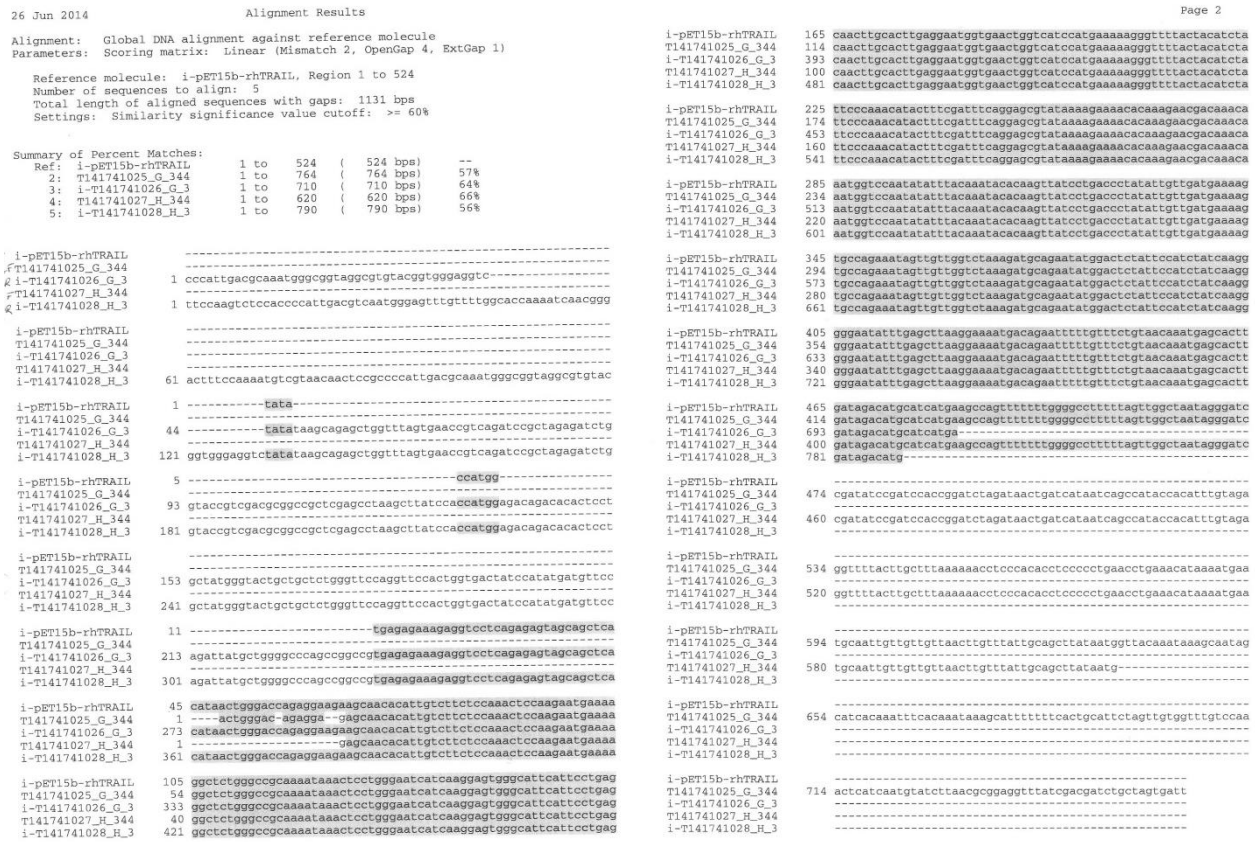
Appendix

A.1. Sequencing

After the cloning of the rhTRAIL DHER gene into the pADTRACK-CMV-IGK-HA-rhTRAIL DHER, two colonies were selected and send to sequencing. The first line corresponds to the rhTRAIL DHER gene from the pET15b-rhTRAIL DHER; the second line is the forward sequencing of the colony pADTRACK-CMV-IGK-HA-rhTRAIL DHER-A; The third line is the reverse sequencing of the colony pADTRACK-CMV-IGK-HA-rhTRAIL DHER-A; the fourth is the forward sequencing of the colony pADTRACK-CMV-IGK-HA-rhTRAIL DHER-B; and the fifth is the reverse sequencing of the colony pADTRACK-CMV-IGK-HA-rhTRAIL DHER-B.



26 Jun 2014 Alignment Results

Alignment: Global DNA alignment against reference molecule
Parameters: Scoring matrix: Linear (Mismatch 2, OpenGap 4, ExtGap 1)

Reference molecule: i-pET15b-rhTRAIL, Region 1 to 524
Number of sequences to align: 5
Total length of aligned sequences with gaps: 1131 bps
Settings: Similarity significance value cutoff: >= 60%

Summary of Percent Matches:

	Ref:					
2:	i-pET15b-rhTRAIL	1 to	524	(524 bps)	--
3:	T141741025_G_344	1 to	764	(764 bps)	57%
4:	i-T141741026_G_3	1 to	710	(710 bps)	64%
5:	T141741027_H_344	1 to	620	(620 bps)	66%
	i-T141741028_H_3	1 to	790	(790 bps)	56%

Page 2

i-pET15b-rhTRAIL
T141741025_G_344
i-T141741026_G_3
T141741027_H_344
i-T141741028_H_3

i-pET15b-rhTRAIL
T141741025_G_344
i-T141741026_G_3
T141741027_H_344
i-T141741028_H_3 61 actttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtac

i-pET15b-rhTRAIL 1 ----------tata----------
T141741025_G_344
i-T141741026_G_3 44 ----------tataataagcagagctggtttagtgaaccgtcagatccgctagagatctg
T141741027_H_344
i-T141741028_H_3 121 ggtgggaggtctatataagcagagctggtttagtgaaccgtcagatccgctagagatctg

i-pET15b-rhTRAIL 5 ----------ccatgg----------
T141741025_G_344
i-T141741026_G_3 93 gtaccgtcgacgcggccgctcgagcctaagcttatccaccatggagacagacacactcct
T141741027_H_344
i-T141741028_H_3 181 gtaccgtcgacgcggccgctcgagcctaagcttatccaccatggagacagacacactcct

i-pET15b-rhTRAIL
T141741025_G_344
i-T141741026_G_3 153 gctatgggtactgctgctctgggttccaggttccactggtgactatccatatgatgttcc
T141741027_H_344
i-T141741028_H_3 241 gctatgggtactgctgctctgggttccaggttccactggtgactatccatatgatgttcc

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T141741027_H_344
i-T141741028_H_3 301 agattatgctggggcccagccggccgtgagagaaagaggtcctcagagagtagcagctca

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T141741027_H_344 1 ----------gagcaacacattgtcttctccaaactccaagaatgaaaa
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i-pET15b-rhTRAIL 105 ggctctgggccgcaaaataaactcctgggaatcatcaaggagtgggcattcattcctgag
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i-pET15b-rhTRAIL 165 caacttgcacttgaggaatggtgaactggtcatccatgaaaaaggttttactacatcta
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i-T141741028_H_3 781 gatagacatg----------

i-pET15b-rhTRAIL
T141741025_G_344 474 cgatatccgatccaccggatctagataactgatcataatcagccataccacatttgtaga
i-T141741026_G_3
T141741027_H_344 460 cgatatccgatccaccggatctagataactgatcataatcagccataccacatttgtaga
i-T141741028_H_3

i-pET15b-rhTRAIL
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i-T141741026_G_3
T141741027_H_344 520 ggttttacttgctttaaaaaacctcccacacctcccccctgaacctgaaacataaaatgaa
i-T141741028_H_3

i-pET15b-rhTRAIL
T141741025_G_344 594 tgcaattgttgttgttaacttgtttattgcagcttatataatggttacaaataaagcaatag
i-T141741026_G_3
T141741027_H_344 580 tgcaattgttgttgttaacttgtttattgcagcttatataatg----------
i-T141741028_H_3

i-pET15b-rhTRAIL
T141741025_G_344 654 catcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaa
i-T141741026_G_3
T141741027_H_344
i-T141741028_H_3

i-pET15b-rhTRAIL
T141741025_G_344 714 actcatcaatgtatcttaacgcggaggtttatcgacgatctgctagtgatt
i-T141741026_G_3
T141741027_H_344
i-T141741028_H_3

A.2. Constituents of enzymatic reactions

The restriction endonuclease digestions were performed to confirm the presence of the rhTRAIL DHER gene (Table A.1).

Table A.1: Restriction endonuclease digestions, with ClaI and EcoRI, of the rhTRAIL DHER gene to determine the presence of the gene.

	ClaI	**ClaI+EcoRI**	**EcoRI**
DNA (µL)	5.5	5.5	5.5
Enzyme (µL)	1	1	1
Enzyme (µL)	-------	1	-------
Buffer (µL)	2 (Buffer 4)	2 (Buffer 4)	2 (Buffer 3)
Demi Water (µL)	11.5	10.5	11.5
Total (µL)	20	20	20

In order to perform the recombination of the shuttle vector with the adenoviral vector, it was necessary to linearize the shuttle vector with the PmeI restriction endonuclease (Table A.2).

Table A.2: Restriction endonuclease digestions of the pADTRACK-CMV-IGK-HA-rhTRAIL DHER with PmeI to linearize the vector.

Component	**PmeI**
DNA (µL)	11
Enzyme (µL)	0.8
Enzyme (µL)	3
Buffer (µL)	0.5
Demi Water (µL)	14.7
Total (µL)	30

Concerning to the restriction endonuclease digestions to verify if the recombination of the pADTRACK-CMV-IGK-HA-rhTRAIL DHER with pAdEasy-1 occurs, were performed digestions with PacI, SfiI and ClaI restriction endonucleases, using as control the plasmid without recombination (Table A.3, A.4).

Table A.3: Restriction endonuclease digestions of the plasmids without recombination.

	pAdEasy-1				pADTRACK-CMV-IGK-HA-rhTRAIL DHER			
	PacI	**SfiI**	**PacI+ClaI**	**ClaI**	**PacI**	**SfiI**	**PacI+ClaI**	**ClaI**
DNA (µL)	2.5	2.5	2.5	2.5	4.7	4.7	4.7	4.7
Enzyme (µL)	1	1	1	1	1	1	1	1
Enzyme (µL)	------	------	1	------	------	------	1	------
Buffer (µL)	2 (Buffer 4)	2(Buffer 2)	2(Buffer 4)	2(Buffer 4)	2 (Buffer 4)	2(Buffer 2)	2 (Buffer 4)	2 (Buffer 4)
Demi Water(µL)	14.5	14.5	13.5	14.5	12.3	12.3	11.3	12.3
Total (µL)	20	20	20	20	20	20	20	20

Table A.4.: Restriction endonuclease digestions of the recombinants plasmids.

	PacI	**SfiI**	**PacI+ClaI**
DNA (µL)	11.2	11.2	11.2
Enzyme (µL)	0.8	0.8	0.8
Enzyme (µL)	------	------	0.8
Buffer (µL)	2 (Buffer 4)	2 (Buffer 2)	2 (Buffer 4)
Demi Water (µL)	6	6	5.2
Total (µL)	20	20	20

A.3.Generation of recombinant adenoviral plasmid in BJ5183 bacteria

The electrocompetent bacteria BJ5183 was transformed with the recombinant plasmid (pADTRACK-CMV-IGK-HA-rhTRAIL DHER + pAdEasy-1) and added in two plates with kanamycin and another plate with ampicillin.

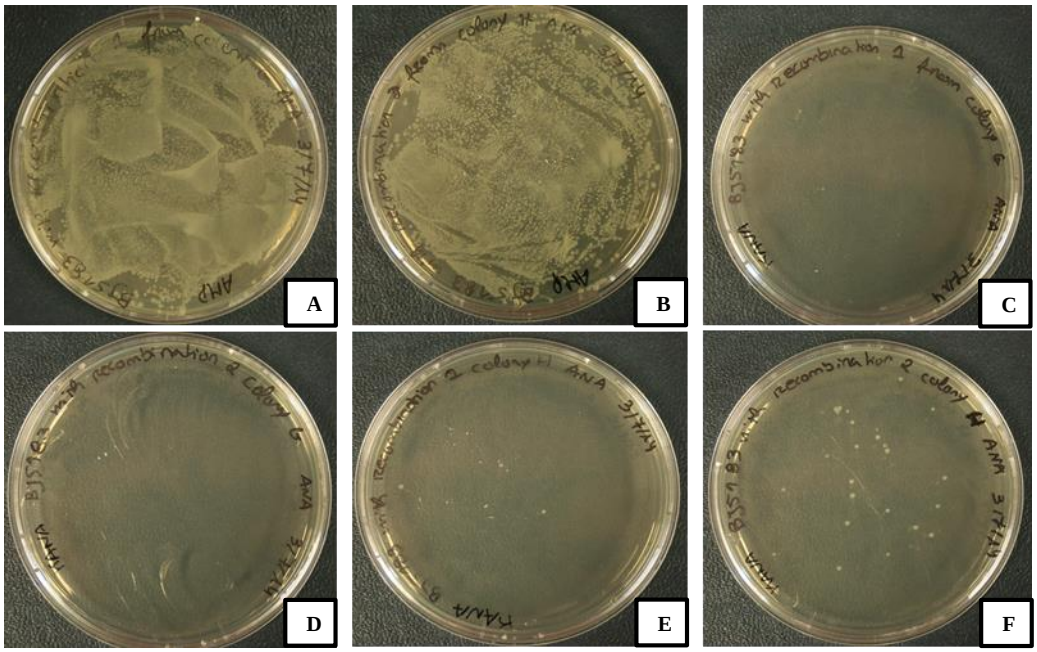

Figure A.1.: Plates containing selective antibiotic for the recombinant plasmids. (A) Plate containing ampicillin for the recombinant plasmid holding the pADTRACK-CMV-IGK-HA-rhTRAIL DHER A; (B) Plate containing ampicillin for the recombinant plasmid holding the pADTRACK-CMV-IGK-HA-rhTRAIL DHER B; (C) Plate with kanamycin for the bacteria suspension before centrifugation containing the recombinant plasmid holding the pADTRACK-CMV-IGK-HA-rhTRAIL DHER A; (D) Plate with kanamycin for the bacteria suspension after centrifugation containing the recombinant plasmid holding the pADTRACK CMV-IGK-HA-rhTRAIL DHER A; (E) Plate with kanamycin for the bacteria suspension before centrifugation containing the recombinant plasmid holding the pADTRACK-CMV-IGK-HA-rhTRAIL DHER B; (F) Plate with kanamycin for the bacteria suspension after centrifugation containing the recombinant plasmid holding the pADTRACK-CMV-IGK-HA-rhTRAIL DHER B.

A.4. Protocols used for molecular techniques

A.4.1. Electroporation protocol

The protocol from electroporation was created and adapted for the equipment and conditions in the Pharmaceutical Gene Modulation Laboratory, Groningen Research Institute of Pharmacy, University of Groningen:

1) Pre-cool cuvettes on ice;
2) Thaw electrocompetent bacteria on ice;
3) Add DNA to bacteria (0.5-1µL);
4) Incubate on ice for 30 seconds;
5) Transfer to electroporation cuvette;
6) Dry outside of cuvette before electroporation;
7) Insert cuvette in electroporation apparatus (setting Ec1 and check time constant on apparatus, around 5ms);
8) Add 500 µL of LB medium at RT into the cuvette as fast as possible;
9) Transfer bacterial suspension to Eppendorf tube
10) Allow bacteria to recover, and express antibiotic resistance at 37ºC in shaker for 1 hour;
11) Plate 50 µL of bacterial suspension on selective agar plate;,
12) Centrifuge remainder of the suspension 1 min at 5000 x g
13) Aspirate all but ~50 µL of the medium. Resuspend cells in the leftover 50 µL. Plate these on another plate.
14) Grow plates during the night at 37ºC.

A.4.2. FuGENE® HD transfection reagent protocol

This protocol was adapted from the recommended protocol of FuGENE® HD Transfection reagent (Promega®, Fitchburg, Wisconsin, USA):

1) One day before the transfection plate the cells (80% confluence for the transfection day);
2) On the transfection day,:

A. Dilute 400 ng DNA + 1.44 µL of FuGENE ®+ DMEM medium until 30uL of final volume, and leave 15min at RT;
B. Aspirate the medium of the cells;
C. Add 475 ml complete DMEM in the cells;
D. Add in each well 25 µL of the respectively mix;
E. Mix by pipetting gently;

Note: It is not necessary to remove serum or change culture conditions and the removal of transfection complex is not needed.

A.4.3. MTS assay protocol

The protocol from MTS assay was created and adapted for the equipment and conditions in the Pharmaceutical Gene Modulation Laboratory, Groningen Research Institute of Pharmacy, University of Groningen:

A. Preparation of MTS solution:
1) Wrap a 50 ml tube with aluminum foil;
2) Add 21 ml of PBS to the container;
3) Weigh out 42 mg of MTS reagent powder and add to PBS;
4) Mix until completely dissolved;
5) Measure the pH of the solution. Optimum pH is between pH 6.0-6.5;
6) Filter-sterilize MTS solution through a 0.2 µm filter into a sterile light protected;
7) Store MTS solution at -20°C.

B. Preparation of PMS solution:
1) Dissolve 9.2 mg of PMS in 10 ml of PBS;
2) Filter sterilize;
3) Store at -20°C in aluminum foil wrapped tube.

C. Assay:
1) Mix per well 20 µL of MTS and 1 µL of PMS per well assayed;
2) Add 100 µL of complete medium in each well that contain the MTS/PMS solution;
3) After 1-4hours read the absorbance in a plate reader equipment (synergy H1 hybrid Multi-Mode Microplate reader, Biotek®,Winooski, VT, USA).

A.4.4. Western Blotting protocol

The protocol for western blotting was created and adapted for the equipment and conditions in the Pharmaceutical Gene Modulation Laboratory, Groningen Research Institute of Pharmacy, University of Groningen:

A. Electrophoresis of the SDS Gel:

1) Take the APS out from -20ºC (keep on ice)
2) Prepare 12.5% running gel in 50 ml falcon tube (4.17 mL 30% AA/bis acrylamide; 2.5mL 1.5M Tris-HCl pH8.8; 50 µL 20% SDS, 3.23mL dH_2O, 5 µL TEMED, 50 µL 10% APS);
3) Pipet the separating gel (with pipet and pipet-boy) till about 1 cm under the green clip;
4) Cover with H_2O saturated with isobutanol (to smoothen surface and prevent oxidation of top layer of gel during polymerization);
5) Let to polymerize for 15-30 min (check the left-over in the tube to see if it has polymerized);
6) Remove isobutanol;
7) To do the stacking gel (4%) (667 µL 30% AA/bis acrylamide; 1250 µL 0.5M Tris-HCl pH6.8; 25 µL 20% SDS,3028 µL dH_2O, 2.5 µL TEMED, 25 µL 10% APS);
8) Pipette the stacking gel till the top (that it flows over a little bit), place comb (avoid bubbles), and let it polymerize (~15 min);
9) Thaw marker and samples (when taken from -20°C). Heat the samples to 95-110ºC, in heating block for 5 minutes;
10) Built electrophoresis chamber (thin glass inside);
11) Fill inner compartment with electrophoresis buffer;
12) Fill outer compartment with electrophoresis buffer until bottom of the gel is immersed;
13) Add proper amount of samples and marker (Page Ruler Plus Prestained Protein Ladder, Thermo Fisher Scientific® Waltham, Massachusetts, USA);
14) Electrophoresis: run at 70V through stacking gel and 150-200V through separating gel for 1,5-2h;

B. Blotting:

1) Take glass plate out of electrophoresis chamber;
2) Gently separate the 2 plates;
3) Place blot cassette in a container with blot buffer (black side down). Place in this order:

i. Sponge drenched in blot buffer
ii. 2 Whatman filter papers
iii. Gel
iv. PVDF membrane (activate prior blotting: 2 min in 100% methanol)
v. 2 Whatman filter papers
vi. Sponge

4) Close cassette, place in blot chamber (mind black to black);
5) Place cooler (-20 °C);
6) Fill with blotting buffer;
7) Blot about 1.5 h at 0,18-0,22 A (~ constant 0.25 A);

A.4.5. Purification of miniprep products

This protocol was used from the centrifugation protocol of PureYield™ Plasmid Miniprep System (Promega®, Fitchburg, Wisconsin, USA):

1) Centrifuge 1.5 ml of bacterial culture for 30 seconds at maximum speed in a micro centrifuge;
2) Discard the supernatant;
3) Add 600 µL of TE buffer and resuspend completely;
4) Add 100 µL of Cell Lysis Buffer, and mix by inverting the tube 6 times (to have a complete lysis the solution should be clear blue and excessive lysis can result in denatured plasmid DNA);
5) Add 350 µL of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting the tube (a yellow precipitate should be formed);
6) Centrifuge at maximum speed in a micro centrifuge for 3 minutes;
7) Transfer the supernatant (~900 µL) to a PureYield™ Minicolumn (do not disturb the cell pellet);
8) Place the minicolumn into a PureYield™ Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 1 minute;
9) Discard the flowthrough, and place the minicolumn into the same PureYield™ Collection Tube;
10) Add 200 µL of Endotoxin Removal Wash to the minicolumn. Centrifuge at maximum speed in a micro centrifuge for 1 minute;
11) Add 400 µL of Column Wash Solution to the minicolumn. Centrifuge at maximum speed in a micro centrifuge for 1 minute;

12) Transfer the minicolumn to a clean microcentrifuge tube, and add 30µl of Elution Buffer directly to the minicolumn matrix (stand for 1 min at RT);
13) Centrifuge at maximum speed in a microcentrifuge for 1 minute to elute the plasmid DNA;
14) Store eluted plasmid DNA at –20°C.

A.4.6. Purification of PCR products

This protocol was used from the DNA purification by centrifugation protocol of PCR Clean-Up system (Promega®, Fitchburg, Wisconsin, USA):

1) Add an equal volume of Membrane Binding Solution to the PCR amplification ;
2) Transfer the prepared PCR product to the SV Minicolumn assembly (incubated for 1minute at RT);
3) Centrifuge the SV Minicolumn assembly in a micro centrifuge at 16,000 × *g* (14,000rpm) for 1 minute. Remove the SV Minicolumn from the Spin Column assembly and discard the liquid in the Collection Tube. Return the SV Minicolumn to the Collection Tube;
4) Wash the column by adding 700µl of Membrane Wash Solution to the SV Minicolumn. Centrifuge the SV Minicolumn assembly for 1 minute at 16,000 × *g* (14,000rpm). Empty the Collection Tube as before and place the SV Minicolumn back in the Collection Tube.
5) Repeat the wash with 500µl of Membrane Wash Solution and centrifuge the SV Minicolumn assembly for 5 minutes at 16,000 × *g*.
6) Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
7) Carefully transfer the SV Minicolumn to a clean 1.5ml microcentrifuge tube.
8) Apply 50µl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip (incubate for 1minute at RT);
9) Centrifuge for 1 minute at 16,000 × *g* (14,000rpm).
10) Discard the SV Minicolumn and store the microcentrifuge tube containing the eluted DNA at 4°C or –20°C.

A.4.7. Protocol to create electrocompetent bacteria

This protocol was created and adapted for the equipment and conditions in the Pharmaceutical Gene Modulation Laboratory, Groningen Research Institute of Pharmacy, University of Groningen:

1) Inoculate 3 or 5 ml LB with 1 colony in 15 ml tube (incubate overnight at 37ºC);
2) Add 5ml of LB to 250ml LB, take sample for A600 reading;
3) Grow for 4-5 hours (calculate, doubling time = 30 min) until A600=0.75 – 0.9;
4) Collect cells in 250 ml flask (wash with 70% EtOH > 100% EtOH > 70% EtOH – allow to dry);
5) Incubate on ice for 30-60 min (preferentially 60 minutes);
6) Centrifuge 4000 x g, 4ºC for 10 min;
7) Wash pellet with 250 ml Wash Buffer (10% Glycerol in 1L MiliQwater);
8) Centrifuge 4000 x g, 4ºC for 30 min;
9) Wash with 125 ml Wash Buffer;
10) Centrifuge 4000 x g, 4ºC for 30 min
11) Discard the supernatant, leaving only 15ml to resuspend and transfer to a 50ml tubes;
12) Wash flask with 15 ml Wash Buffer;
13) Centrifuge 2500 g, 4ºC for 10 minutes;
14) Aspirate to < 1 ml;
15) Resuspend pellet in remaining WB;
16) Aliquot 20 ml in prechilled tubes – 80ºC (for immediate freezing);
17) Store aliquots at -80ºC.

A.5. Tables from the assays with SW948, H460, HUH-7 and HepG2

The results obtained after the experiments with the combination of HDAC and HAT inhibitors with rhTRAIL variants, for the different cells: SW948, H460, HUH-7 and HepG2.

Table A.5: The cells SW948 containing the rhTRAIL variants alone, and in combination with HDAC and HAT inhibitors. The values in bold correspond to the values described in the content of the thesis.

					SAHA		Entinostat		C646		MG149	
			Concentration rhTRAIL (ng/mL)	Without inhibitors (%)	1μM (%)	5μM (%)	0.01μM (%)	0.05μM (%)	10μM (%)	50μM (%)	10μM (%)	50μM (%)
SW948	rhTRAIL DHER	48 hours	0	100.00 ± 53.03	62.60 ± 12.03	16.17 ± 3.38	63.17 ± 5.57	10.45 ± 1.46	78.23 ± 8.27	16.36 ± 5.96	104.24 ± 4.22	42.21 ± 36.43
			10	79.21 ± 3.96	22.57 ± 5.03	5.35 ± 3.20	16.70 ± 2.01	3.92 ± 6.98	52.68 ± 6.62	11.87 ± 10.17	**80.39 ± 20.04**	46.94 ± 2.62
			100	**71.04 ± 1.56**	20.30 ± 1.04	5.78 ± 1.66	20.73 ± 1.13	5.73 ± 1.53	53.08 ± 2.10	8.17 ± 2.31	86.19 ±8.32	45.62 ± 4.84
		72 hours	0	100.00 ± 50.49	**56.96 ± 3.72**	5.76 ± 3.64	62.66 ± 17.75	59.58 ± 11.01	77.63 ± 21.84	54.82 ± 15.87	59.43 ± 13.65	53.43 ± 31.87
			10	**21.80 ± 6.09**	**9.93 ± 4.17**	3.40 ± 0.17	9.58 ± 5.25	8.57 ± 10.75	12.99 ± 1.32	9.06 ± 2.95	12.55 ± 0.82	16.32 ± 3.58
			100	23.01 ± 2.82	8.35 ± 1.40	3.36 ± 2.80	9.48 ± 0.38	15.51 ± 3.96	10.61 ± 3.58	10.26 ± 3.46	11.70 ± 1.64	16.10 ± 4.67
	rhTRAIL 4C7	48 hours	0	100.00 ± 53.03	62.60 ± 12.03	16.17 ± 3.38	63.17 ± 5.57	10.45 ± 1.46	78.23 ± 8.27	16.36 ± 5.96	104.24 ± 4.22	42.21 ± 36.43
			10	91.44 ±6.35	34.25 ± 1.63	8.07 ± 3.12	16.48 ± 4.01	4.22 ± 7.48	46.47 ± 2.87	6.34 ± 2.24	**89.47 ± 3.76**	28.90 ± 7.05
			100	**87.45 ± 6.49**	37.60 ± 11.26	8.02 ± 3.41	21.54 ± 0.94	5.34 ± 1.05	50.31 ± 5.34	6.47 ± 5.01	87.84 ±8.16	31.82 ± 10.10
		72 hours	0	**100.00 ± 50.49**	29.72 ± 12.76	2.89 ± 0.71	38.72 ± 15.93	46.22 ± 4.41	32.77 ± 18.03	40.01 ± 5.15	40.45 ± 13.41	41.36 ± 10.10
			10	80.37 ± 36.12	2.84 ± 1.50	2.04 ± 1.27	1.61 ± 1.06	0.96 ± 1.24	2.04 ± 0.42	1.26 ± 0.60	1.09 ± 0.70	1.41 ± 1.33
			100	**4.26 ± 1.50**	1.09 ± 2.78	1.29 ± 0.55	2.03 ± 2.17	0.48 ± 0.78	1.31 ± 0.58	1.63 ± 0.15	0.98 ± 1.39	1.41 ± 0.25
	rhTRAIL WT	48 hours	0	100.00 ± 53.03	62.60 ± 12.03	16.17 ± 3.38	63.17 ± 5.57	10.45 ± 1.46	78.23 ± 8.27	16.36 ± 5.96	104.24 ± 4.22	42.21 ± 36.43
			10	97.04 ± 10.48	25.18 ± 5.42	4.70 ± 0.49	22.48 ± 3.59	4.05 ± 7.33	56.19 ± 5.51	5.06 ± 0.97	95.42 ± 8.34	32.32 ± 6.73
			100	78.98 ± 29.80	20.72 ± 0.67	4.02 ± 0.19	18.77 ± 4.87	4.47 ± 0.58	44.44 ± 8.49	4.27 ± 0.65	87.08 ± 14.07	29.90 ± 15.27
		72 hours	0	100.00 ± 50.49	37.01 ± 7.93	1.56 ± 1.78	33.95 ± 5.21	34.55 ± 15.13	37.53 ± 4.55	39.06 ± 14.30	43.47 ± 12.75	30.20 ± 31.21
			10	7.40 ± 2.82	7.88 ± 0.13	0.79 ± 0.48	8.05 ± 1.40	3.60 ± 4.83	5.22 ± 0.72	4.48 ± 2.32	5.32 ± 0.61	6.39 ± 4.79
			100	7.32 ± 6.43	4.61 ± 0.95	0.97 ± 1.77	8.05 ± 1.40	3.60 ± 4.83	4.46 ± 6.36	1.69 ± 2.92	4.81 ± 2.26	6.10 ± 1.71

Table A.6: The cells H460 containing the rhTRAIL variants alone, and in combination with HDAC and HAT inhibitors. The values in bold correspond to the values described in the content of the thesis.

					SAHA		Entinostat		C646		MG149	
			Concentration rhTRAIL (ng/mL)	Without inhibitors (%)	1μM (%)	5μM (%)	0.01μM (%)	0.05μM (%)	10μM (%)	50μM (%)	10μM (%)	50μM (%)
H460	rhTRAIL DHER	48 hours	0	100.00 ± 22.58	92.77 ± 8.75	48.88 ± 7.03	104.06 ± 4.67	87.88 ± 7.44	85.31 ± 16.33	98.35 ± 8.63	95.45 ± 10.22	48.22 ± 10.29
			10	87.13 ± 4.35	23.00 ± 6.27	8.92 ± 4.35	62.60 ± 47.17	24.47 ± 44.59	57.11 ± 9.13	6.97 ± 2.29	70.12 ± 3.53	24.07 ± 1.89
			100	64.73 ± 32.17	14.08 ± 7.35	5.46 ± 4.79	70.46 ± 12.90	18.97 ± 21.83	32.64 ± 16.65	5.18 ± 0.71	73.76 ± 7.42	17.29 ± 9.32
		72 hours	0	100 ± 64.68	90.42 ± 10.27	38.95 ± 10.80	100.26 ± 20.07	107.86 ± 27.78	99.24 ± 11.74	71.36 ± 70.82	109.13 ± 32.89	86.14 ± 15.77
			10	**67.52 ± 1.10**	66.75 ± 13.07	9.18 ± 4.82	**74.66 ± 2.68**	45.50 ± 60.07	68.15 ± 5.32	52.80 ± 3.38	**77.79 ± 14.25**	61.14 ± 5.55
			100	73.10 ± 5.66	56.35 ± 5.83	7.43 ± 0.61	69.30 ± 12.37	65.05 ± 9.58	63.49 ± 13.69	42.53 ± 19.09	63.92 ± 4.58	62.91 ± 7.41
	rhTRAIL 4C7	48 hours	0	100.00 ± 22.58	92.77 ± 8.75	48.88 ± 7.03	104.06 ± 4.67	87.88 ± 7.44	85.31 ± 16.33	98.35 ± 8.63	95.45 ± 10.22	48.22 ± 10.29
			10	**14.65 ± 6.65**	5.73 ± 2.23	3.25 ± 0.03	**16.66 ± 9.44**	2.20 ± 4.13	6.83 ± 1.03	1.74 ± 0.72	19.13 ± 4.91	10.94 ± 1.86
			100	6.61 ± 3.53	4.82 ± 0.19	2.18 ± 0.25	8.30 ± 4.46	2.66 ± 3.11	3.78 ± 0.67	1.22 ± 0.82	10.52 ± 4.80	6.44 ± 5.90
		72 hours	0	100 ± 64.68	69.15 ± 18.15	29.40 ± 4.18	76.50 ± 14.45	81.15 ± 7.42	76.53 ± 15.45	84.76 ± 21.86	79.59 ± 14.82	64.16 ± 42.33
			10	**13.17 ± 1.63**	11.94 ± 3.55	2.62 ± 1.59	11.60 ± 1.12	4.54 ± 6.23	8.18 ± 2.15	7.40 ± 2.00	7.69 ± 3.08	7.99 ± 1.06
			100	7.14 ± 1.98	9.59 ± 0.46	2.09 ± 0.93	9.63 ± 1.06	6.23 ± 1.73	4.97 ± 4.88	5.22 ± 0.85	7.61 ± 1.24	8.82 ± 3.13
	rhTRAIL WT	48 hours	0	100.00 ± 22.58	92.77 ± 8.75	48.88 ± 7.03	104.06 ± 4.67	87.88 ± 7.44	85.31 ± 16.33	98.35 ± 8.63	95.45 ± 10.22	48.22 ± 10.29
			10	68.83 ± 7.44	31.13 ± 1.55	17.82 ± 3.46	66.25 ± 11.58	18.70 ± 34.24	37.25 ± 4.17	29.43 ± 7.67	68.39 ± 6.58	23.39 ± 8.83
			100	49.64 ± 16.59	24.78 ± 9.77	10.98 ± 3.24	43.31 ± 7.67	14.41 ± 2.52	22.49 ± 6.92	20.19 ± 2.34	52.46 ± 12.06	18.25 ± 14.59
		72 hours	0	**100.00 ± 64.68**	72.80 ± 16.71	21.75 ± 25.52	82.74 ± 18.61	101.91 ± 15.09	90.49 ± 24.11	98.62 ± 9.26	89.31 ± 16.96	83.92 ± 9.82
			10	**61.16 ± 10.66**	44.05 ± 10.27	6.85 ± 1.18	65.59 ± 6.59	28.42 ± 37.51	58.50 ± 3.35	46.04 ± 6.65	59.66 ± 13.53	68.36 ± 20.61
			100	50.44 ± 4.90	42.15 ±11.20	5.88 ± 3.93	48.68 ± 16.72	32.30 ± 18.36	47.82 ± 13.70	45.48 ± 11.35	45.70 ± 10.61	44.72 ± 12.11

Table A.7: The cells HUH-7 containing the rhTRAIL variants alone, and in combination with HDAC and HAT inhibitors. The values in bold correspond to the values described in the content of the thesis.

					SAHA		Entinostat		C646		MG149	
			Concentration rhTRAIL (ng/mL)	Without inhibitors (%)	1μM (%)	5μM (%)	0.01μM (%)	0.05μM (%)	10μM (%)	50μM (%)	10μM (%)	50μM (%)
HUH-7	rhTRAIL DHER	48 hours	0	100.00 ± 5.22	75.83 ± 6.96	24.09 ± 3.50	96.19 ± 8.52	77.47 ± 5.84	72.07 ± 2.49	38.83 ± 4.43	102.54 ± 8.54	54.84 ± 8.08
			10	86.54 ± 5.54	38.91 ± 2.68	10.77 ± 1.12	74.41 ± 2.38	26.44 ± 25.41	27.64 ± 2.95	22.94 ±2.90	84.86 ± 4.20	53.33 ± 0.50
			100	78.58 ± 11.70	32.64 ± 2.37	9.95 ± 0.57	70.24 ± 7.87	32.74 ± 6.28	10.54 ± 1.32	15.74 ± 2.84	75.82 ± 10.54	42.40 ± 8.70
		72 hours	0	100.00 ± 17.79	48.70 ± 2.68	13.80 ± 2.30	84.89 ±13.85	78.66 ± 3.14	72.31 ± 3.95	20.26 ± 1.13	97.17 ± 3.64	54.24 ± 1.62
			10	**65.84 ± 1.23**	42.96 ± 3.56	4.93 ± 1.21	90.45 ± 1.90	56.25 ± 25.38	76.88 ± 3.33	19.31 ± 3.45	**110.77 ± 5.90**	**53.31 ± 5.27**
			100	53.75 ± 4.23	21.71 ± 5.32	2.57 ± 1.95	76.85 ± 3.49	57.63 ± 4.81	72.60 ± 11.07	14.02 ± 2.22	93.00 ± 7.16	48.10 ± 3.37
	rhTRAIL 4C7	48 hours	0	100.00 ± 5.22	75.83 ± 6.96	24.09 ± 3.50	96.19 ± 8.52	77.47 ± 5.84	72.07 ± 2.49	38.83 ± 4.43	102.54 ± 8.54	54.84 ± 8.08
			10	99.93 ± 7.93	62.74 ± 2.19	18.14 ± 3.48	89.97 ± 1.13	49.19 ± 47.33	57.02 ± 2.74	20.17 ± 1.04	92.45 ± 1.19	60.64 ± 8.78
			100	93.06 ± 3.81	60.56 ± 6.33	16.04 ± 0.82	87.80 ± 13.75	56.10 ± 10.10	56.45 ± 8.83	14.98 ± 2.68	87.46 ± 12.81	45.50 ± 11.17
		72 hours	0	100.00 ± 17.79	48.70 ± 2.68	13.80 ± 2.30	84.89 ± 13.85	78.66 ± 3.14	72.31 ± 3.95	20.26 ± 1.13	97.17 ± 3.64	54.24 ± 1.62
			10	**65.22 ± 4.18**	**16.13 ± 1.36**	6.78 ± 0.35	**44.14 ± 0.84**	27.79 ±12.76	**41.63 ± 2.33**	13.10 ± 1.30	**54.19 ± 2.75**	23.41 ± 1.57
			100	49.50 ± 4.65	21.49 ± 0.26	5.16 ± 0.26	60.48 ± 6.61	51.09 ± 6.81	47.21 ± 2.89	8.26 ± 3.21	71.38 ± 4.26	35.83 ± 3.14
	rhTRAIL WT	48 hours	0	100.00 ± 5.22	75.83 ± 6.96	24.09 ± 3.50	96.19 ± 8.52	77.47 ± 5.84	72.07 ± 2.49	38.83 ± 4.43	102.54 ± 8.54	54.84 ± 8.08
			10	98.05 ± 3.42	52.56 ± 7.46	11.13 ± 2.00	82.34 ± 0.47	36.45 ± 35.30	62.12 ± 8.14	30.83 ± 1.25	89.57 ± 8.34	36.75 ± 7.54
			100	89.92 ± 7.52	42.90 ± 6.85	11.10 ± 1.32	83.85 ± 10.65	45.66 ± 3.55	52.27 ± 1.40	17.11 ± 1.67	84.30 ± 13.90	38.50 ± 2.04
		72 hours	0	100.00 ± 17.79	48.70 ± 2.68	13.80 ± 2.30	84.89 ± 13.85	78.66 ± 3.14	72.31 ± 3.95	20.26 ± 1.13	97.17 ± 3.64	54.24 ± 1.62
			10	**94.51 ± 0.99**	**26.07 ± 4.30**	8.89 ± 0.58	94.88 ± 2.28	42.68 ± 19.31	**63.28 ± 3.97**	19.35 ± 3.12	89.89 ± 4.15	40.37 ± 0.83
			100	86.83 ± 4.75	25.52 ± 2.69	5.86 ± 1.82	75.26 ± 4.87	70.71 ± 6.51	91.22 ± 21.03	13.17 ± 4.19	85.75 ± 2.07	46.77 ± 5.89

Table A.8: The cells HepG2 containing the rhTRAIL variants alone, and in combination with HDAC and HAT inhibitors. The values in bold correspond to the values described in the content of the thesis.

					SAHA		Entinostat		C646		MG149	
			Concentration rhTRAIL (ng/mL)	Without inhibitors (%)	1μM (%)	5μM (%)	0.01μM (%)	0.05μM (%)	10μM (%)	50μM (%)	10μM (%)	50μM (%)
HepG2	rhTRAIL DHER	48 hours	0	100.00 ± 24.89	40.88 ± 2.64	10.08 ± 2.37	79.71 ± 19.08	40.31 ± 9.52	74.46 ± 29.64	18.60 ± 1.87	86.64 ± 18.65	32.42 ± 15.23
			10	**65.83 ± 8.37**	14.65 ± 1.22	5.53 ± 0.74	11.17 ± 2.00	5.06 ± 6.76	50.97 ± 3.89	7.88 ± 3.27	**77.62 ± 6.66**	48.48 ± 3.27
			100	52.88 ± 9.15	15.52 ± 2.98	5.31 ± 1.95	9.51 ± 3.12	4.36 ± 0.88	51.84 ± 25.97	5.93 ± 3.62	73.46 ± 42.62	45.80 ± 19.40
		72 hours	0	100.00 ± 60.41	105.05 ± 6.72	45.95 ± 6.56	117.57 ± 18.68	126.04 ±8.64	106.35 ± 11.51	148.96 ±19.17	112.88 ± 10.18	126.19 ± 11.05
			10	**114.99 ± 12.21**	67.74 ± 59.34	1.37 ± 3.21	123,61 ± 23.99	54.16 ±36.23	109.39 ± 11.64	93.66 ± 74.65	**140.91 ± 26.95**	127.47 ± 22.12
			100	118.77 ± 26.47	61.49 ± 25.51	4.83 ± 1.02	107.73 ± 5.70	104.98 ± 8.08	124.14 ± 8.06	120.23 ± 7.61	118.07 ± 7.00	108.08 ± 5.67
	rhTRAIL 4C7	48 hours	0	100.00 ± 24.89	40.88 ± 2.64	10.08 ± 2.37	79.71 ± 19.08	40.31 ± 9.52	74.46 ± 29.64	18.60 ± 1.87	86.64 ± 18.65	32.42 ± 15.23
			10	10.04 ± 5.48	4.30 ± 0.93	5.71 ± 2.27	5.88 ± 1.41	3.91 ± 4.89	5.45 ± 0.87	5.43 ± 1.95	6.91 ± 3.11	4.95 ± 2.03
			100	6.32 ± 0.78	4.27 ± 0.80	4.83 ± 1.22	5.63 ± 0.12	5.33 ± 1.80	5.71 ± 3.20	6.38 ± 0.93	4.89 ± 0.84	4.79 ± 1.89
		72 hours	0	100.00 ± 60.41	107.22 ± 10.12	65.54 ± 14.17	113.03 ± 4.17	147.13 ±20.64	109.83 ± 17.84	120.84 ± 3.40	114.81 ± 10.88	122.85 ± 8.58
			10	**146.31 ± 21.45**	108.96 ± 12.74	38.31 ± 6.71	125.76 ± 10.58	79.05 ± 52.26	125.93 ± 6.57	132.26 ± 21.11	**134.65 ± 6.60**	**123.49 ± 12.22**
			100	117.81 ± 14.36	113.17 ± 33.00	32.49 ± 1.53	110.48 ± 7.89	117.11 ± 13.39	115.97 ± 13.43	115.52 ± 13.24	136.60 ± 20.64	111.59 ± 10.17
	rhTRAIL WT	48 hours	0	100.00 ± 24.89	40.88 ± 2.64	10.08 ± 2.37	79.71 ± 19.08	40.31 ± 9.52	74.46 ± 29.64	18.60 ± 1.87	86.64 ± 18.65	32.42 ± 15.23
			10	31.98 ± 4.68	31.38 ± 9.13	7.44 ± 4.51	22.56 ± 5.79	5.00 ± 6.05	16.71 ± 1.30	6.55 ± 1.91	21.56 ± 1.57	16.68 ± 2.54
			100	18.43 ± 5.12	12.37 ± 2.86	3.70 ± 0.61	7.61 ± 0.76	4.27 ± 1.23	5.61 ± 1.71	4.90 ± 1.36	8.42 ± 1.01	7.07 ± 0.58
		72 hours	0	100.00 ± 60.41	115.85 ± 18.41	42.42 ± 3.99	122.86 ± 8.86	131.81 ± 13.38	105.94 ± 16.90	153.54 ±25.45	103.57 ± 15.88	129.33 ± 19.22
			10	**119.71 ± 8.36**	85.01 ± 16.88	6.87 ± 1.49	**87.96 ± 3.33**	81.01 ± 57.28	**129.00 ± 24.80**	108.71 ± 7.90	**113.81 ± 8.51**	95.05 ± 4.69
			100	140.58 ±45.95	66.81 ± 3.35	61.11 ± 34.39	88.04 ± 4.05	105.03 ± 11.37	127.62 ± 25.82	102.38 ± 1.73	109.29 ± 6.72	97.97 ± 3.66