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Measuring DNA lesions induced by chemotherapeutic agents – DNA repair and DNA Damage

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Resumo

Diariamente, as células estão expostas a uma variedade de agentes que podem conduzir a lesões no DNA e a sua acumulação poderá ser um indutor da cancerigénese induzindo a acumulação de mutações que originam erros genéticos. Lesões no DNA podem surgir devido a vários factores externos ou internos às células assim como de ineficiências em processos de reparação de DNA. Os agentes antineoplásicos têm como alvo danificar o DNA e são um dos tipos de terapêutica mais utilizados no tratamento do cancro. A primeira linha de defesa contra as lesões no DNA está atribuída à via de reparação BER.

O objectivo principal deste estudo foi a avaliação do papel da via BER através da avaliação de lesões no DNA induzidas por agentes antineoplásicos. Três genes que actuam na BER foram selecionados e individualmente silenciados em células HeLa: *APE1*, *PARP1* e *XRCC1*. Estas linhas silenciadas foram expostas, individualmente, a três agentes antineoplásicos diferentes utilizados na terapia do cancro: doxorubicina (DOX); paclitaxel (PAX); e 5-fluorouracilo (5-FU). Seguidamente foi quantificada a lesão induzida por esses agentes através do ensaio do cometa.

Os nossos resultados mostram que, globalmente, as linhas silenciadas quando comparadas com a linha controlo apresentam maior resistência à exposição das drogas, observada pela diminuição de lesão quantificada. Adicionalmente, as lesões provocadas por 5-FU e PAX, medidas em % de DNA na cauda, apresentaram um comportamento semelhante ao obtido para o controlo negativo. No entanto, os efeitos medidos após a exposição a H_2O_2 demonstraram o contrário, com as linhas silenciadas ao apresentar maior lesão do que a linha controlo, o que pode ser representativo do tipo de lesão associada.

Em conclusão, o impacto do silenciamento de cada gene em testes genotóxicos não ficou totalmente esclarecido sendo que serão necessários futuros testes (e.g. ensaios de apoptose) de maneira a melhor entender o papel dos genes da via BER em lesões induzidas no DNA.

Palavras-chave: Via de reparação BER; Ensaio do cometa; danos no DNA; células HeLa; Reparação de DNA; Agentes antineoplásicos.

Abstract

DNA-damaging chemotherapy is amongst the main kinds of cancer treatment. Conversely, our cells are exposed to a wide variety of factors that may cause DNA lesions which, by themselves, may be precursors of cancer development. Cancer genomes, as a whole, accumulate mutations ('driver' and 'passenger' mutations) which may amount to more than 1000 in cancer-associated genes. These mutations may ensue from a multiplicity of exogenous and endogenous DNA damaging agents, as well as by less proficient DNA repair processes. The first line of cell defense against DNA damage is attributed to BER pathway.

The main aim of this study was evaluate the role of BER pathway through the measure of DNA lesions induced by chemotherapeutic agents. Three genes acting on BER pathway were individually silenced in HeLa cells: *PARP1*, *XRCC1* and *APE1*. These silenced cell-lines were then exposed individually to three different chemotherapeutic agents used in cancer therapy: doxorubicin (DOX); paclitaxel (PAX); and 5-fluorouracil (5-FU), and the DNA damage induced by these agents was measured by comet assay.

Our results showed that, globally, silenced cell lines were slightly resistant to drugs exposure than wild-type ones. Furthermore, the effect of DNA lesion measured by % DNA in tail induced by 5-FU and PAX was similar to the one reported for negative control in all cell lines. However, the effect measured for H_2O_2 (Positive Control) exposure was the inverse, the silenced cell lines showed much more lesion that the wild-type, which might be representative of the lesion type.

In conclusion, the impact of each gene silencing on the genotoxicity of individual agents was not fully clear. Additional approaches able to measure other end-points (e.g. apoptosis) are fundamental to better understand the role of BER genes in DNA induced lesion.

Keywords: BER pathway; Comet assay; DNA damage; HeLa cells; DNA repair; Chemotherapeutic agents.

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List of Abbreviations, genes, proteins and chemicals

All genes names are denoted in accordance with HUGO Gene Nomenclature Committee. All protein names are denoted in accordance with The Universal Protein Resource UniProt.

%DNA head - percentage DNA in head

%DNA tail - percentage DNA in tail

3'OH - 3'-hydroxyl

5'dRP - 5' deoxyribose phosphate

5-FU – 5-fluorouracil

AIF - Apoptosis-inducing factor

ALS - alkali-labile sites

AP site - abasic site

APE1 – AP endonuclease 1

APE2 – AP endonuclease 2

BER – Base Excision Repair

BSA – Bovine Albumin Serum

DMEM - Dulbecco's Modified Eagle's Medium

DMSO - Dimethyl sulfoxide

DOX – Doxorubicin

DSBs – Double strand breaks

dTMP - Deoxythymidine monophosphate

dUMP - Deoxyuridine monophosphate

EDTA - Ethylenediaminetetraacetic acid

FDA - Food and Drug Administration

FdUTP - Fluorodeoxyuridine triphosphate

FEN1 - Flap endonuclease 1

FISH - Fluorescence in situ hybridization

FUTP - Fluorouridine triphosphate

GC-MS - Gas chromatography–mass spectrometry

HR – Homologous Recombination

KD - knock down

kDa – kilodaltons

LB - Laemmli buffer

LigIII - DNA ligase III

LMP - low melting point

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD+ - Nicotinamide adenine dinucleotide

NC - negative control

NER – Nucleotide Excision Repair

NHEJ – Non-Homologous End Joining

nM – nano molar

NMP - normal melting point

OTM - Olive Tail Moment

PAR - Poly(ADP-ribose)

PARG - Poly(ADP-ribose) glycohydrolase

PARP1 – poly(ADP-ribose) polymerase 1

PAX – Paclitaxel

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

PNKP - polynucleotide kinase

pol β - DNA polymerase β

PUA - α,β-unsaturated aldehyde

PVDF - polyvinylidene fluoride membrane

qRT-PCR - Quantitative real time PCR

ROS – Reactive Oxygen Species

rRNA - Ribosomal ribonucleic acid

RT - Room temperature

SBs - Strand breaks

SCGE - Single cell gel electrophoreses assay

SDS - Sodium dodecyl sulfate

SiRNA - Small interfering RNA

SSBs – Single Strand Breaks

TM - Tail moment

TS – Thymidylate synthase

UV – Ultraviolet

WST - Water-soluble Tetrazolium

WT – Wild type

XRCC1 – X-ray cross complementing protein 1

XTT - 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

1. Introduction

Cells are permanently exposed to reactive agents, either chemical or physical, and cellular components are hence permanently damaged and replaced. DNA is the only biological molecule that relies solely on repair of existing molecules, without any resynthesizes. It also can accumulate damage over a lifetime and is uniquely represented by one single copy in most cells. As a target of multiple endogenous and exogenous agents capable of causing DNA lesion it can be said that DNA is the largest molecule capable of accumulating numerous lesions and yet be kept intact (at best in germline and proliferating cells) (Hoeijmakers 2009). The bases in DNA are highly vulnerable to chemical modification, which can cause numerous lesions. This lesions can be converted into mutations by means of defective repair. When this happens their effect and changes are permanent and continually exert their effect on the respective descendant cells. One of the possible consequence of this mutations, besides cell death, is the loss of tumor-suppressor genes and the improper activation of oncogenes, which will trigger an uncontrolled cellular proliferation and the development of malignant cells (Hoeijmakers 2009).

DNA integrity is threatened from three sides. First, as (by)products of our own cell metabolism that generates reactive oxygen (e.g. one of the results of respiration) and nitrogen species, lipid peroxidation products, endogenous alkylating agents, estrogen and cholesterol metabolites and reactive carbonyl species are formed, all of which are able to damage DNA. The second side comes from the spontaneous reactions (of which mostly consist of hydrolysis), intrinsic to the chemical nature of DNA. Hydrolysis of nucleotide residues leave a non-instructive abasic site (AP site) and are capable of causing deaminations. Lastly, the third side is due to the damage caused by exogenous agents. Environmental agents such as the ultraviolet (UV) component of sunlight, and genotoxic agents like cigarette smoke, can cause alterations in the DNA structure, although this damage is somewhat avoidable with the appropriate protections (Hoeijmakers 2009; Hoeijmakers 2001).

It is estimated that the number of single strand breaks (SSBs) and the spontaneous base losses in nuclear DNA are around 10^4 per cell per day. If we account all the other types of spontaneous damage, the total number should rise to 10^5 lesions per cell per day. This number of lesions can be obtained with a single day of sun exposure, inducing up to 10^5 photoproducts in each exposed keratinocyte. These DNA injuries can lead to mutations that, as a consequence, cause the appearance of malignant cells leading to cancer. In other scenarios these mutations can cause also cell death or senescence. The outcome of this types of lesions can lead to cell-cycle arrest or cell death, and in some cases the long term effects can result in irreversible mutations contributing to oncogenesis (Hoeijmakers 2001).

In order to prevent lesions on the cell's vital genetic information, a series of different DNA repair systems are used. Since the problem of DNA damage has existed from the beginning of life it is only natural that the DNA repair systems had arisen at the same time. This explains why all known repair pathways are highly conserved. In mammals exist, at least, four mains (sometimes overlapping) damage repair pathways – Nucleotide Excision Repair (NER), Base Excision Repair (BER), homologous recombination (HR) and non-homologous end joining (NHEJ) (Figure 1).

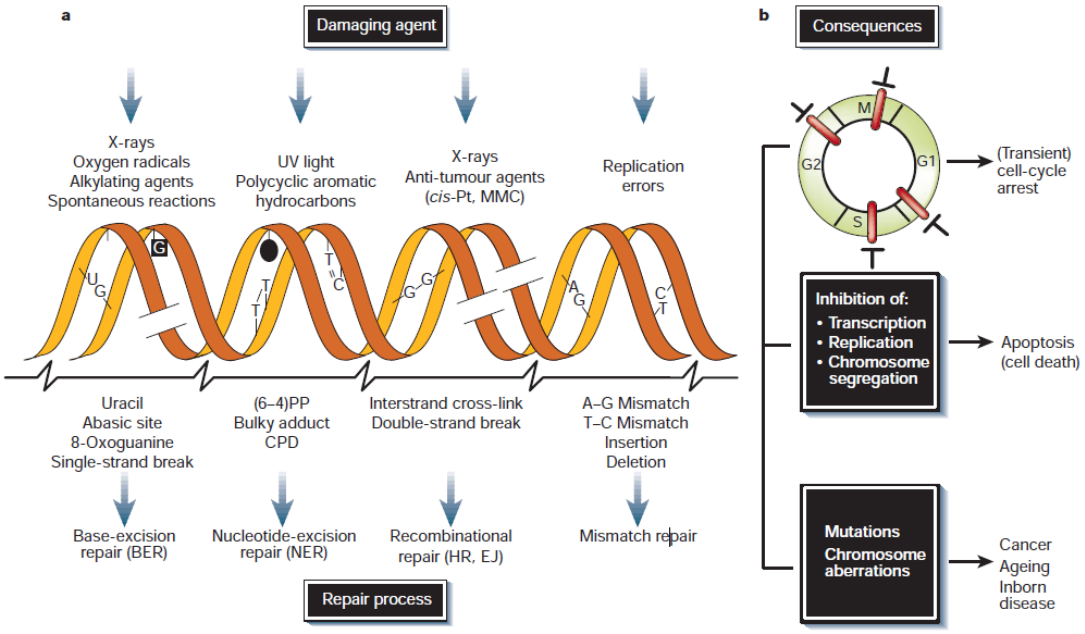

Figure 1 DNA damage, repair mechanisms and consequences. a. General DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and DNA mechanism responsible for the removal of the lesions (bottom). **b.** Critical effects of DNA damage on cell-cycle progression. Adapted from Hoeijmakers 2001

NER pathway treats helix-distorting lesions that can interfere with base pairing and obstruct normal replication and transcription, with the majority of the lesions arising from exogenous sources (Figure 1) (Hoeijmakers 2001). The BER pathway targets small chemical alterations mostly from endogenous origin. These alterations result from reactive oxygen species (ROS), methylations, deaminations, and hydroxylations meaning that although the effects of these alterations, may or may not, affect the same mechanism as the lesions treated by NER, these lesions can result in severe mutagenesis leading to oncogenesis. As such, BER is particularly relevant for preventing this mutagenesis (Hoeijmakers 2001). The lesions associated to these two repair pathways only affect one of the DNA strands. On the other hand, homologous

recombination and non-homologous end joining are designed to repair double strand breaks (DSBs). NHEJ acts during the S and G2 phase of replication, provided that a second copy of the sequence exists, for the aligning of the breaks. NHEJ repair system acts during G1 phase of the cell cycle, and it is used in situations where a second copy of the genetic material is not available as a template (Hoeijmakers 2001).

The focus of this work will be on the Base Excision Repair pathway as a way of understanding the importance of specific proteins involved in this pathway, when the cell DNA is exposed to different chemotherapeutic agents. In this study four independent HeLa cell lines were used. One cell line acted as a control cell line (wild-type), were no alteration was made in the genetic material of the cell. The remaining three cell lines have 3 specific genes silenced. One cell line had the poly(ADP-ribose) polymerase 1 (*PARP1*) gene silenced, another line had the AP endonuclease (*APE1*) gene silenced, and the last cell line had the X-ray cross complementing protein 1 (*XRCC1*) silenced. All of the silenced genes play major roles in the BER pathway, however the importance of each gene in repairing DNA lesions specific to different chemotherapeutic agents is not fully known. The cell lines were thus exposed to three different widely used chemotherapeutic agents: doxorubicin (DOX), 5-fluorouracil (5-FU), and paclitaxel (PAX). These agents are often used as a first line of cancer treatment, making it imperative the measurement of the DNA lesions for a better understanding of how these specific proteins impact the activity of the DNA repair mechanism of the BER pathway.

1.1. Base Excision Repair

Base excision repair (BER) is a highly conserved pathway from bacteria to humans and is responsible for repairing the vast majority of endogenous DNA damage including deaminations, depurinations, alkylations, oxidations, as well as single-strand breaks (SSBs), a total of about 30,000 per human cell per day (Wallace 2014). The first step in BER is recognition and removal of an altered base in DNA by DNA glycosylases. These enzymes remove the altered base by cleaving the N-glycosyl bond releasing the damaged base, and can either be monofunctional or bifunctional. If the enzyme is monofunctional, an abasic site (or AP site) results. This location in the DNA is characterized by the absence of a purine or a pyrimidine base. The AP sites are recognized by an apurinic endonuclease (APE1) which will cleave the site leaving a nick with a 3'-hydroxyl (3'OH) and 5' deoxyribose phosphate (5'dRP) termini (Wallace, Murphy, and Sweasy 2012). The 5'dRp at the nick is removed by the lyase activity of the DNA polymerase β (Pol β). DNA Pol β then inserts the missing base, with the resulting nick being sealed by a DNA ligase III complexed to an X-ray cross complementing enzyme 1 (XRCC1). If the glycosylase is bifunctional its associated lyase activity cleaves the DNA backbone and leaves either an α,β-

unsaturated aldehyde (PUA) or a phosphate group attached to the 3' end of the break. The PUA sites are removed by the diesterase activity of APE1 creating a 3' hydroxyl substrate for Pol β. If the result is a phosphate group, this one is removed by the phosphatase activity of polynucleotide kinase (PNKP) (Wallace, Murphy, and Sweasy 2012; Wallace 2014).

The BER pathway can follow up to two paths, the short patch or the long patch. In the short patch (also known as single nucleotide BER), the DNA Pol β inserts the missing base, with the resulting nick being sealed by a DNA ligase III (LigIII) complexed to XRCC1 (Figure 2) (Wallace, Murphy, and Sweasy 2012).

In long patch BER a different number of polymerases can take over, including the replicative polymerases. The damage-containing strand is displaced and removed by flap endonuclease 1 (FEN1). This enzyme will remove this flap from the DNA, leaving behind a nick in the DNA. 2-13 nucleotides are removed from the original site of damage, with the nick being sealed by Pol β, resulting in the addition of several nucleotides. XRCC1 and LigIII also seal the nick (Wallace, Murphy, and Sweasy 2012). The choice of whether repair is accomplished via short or long patch BER is mainly dependent on whether the abasic sugar is oxidized or reduced, as DNA Pol β cannot eliminate a modified sugar. This means that if the 5' sugar is modified, it is not removed by Pol β and long patch BER is initiated (figure 2) (Wallace, Murphy, and Sweasy 2012).

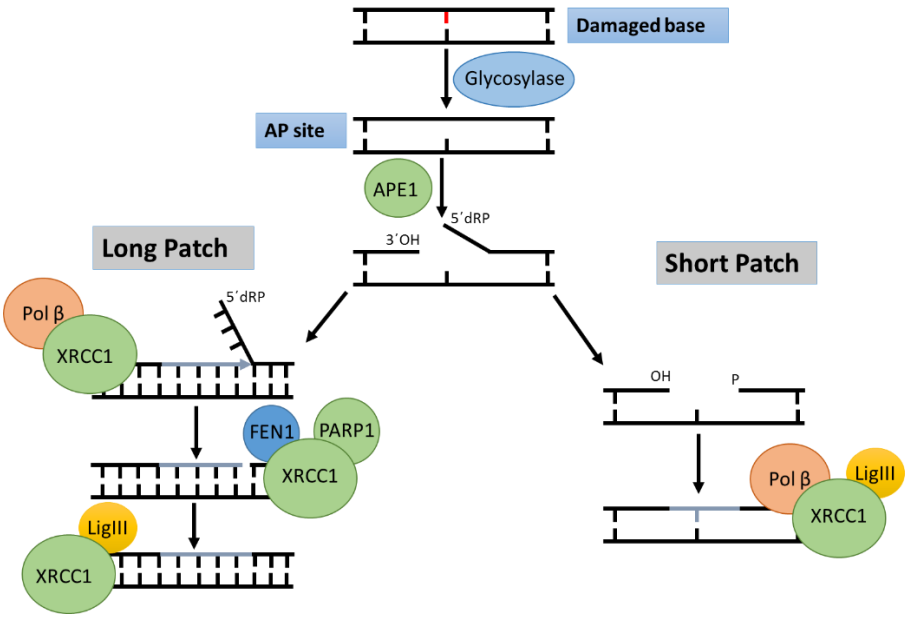

Figure 2: Schematic representation of the BER pathway.

1.1.1. AP endonuclease (APE1)

AP endonucleases (or apurinic/apyrimidinic endonucleases) are enzymes with the role of creating a nick in the phosphodiester backbone of the AP site. In the BER pathway these enzymes have the role of identifying and cleaving the AP site leaving a 3' OH and a 5' dRP. When an AP site is created by DNA glycosylase during BER, recruitment of APE1 may displace the DNA glycosylase from the abasic site and then both, recruit DNA Pol β and cleaves the abasic site (Wallace, Murphy, and Sweasy 2012), furthermore it is also known as a redox active enzyme.

In humans there are two AP endonucleases, APE1 and APE2. Human apurinic/apyrimidinic endonuclease 1 (APE1) is a ubiquitous multifunctional protein, a key player in BER pathway (Abbots and Madhusudan, 2010), responsible for the repair of cytotoxic abasic sites. AP endonuclease APE1 (also known as Hap1, Apex and Ref-1) displays the major AP endonuclease activity in mammalian cells and cleaves the 5' sugar phosphate backbone of abasic sites. This enzyme accounts for over 95% of the AP site processing in mammalian cells (Mol, Hosfield, and Tainer 2000).

Four different types of AP endonucleases have been classified according to their sites of incision and their products after cleavage. Class I and class II endonucleases, nick the DNA at the phosphate groups 3' and 5' resulting in a 3'-hydroxyl group and a 5'-deoxyribose phosphate group. Class III and class IV AP endonucleases also cleave the phosphate groups 3' and 5' resulting in a 3'-phosphate and a 5'-OH groups. This way the AP endonucleases present in humans are classified as class II endonucleases like majority of this enzymes (Marenstein, Wilson, and Teebor 2004).

1.1.2. Poly(ADP-ribose) polymerase 1 (PARP-1)

Poly(ADP-ribose) polymerases belong to a family of proteins involved in a series of cellular processes from DNA repair to programmed cell death. In this family of enzymes, the most abundant and studied protein is the poly(ADP-ribose) polymerase 1 (PARP-1). PARP1 is an abundant nuclear protein that can function as a molecular nick sensor and is important for genetic stability and for cellular resistance to extracellular or intracellular agents that may disrupt the DNA (Wallace, Murphy, and Sweasy 2012; Amé, Spenlehauer, and de Murcia 2004; Satoh and Lindahl 1992).

At low levels of genotoxic damage PARP-1 promotes the survival of the cell. However if the DNA is exposed to a lethal level of damage PARP-1 triggers cell death by promoting nuclear translocation of apoptosis-inducing factor (AIF). This is most likely accomplished via excessive

depletion of cellular NAD^+. When PARP-1 binds to sites of SSBs it results in dimerization of the enzyme triggering an elongation and transfer of a long linear or branched chains of poly(ADP-ribose) (PAR) onto various nuclear acceptors (heteromodification). This process may include PARP-1 itself (automodification) at the expense of NAD^+. Once a SSB is detected and PARP-1 binds to the DNA it acts as a signal for other DNA-repairing enzymes such as DNA ligase III, DNA polymerase β and scaffold proteins such as XRCC1. After repairing the DNA damage, the PAR chains are degraded via Poly(ADP-ribose) glycohydrolase (PARG) and the complex is released. This rapid dissociation of PARP-1 from the breaks is important to allow subsequent access to the break by other DNA repair proteins (Godon et al. 2008).

1.1.3. X-ray cross complementing protein 1 (XRCC1)

The XRCC1 protein plays a major role in enabling the repair of SSBs and in the BER pathway, due to its ability to interact with multiple core enzymes. XRCC1 acts as a scaffold protein and has no known enzymatic activity of its own, but scaffolding allows these repair enzymes to then carry out their enzymatic steps in repairing DNA. This enzyme is known to interact with proteins like Pol β, PARP-1, LigIII and APE1 and it has three globular domains connected by two linker segments of approximately 120 to 150 residues. The N-terminal domain binds to the DNA polymerase β, the C-terminal domain interacts with Lig III and the central domain contains a PAR binding motif (Wallace, Murphy, and Sweasy 2012; Caldecott 2003; London 2015). In the BER pathway, XRCC1 attaches to LigIII, where it appears to be required for the stability of cellular LigIII, preventing the degradation by proteosome. Besides preventing degradation XRCC1 also has the function of guiding the DNA ligase to the sites of stranded breakage (Caldecott 2003).

XRCC1 can interact with PARP-1 by distinguishing between “inactive” and “active” PARPs and this is accomplished by a degenerate consensus motif for interactions with PAR (Caldecott 2003).

1.2. Chemotherapeutic agents

1.2.1. Doxorubicin

Doxorubicin (DOX) is a frontline drug capable of intercalating with base pairs of DNA's double helix. It has been used in the treatment of cancer for over 35 years. This nonselective chemotherapeutic agent is a class I anthracycline with aglyconic and sugar moieties. In its unaltered form, DOX is considered to be one of the most potent approved chemotherapeutic drugs. The cellular internalization of this drug is via passive diffusion, and after which it binds to target enzymes such as topoisomerase I and II creating a range of different cytotoxic effects, such as covalent cleavage complexes, leading to apoptosis induction. However, DOX can also intercalate itself in the DNA, inhibiting both DNA and RNA polymerase, resulting in the cessation of DNA replication and RNA transcription. DOX acts by forming a proteasome complex (binding itself to the 20S subunit of the proteasome) and being translocated through the nuclear pore into the nucleus. Once in the nucleus DOX dissociates itself from the proteasome and binds to the DNA. This binding happens due to the higher affinity of DOX to nuclear DNA over the proteasome. It is estimated that one molecule of doxorubicin intercalates with the DNA every fifth base pair. When intercalated in the DNA it leads to a generation of free radicals, such as ROS, resulting in an increase of DNA lesions and consequently cell death. These free radicals are associated with the dangerous side effects of DOX toxicity and unspecific mechanism of action, making the property that makes DOX such a potent anticancer drug, the same that causes the high levels of toxicity associated with this chemotherapeutic agent (Tacar, Sriamornsak, and Dass 2013).

1.2.2. Paclitaxel

Paclitaxel (PAX) is a taxane extremely important in the treatment of cancer, due to its ability to target tubulin, altering the mitotic spindle function. This alteration disrupts chromosome segregation and inhibits mitotic progression therefore prevents cell division. PAX inhibits the detachment of the microtubules suppressing their dynamic behavior and through this action inhibits mitosis (Ganguly, Yang, and Cabral 2010). Paclitaxel gains access to the microtubules through diffusion. Once in the microtubule PAX binds to the β-subunit of the tubulin which is on inside surface of the microtubule. For each microtubule there is estimated to be 10,000 tubulin molecules of which each one has a paclitaxel binding site, making the binding of PAX a nearly 1:1 stoichiometric ratio (Jordan and Wilson 2004). PAX will lead to the stabilization of microtubules and it does so by preventing the formation of mitotic spindles. Without the formation of microtubules, the chromosome loses its bi-orientation and a consequent mitotic arrest preventing the cell proliferation (Khongkow et al. 2016).

1.2.3. 5-Fluorouracil

5-Fluorouracil (5-FU) is an antimetabolite drug capable of inhibiting essential biosynthetic processes and be incorporated into the DNA and RNA. 5-FU is used in the treatment of a wide range of cancers and its mechanism of action has been associated with the drugs capacity to be misincorporated in macromolecules, such as DNA and RNA, while at the same time inhibiting the nucleotide synthetic enzyme thymidylate synthase (TS). As such 5-FU is considered to be an analogue of the nucleobase uracil, with the exception that this drug contains a fluorine atom at the C-5 position instead of a hydrogen. The cellular internalization of 5-FU is the same transport mechanism as uracil – passive diffusion. When inside 5-FU is converted to several active metabolites such as: fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These metabolites disrupt RNA synthesis and the action of TS.(Longley, Harkin, and Johnston 2003) The action of TS is of extreme importance due to its catalysis activity. TS catalyzes the reduction of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction is responsible for a new source of thymidylate, which is necessary for the replication and repair of the DNA. Furthermore, when metabolite FUTP is incorporated into RNA it results in a high toxicity to RNA metabolism, with the inhibition of the process of maturation of pre-rRNA into rRNA. Misincorporation of 5-FU can also disrupt post-transcriptional modifications of tRNA. This properties of 5-FU make it an important chemotherapeutic agent that has been used over the last 20 years (Longley, Harkin, and Johnston 2003).

1.3. Comet assay

Human cells are constantly exposed to a series of harmful factors (which have the proficiency of causing DNA damage) create the necessity of a method which is capable of measuring the extension of this damage. This method should be sensitive, rapid, simple, and able to assess the damage in both proliferating as well as non-proliferating cells (Gunasekarana, Raj, and Chand 2015).

There are several assessment methods that match this description (Table 1). Frequently the activity of the DNA repair machinery is determined at the level of transcription by using DNA microarray techniques or by Real Time-PCR for selected genes involved in the different repair pathways, however the activity of an enzyme does not just depend on the rate of transcription. BER enzymes are thought to be highly constitutive due to the fact that they deal with the oxidized bases produced as an inevitable presence of ROS (one possible consequence of respiration). As

such, the amount of protein present will not be representative of the DNA repair activity (Azqueta et al. 2014).

The comet assay (or single cell gel electrophoreses assay - SCGE) differentiates itself for being able to detect low levels of DNA damage and for obtaining results in a relatively short period of time. This assay combines the simplicity of biochemical techniques for detecting DNA single strand breaks (strand break and incomplete excision repair sites), alkali-labile sites, and cross-linking, with the single cell approach typical of cytogenetic assay. The comet assay technique might be use in a wide range of cells, ranging between peripheral blood mononuclear cells to even biopsy tissues. Advantages of this technique are: i) the possibility of cell to cell approach as a result of a single cell electrophoreses; ii) its applicability to a variety of different types of cells; iii) there is no *in vitro* cultivation step required; iv) it is possible to have an estimation of the global repair capacity after one *in vitro* challenging experiment; v) it's very simple, fast and cheap to reproduce; vi) and can give some indications on apoptosis of the cells, although it doesn't permit to assess the pathway of apoptosis followed by the cell. Even though this technique is granted of some limitations such as: the inability of revealing DNA damage caused by small deletions, the uncertainty of if the lesion tested corresponds to the fixed mutations. However, even with these disadvantages the comet assay is a versatile tool of choice to assess the DNA damage and repair efficiency of the damaged cells (Gunasekarana, Raj, and Chand 2015).

The principle behind the comet assay is the movement of negatively charged damaged low molecular weight DNA fragments towards the positive electrode (anode), during the electrophoresis. As a result, a comet-like image appears with the intensity of the tail depending on the frequency of breaks which relax supercoiling, allowing migration of the DNA loops containing the breaks. The head of the comet corresponding to the nucleus (where most of the undamaged DNA is) and the tail of the comet corresponding to the damaged DNA of the cell. In this study a variant of the comet assay is used. The method used is denominated by "alkaline" comet assay and in its standard version it allows the detection of DNA strand breaks (SBs) and alkali-labile sites (ALS). This method was first published by Singh et al. (1988) in which the authors submitted the cells to an alkaline treatment allowing the DNA helix to unwind, and as a consequence converting the ALS to breaks (Azqueta et al. 2014). The use of alkaline pH helps denature DNA and unmasks single-stranded interruptions present in the DNA (Caldecott 2003). As a first step of this assay the cells are treated with different types of chemicals or exogenous agents capable of causing DNA lesions. Afterwards the cells are placed in microscope slides previously embedded in agarose and immersed in a lysis solution in order to obtain the nucleoids. Cells are then submitted to an alkaline treatment followed by an electrophoreses (Caldecott 2003).

Once the electrophoresis is over the cells are stained with an intercalating nucleic acid stain allowing the comets to be captured via fluorescent microscopy. Posteriorly the images captured are analyzed in a separated software, scoring the percentage DNA in the head and percentage DNA in the tail. This results are then grouped and analyzed using a statistical software for a better comprehension of the results obtained.

Table 1:Different assays to assess the damage on DNA - Adapted from Gunasekarana et. al 2015

Procedure	Advantages	Limitations
PCR	Easy to measure gene-specific DNA damage	Cannot quantify and recognize the kind of damage
FISH	Non-isotope labeling	Well-equipped laboraty facility
GC-MS	Sensitive to detect oxidative DNA damage	Over estimation of damage
Immunological Assay	Requires less amount of DNA	Needs costly equipment
Comet Assay	Detects damage in individual, non-proliferating cell	Damage due to small deletions cannot be detected
	Gives some indications on apoptosis	

2. Materials and methods

2.1. HeLa SilenciX

HeLa cells were purchased from Tebu-Bio, more explicitly, the Silencix lines. These are cellular models with ready-to-use knock down (KD) that are based on a unique siRNA delivery system. The cell lines bought were the following: Control HeLa SilenciX, PARP1$^-$ HeLa SilenciX (97% KD efficiency), XRCC1$^-$ HeLa SilenciX (87% KD efficiency) and APE1$^-$ HeLa SilenciX (70% KD efficiency). HeLa cells were cultured in DMEM High Glucose (Sigma-Aldrich D6046), 10% fetal bovine serum (FBS; Sigma-Aldrich F7524), 0.25% hygromycin B (Sigma-Aldrich I9278) and 1% penicillin-streptomycin (with 10,000 units penicillin and 10 mg streptomycin per mL) (Sigma-Aldrich P0781). All cell lines were incubated at 37 °C with 5% CO2 in a humidified chamber

2.2. Protein quantification

Protein quantification was done by Bradford assay using protein assay dye reagent concentrate (Bio-Rad # 500-00006) and bovine serum albumin (BSA) standard (Bio-Rad #500-0206). Standard concentrations of protein (0; 1; 2; 4; 8; 16; 32; 64| µg) were prepared in deionized water to a final volume of 800 µL. Then, 200 µL of protein assay dye reagent concentrate were added. The samples were then loaded in a 96-well plate and read in a plate-reader spectrophotometer at 595 nm. The same procedure was done using the protein extracts and then by linear regression the exact concentration determined.

2.3. Western Blot

Western Blot is an analytic technique, used in molecular biology to detect specific proteins in a sample or extract. This method utilizes a gel electrophoreses to separate the proteins in the extract. This separation can be accomplished using different characteristics of the proteins, either it being its isoelectric point or molecular weight. The nature of the separation will depend however on the treatment of the sample and the nature of the gel. This methodology separated proteins by molecular weight, and relies on the fact that when placed in the gel and submitted to a voltage the proteins will migrate in the gel accordingly to its molecular weight (in kilodaltons - kDa). This means that smaller proteins migrate faster through the gel and thus separating different proteins according to their size

Samples were diluted in Laemmli buffer 2x (LB) (4% (w/v) SDS 10%; 20% (20/20 glycerol 50%; 0.02 (w/v) bromophenol blue; 125 Mm Trist.HCl pH 6.8; and 10% (v/v) 2-Mercaptoethanol) with a ratio of 1:1 and heated at 95 ˚C for 5 minutes. set. The samples were then loaded into precast gels wells (4-20% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad #4561093S**)**,

along with a protein ladder (Page Ruler™), using a Hamilton pipet. Afterwards an electrophoreses was performed at 100V (400mA) for 1h30 in running buffer (25mM Tris, 250mM glycine, 0.1% SDS). Gels were equilibrated in transfer buffer 1x (500nM Glycine, 50nM TrisHCl, 0.01% SDS, 20% methanol**)** for 20 minutes as well as the polyvinylidene fluoride membrane (PVDF). The gel was transferred to the PVDF membrane in transfer buffer 1x for 1 hour at 100V (400mA) . Membranes were then cut and washed twice in double-distilled water (ddH_2O) for 5 min each, and blocked with blocking buffer from WesternDot™ 625 Goat Anti-Mouse Western Blot Kit (# W101132**)** during 1h at room temperature (RT). Membranes were exposed to an appropriated primary antibody (Thermo Fisher #13B 8E5C2 / Thermo Fisher #7A10 / Thermo Fisher #33-2-5**),** for each protein tested and β-actin (Santa Cruz # sc-47778**)**, diluted in wash buffer provided with the kit (1:1000 in wash buffer) over night at 4˚C. After the primary exposure the membranes were washed with wash buffer five times for 5 minutes and then incubated with secondary antibody (1:1000 in wash buffer) provided with the kit for 1h at RT followed by three washings with wash buffer. To ensure a correct staining, the membranes where submerged with a streptavidin conjugate (1:2000 in blocking buffer) for 1h at RT and followed by 3 successive washes with wash buffer where it remained. The membrane was visualized under ultra-violet light and photographed in a ChemiDoc^{tm} Imaging System by Bio Rad.

2.4. Quantitative real time PCR (qRT-PCR)

In order to confirm the gene silencing of commercial cell lines under study we performed a real-time PCR quantification. To carry out the qRT-PCR several steps should be performed:

1) cells disruption and homogenization; *2) total RNA purification*; *3) syntheses of cDNA* and *4) qPCR reaction*.

1) Cells disruption and homogenization – cells were grown in cell culture flasks and trypsinized in normal conditions as previously described (section 2.1), the number of cells was determined, followed by two washing steps with cold PBS centrifuge at 500 rpm for 10 minutes each; the next step was disruption of the cells by adding Buffer RLT Plus (Qiagen) in the proportion recommended in manufacturer's instructions (350 µl RLT per 1 x 10^5 cells), the final step was vortexed the lysate to guarantee a complete homogenization.

2) Total DNA/RNA purification – the lysate obtained in step 1) was used to proceed to RNA collection. This process was carried out through using AllPrep DNA/RNA mini kit (Qiagen #80204). The lysate obtained in step 1) was loaded into AllPrep DNA spin column and centrifuged for 30 seconds at ≥ 10000 rpm. The flow-through was used for RNA purification and the DNA spin column stored at room temperature until further use. One volume of 70% ethanol was added to the flow-through and mixed well by pipetting. Up to 700 µl of the sample, including any precipitate that may have formed, was loaded into an RNeasy spin column and centrifuged

for 15 seconds at ≥10000 rpm, discarding the flow-through. A wash was done by adding 700 µl of buffer RW1 to the RNeasy spin column and centrifuged for 15 seconds at ≥10000 rpm. The flow-through was discarded. A second wash with 500 µl of RPE buffer was done, followed by a centrifugation for 15 seconds at ≥10000 rpm. The flow-through was also discarded. A third wash with 500 µl of RPE buffer was done, followed by a centrifugation for 2 minutes at ≥10000 rpm. The flow-through was again discarded. An additional centrifugation at full speed for 1 minute was done to eliminate any possible carryover of Buffer RPE. Next, 30 µl of nuclease-free water was added directly to the spin column membrane and centrifuged for 1 min at ≥10000 rpm to elute the RNA. The eluate RNA obtained from previous step was centrifuged once again, at the same speed and time, to increase the RNA yield. This RNA was then stored at -80 °C until further use.

At last, the genomic DNA was purified by adding 500 µl of AW1 buffer to the AllPrep DNA spin column that was stored before and centrifuged for 15 seconds at ≥10000 rpm. The flow-through was discarded and 500 µl of AW2 buffer were added to the column and centrifuged for 2 minutes at full speed. After discarding the flow-through, 100 µl of elution buffer was added to the column and incubated for 1 minute at room temperature. Then, the column was centrifuged for 1 minute at ≥10000 rpm. The genomic DNA was then stored at -80 °C until further use.

All samples were quantified using a NanoDrop™ spectrophotometer.

3) Syntheses of cDNA – The synthesis of cDNA was done using the High Capacity RNA-to-cDNA kit (ThermoFisher # 4387406). The eluted RNA obtained in step 2) was firstly diluted to the concentration of 1 µg per 20 µl. Each reaction tube was prepared for a final volume of 20 µl, mixing 10 µl of 2x RT Buffer, 1 µl of 20x Enzyme mix, RNA sample volume define according the concentration and nuclease-free water to the final volume. A negative control reaction was also performed, to guarantee that samples used correspond to RNA. For this negative control reaction, all components described above except the Enzyme mix, were mixed together in the same proportion. All samples reaction was incubated for 37 ºC for 60 minutes, the reaction was then stopped by heating to 95 ºC for 5 minutes and cooled to 4 ºC. The cDNA was then stored at -20 ºC until further use. This incubation was performed in a thermal cycler (9700 ABI).

4) qPCR reaction – Using the cDNA of each sample obtained in step 3), was carried out the real-time PCR quantification reaction. This methodology was performed in order to confirm the expression of *APE1*, *XRCC1* and *PARP1* genes in silenced cell lines used. For that, were prepared single reactions for each sample in which were mixed 5 µl of TaqMan® Universal Master Mix II, 4.5 µl of cDNA corresponding to 20 ng, and 0.5 µl of 20x TaqMan® Assay (specific for each gene). Each sample was performed in triplicate and the results were confirmed twice in

independent experiments. GAPDH was used as endogenous control. The qPCR reactions were performed in a 96 well-plate, and can be seen the arrangement of each plate in the Table below.

Table 2: Plate arrangement for qPCR reactions

	1	2	3	4	5	6	7	8	9	10	11	12
A	-RT	-RT	-RT	-RT		NC	WT	APE1	PARP1	XRCC1	NTC	
B	WT	APE1	PARP1	XRCC1		NC	WT	APE1	PARP1	XRCC1	NTC	
C	WT	APE1	PARP1	XRCC1		NC	WT	APE1	PARP1	XRCC1	NTC	
D												
E												
F	WT	APE1	WT	PARP1	WT	XRCC1				NTC	NTC	NTC
G	APE1	APE1	PARP1	PARP1	XRCC1	XRCC1				NTC	NTC	NTC
H	APE1	APE1	PARP1	PARP1	XRCC1	XRCC1				NTC	NTC	NTC

NC – Negative Control; WT – Wild-Type HeLa cell line; APE1^{-} – HeLa silenced for APE1 gene; PARP1^{-} - HeLa silenced for PARP1 gene; XRCC1^{-} – HeLa silenced for XRCC1 gene; NTC – Non template control; -RT – negative control samples obtained through cDNA synthesis.

2.5. Cell viability assay (MTT assay)

The MTT assay is colorimetric assay used for assess cell metabolic activity. This assays consist on the reduction of monotetrazolium salts from weakly colored aqueous solutions to brightly colored derivatives know as formazans (Berridge et. al, 2005). MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is a monotetrazolium salt that when exposed to metabolic active cells is converted into an insoluble formazan with a purple coloration. Other examples of monotetrazolium salts used in colorimetric assays are: XTT; MTS and WST-1. With MTT and MTS being the most used in this types of assays and with the difference residing in the solubilization of the salts. When monotetrazolium salts enter the cells they are reduced by NAD(P)H-dependent oxireductases and dehydrogenases of metabolic active cells, resulting in a production of formazans that can variate from insoluble (MTT) to water-soluble (MTS) formazans. This allows to established a correlation between the colour intensity of the formazan dye and the number of viable cells. The MTT assay requires the salt to be solubilized before the spectrophotometric analysis, and this step might limit some applications.

Cell viability assay was assessed using the MTT assay. Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt MTT into insoluble purple formazan crystals at a rate that is proportional to cell viability. The cells were trypsinyzed and placed in a 96 well plate, with $6x10^3$ cells for PARP1^{-}, XRCC1^{-}, and control cell lines and $8x10^3$ for the APEX cell line. Then they were left to incubate for a period of 24h at the standard conditions (37°C with 5% CO_2). The cells were then exposed to six different chemotherapeutic agents for 48h (table 3). After the exposure period the cells were incubated with the MTT diluted in mean at a concentration of 0.5 mg/ml for 3h. At the end of incubation time the MTT was removed and

the crystals were dissolved in DMSO obtaining a gradient of purple. The absorbance was determined via a microplate reader at 595nm. This procedure was performed for all cell lines obtaining different cell viability curves.

Table 3: Concentrations tested in the MTT assay for each chemotherapeutic agent. Each cell line was exposed to the agent during a period of 48h. Afterwards the cells were submerged in the monotetrazolium salt MTT were crystals were formed. These were diluted in DMSO and the plate was read at 595nm in a microplate reader.

DOX (nM)	PAX (nM)	5-FU (µM)
1	0.01	10
5	0.1	20
10	0.5	50
50	1	75
100	5	100
500	10	200

2.6. Comet assay

Single cell gel electrophoreses is a quantitate DNA damage assay able to evaluate DNA damage in individual cells by lysing and embedding them in agarose (Rojas, Lopez, and Valverde 1999). The technique applied on this work uses alkaline electrophoreses (pH>13) and it was first introduced by Singh et al 1998. This version of the assay allows the detection DNA SSBs and alkali labile. A single cell suspension is embedded in low melting point (LMP) agarose in an agar gel sandwich. The cells are submitted to a treatment with a lysis solution that enables the removal of the cell contents except the nuclear material. With the remaining DNA supercoiled the slides are submersed in an alkaline electrophoreses solution which will allow the unwinding of the DNA increasing the display of the DNA damage and also allowing the migration of the damaged DNA when submitted to an electrical current, giving the cell the appearance of a “comet”.

The Comet assay was performed under alkaline conditions according to the procedures of Singh et al. (1988). HeLa cells were trypsinized and resuspended obtaining a cell suspension of 60000 cells per well. The cells were incubated in a 12 wells plate with DMEM high glucose medium where then they were exposed to a variety of chemotherapeutic agents with different concentrations (Doxorubicin 500-1000nM; Paclitaxel 1nM and 5-fluorouracil 200µM). Non-treated cells were used as a negative control (white) and cells exposed to H_2O_2 (100µM) were used as a positive control. The exposure time was 1h and afterwards the cells were washed twice, once with fresh culture medium, and the second time with PBS, with centrifugations of 2000rpm for 5 min, between washings. Then, the cells were dissolved in 0.5% low melting point (LMP) agarose, and spread onto a glass microscope slide pre-coated with 1% normal melting point

(NMP) agarose. After this step the agarose was allowed to stand for 20min at 4°C. The slides were then left over-night on a cold lysis solution (2.5M NaCl, 10mM Tris, 100mM EDTA, 1% Triton, pH 10) being washed afterwards with fresh water and allowed to stand in the water for 10 min. Then the slides were covered with fresh electrophoresis buffer (10M NaOH, 200mM EDTA, pH > 13) and left there for 20 min allowing DNA unwinding to occur. Electrophoresis was conducted for 20 min at 25V (400mA). Slides were then neutralized (0.4M Tris, pH 7.5), dried with ethanol and stained with GelRed (3x). The cells were observed with a 400x amplification with a fluorescent microscope (Leica DMLB). Approximately 50 cells were randomly selected and analyzed via the CometScore software. The percent of DNA in tail (%Tail DNA) of comets was measured to assess the extent of DNA damage.

2.7. Statistical analysis

Data was analyzed with GraphPad Prims 5 software. In order to determine if our variables have a Gaussian distribution we performed Kolmogorov-Smirnov test. To determine the equality of variances we performed Levens's test. Since our variables violate the assumptions of parametric tests we used Kruskal–Wallis test to compare three or more variables. To look deeper and find which groups are different from which other groups, we used Dunn's multiple comparison test. All graphs were made with GraphPad Prism 5 software. The values for MTT graphs are represented with mean expression ±standard error of the mean (SEM).

3. Results

3.1. Western Blot

In this work, western blot was used to corroborate the silencing of the cell lines, in order to perform the consequent assays without the uncertainty that the cell lines were indeed silenced for the selected genes. The necessity for this kind of technique provides from the fact that the HeLa SilenciX cell lines are a commercial line and therefore there was a need for a confirmation of the silencing.

As stated previously (section 2.1) every cell line had a different efficiency on the knock down of the expression of the respective genes. Therefore 4 different western blots were performed in order to gauge the silencing of the genes. The first western was a positive control were all the protein extracts of the cell lines were incubated with a primary antibody for β-actin. β-actin was used due to its a housekeeping characteristic, meaning that the expression of the protein is universal (mainly because of its importance in the maintenance of basic cellular functions). The following westerns aim to confirm the expression of target proteins, therefore three more westerns were performed (Figure 3).

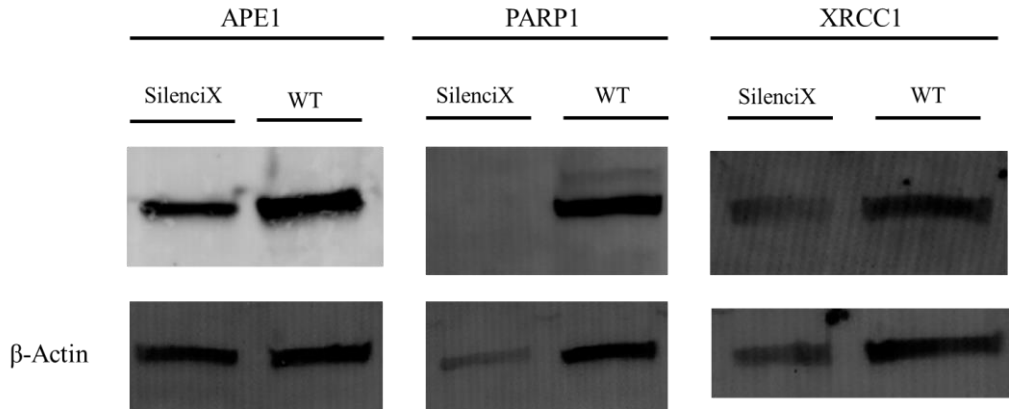

Figure 3: Confirmation of the silenced genes by western blot.

3.2. Quantitative real time PCR (qRT-PCR)

RT-PCR was performed in order to confirm the KD efficiency of the HeLa cell lines acquired. Relative expression of RNAs was determined by $2^{-\Delta Ct}$, where ΔCt is Ct (gene of interest) – Ct (endogenous gene) (figure 4). Mean relative expressions of APE1, PARP1 and XRCC1 corresponded to a silencing rate of 77%, 96% and 85% respectively.

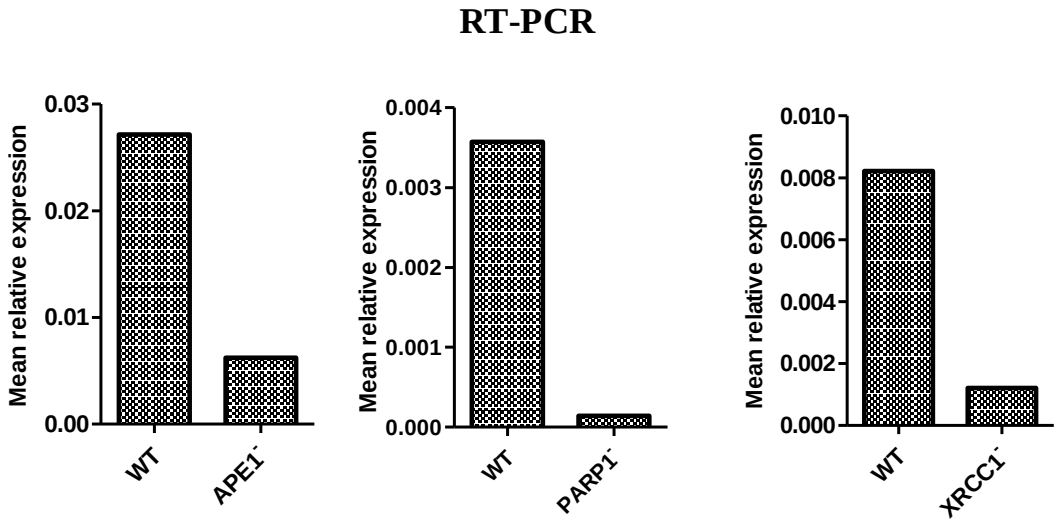

Figure 4: Mean relative expressions of the HeLa SilenciX® genes. The mean relative expressions where obtain via $2^{-\Delta Ct}$ method. The KD efficiency was confirmed where the cell lines for APE1, PARP1 and XRCC1 presented a silencing rate of 77%, 96% and 85% respectively.

3.3. MTT assay

As stated previously, six different concentrations were tested for each one of the three different drugs present in this study (Table 3). Each chemical was tested versus each cell line (total of 4 different cell lines). Every assay had present a negative control (NC) were the cells weren't exposed to any concentration of chemical. Since the negative control was not exposed to any concentration it was associated with 100% of cell viability (Figure 5, 6 and 7). In MTT assay for DOX (Figure 5) it is possible to observe that although six different concentrations were tested, only one concentration dropped below the 50% viability mark. The objective of this assay was to select the concentration that would reveal a significant number of lesions without obtaining a large percentage of cell death. Only 500nM was seemed to accomplish the objective of this assay. As such, the concentration of 500nM was selected as the concentration to use for the subsequent comet assay methodology. Few results have been published with the same concentration interval that we used (Koo et al. 2015). Additional, the ones that show the same concentration as ours are associated with a higher cell viability but were tested on other cell lines (MCF-7) (Tomankova et al. 2015)

The 5-FU was exposed at concentrations of micro molar (μM) unlike the other two chemicals tested (Figure 6). This is due to the mechanism of action of 5-FU and therefore a higher

concentration was needed to find a decrease of 50% viability. As such, of all the six different concentrations tested, four were able to get in the range of the objective of 50% cell viability. The possible concentrations were: 50, 75, 100 and 200μM. Only one concentration could be selected to proceed to comet assay, therefore the concentration of 200 μM was chosen since it was the only one that lowered 50% cell viability in the four cell lines. Some results have been published regarding cell viability assay with 5-FU. However, data published until now was not concordant concerning the cell viability related to the concentration of 200 μM in HeLa (Ahmed and Jamil 2011; Kim et al. 2013)

When tested for an exposure of 48h to paclitaxel (Figure 7) all cell lines had a similar response. Three of four cell lines presented a decrease in cell viability when exposed to a concentration of 5nM of PAX (with the exception being the cell line silenced for the *PARP1* gene), ranging from percentages between 60-80% to 10-35%. This indicates that when exposed to a concentration of 5nM the cells are no longer viable after 48h. Since the comet assay will evaluate the percentage of DNA lesion present in the cells, it would not be wise to use a concentration with a lower percentage than 30%, and therefore the selected concentration to be used in PAX exposure after the evaluation of the MTT assays was 1nM. Data from literature corroborates the chosen concentration and its even evident that this concentration is used on other cell lines aside from HeLa (Peng et al. 2014; Khongkow et al. 2016).

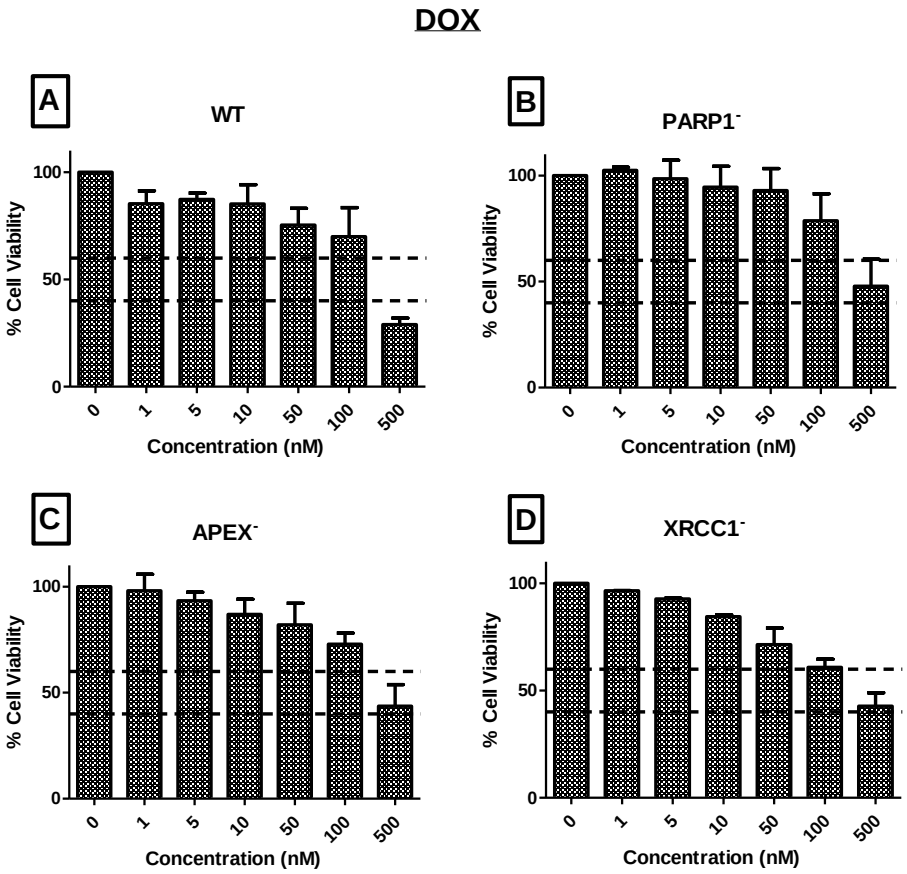

Figure 5: MTT assays for Doxorubicin after 48h of exposure to the cell lines A) HeLa SilenciX WT cell line; B) HeLa SilenciX PARP1$^-$ cell line; C) HeLa SilenciX APE1$^-$ cell line; D) HeLa SilenciX XRCC1$^-$ cell line. After the formation of the formazan crystals these were destroyed and diluted in DMSO enabling a reading of the absorbance at 595nm. Drug concentration of 500nM was chosen as a representative of rate of cell viability were it would be able to find DNA lesion without verifying to much cell death.

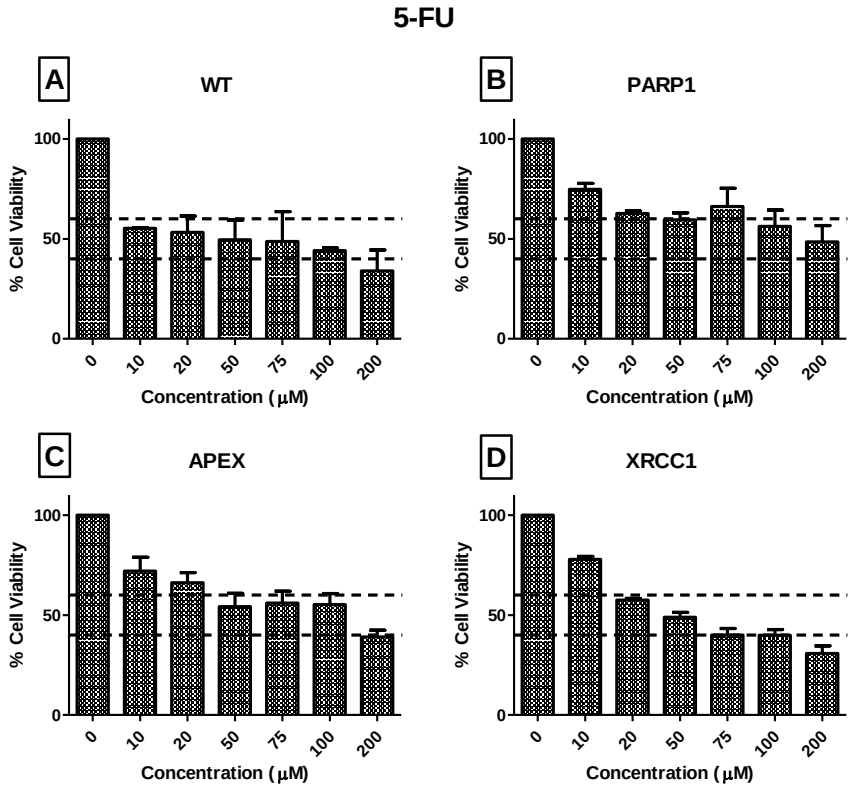

Figure 6: MTT assays for 5-Fluoruracil after 48h of exposure to the cell lines A) HeLa SilenciX WT cell line; B) HeLa SilenciX PARP1$^-$ cell line; C) HeLa SilenciX APE1$^-$ cell line; D) HeLa SilenciX XRCC1$^-$ cell line. After the formation of the formazan crystals these were destroyed and diluted in DMSO enabling a reading of the absorbance at 595nm. Drug concentration of 200μM was chosen as a representative of a rate of cell viability were it would be able to find DNA lesion without verifying to much cell death

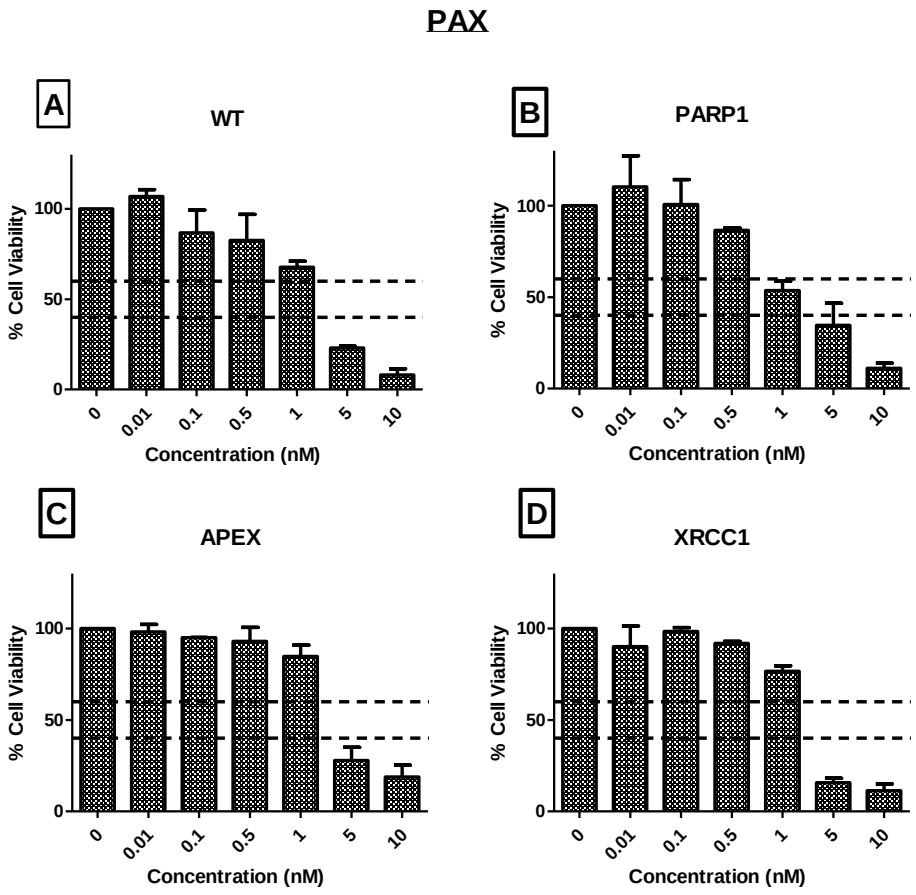

Figure 7: MTT assays for Paclitaxel after 48h of exposure to the cell lines A) HeLa SilenciX WT cell line; B) HeLa SilenciX PARP1^{-} cell line; C) HeLa SilenciX APE1^{-} cell line; D) HeLa SilenciX XRCC1^{-} cell line. After the formation of the formazan crystals these were destroyed and diluted in DMSO enabling a reading of the absorbance at 595nm. Drug concentration of 1nM was chosen as a representative of a rate of cell viability were it would be able to find DNA lesion without verifying to much cell death

3.4. Comet assay

Comet assay was performed in order to ascertain the importance of the silenced genes in BER pathway, HeLa cell line were exposed to different chemotherapeutic agents for 60 minutes after which was carried-out the procedure to assess the percentage of DNA damage. Unlike the MTT assay the cell lines were only exposed to 1 hour of chemotherapeutic agents due to the fact that if left for 48h the mechanism of DNA repair (possibly aside from BER) would start repairing the DNA lesions caused by the drugs. The chemicals concentration used were determined using the MTT assay (section 3.3) and compared with its counterpart without treatment (no addition of any drug). CometScore software was used to measure the percentage of DNA in the tail of the "comets" formed. The "comets" were measured with four different parameters: percentage DNA in head (%DNA head); percentage DNA in tail (%DNA tail); tail moment (TM) and olive tail moment (OTM) (Figure 8). Percentage DNA in the head and tail of the comet correspond to the percentage of DNA remaining in each section of the cell after the electrophoreses (Figure 9)

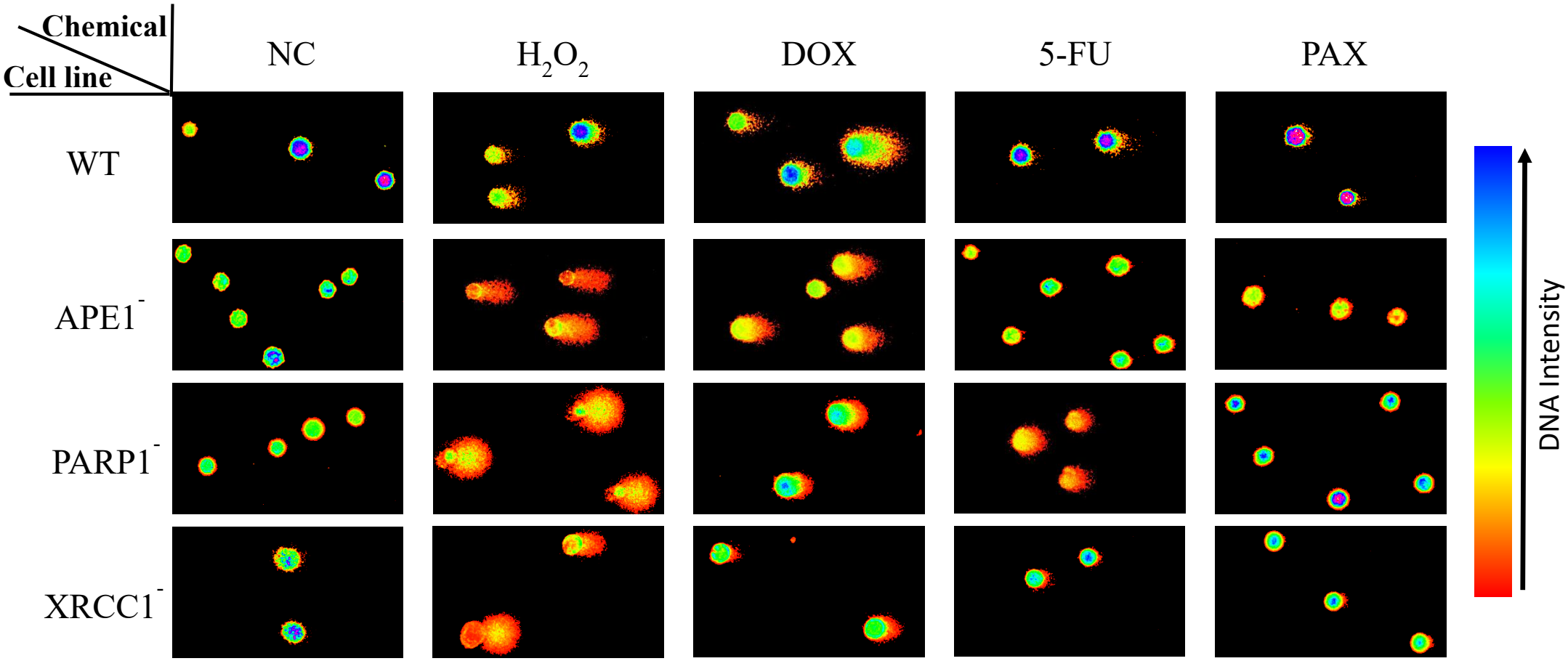

Figure 8: Independent comet assays on HeLa SilenciX cell lines. Results obtained after 1h of exposure to: 100µM of H_2O_2 (Positive Control); 500nM of DOX; 200µM of 5-FU and 1nM of PAX. The capture of the images was obtained with a Leica DMLB and the quantification of the captures was attained via CometScore software where the %DNA in tail, %DNA in the head; Olive tail moment and tail moment were quantified in order to evaluate the comets.

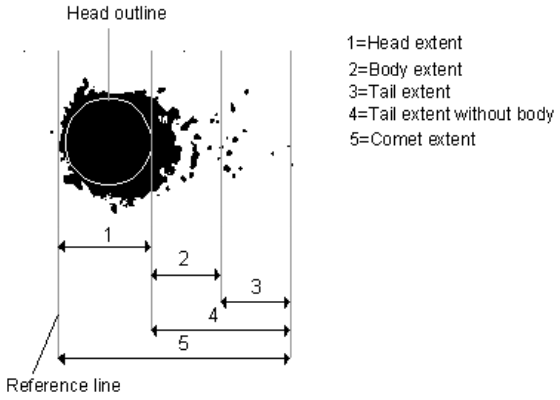

Figure 9: Representative image of each section of a cell after the comet assay.

Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail and can be calculated through this formula (Mozaffarieh et al. 2008):

$$TM = tail\ lenght\ \times \%\ of\ DNA\ in\ the\ tail$$

In addition to the measure of TM the software also calculated the OTM, in which this parameter represents the product of the percentage of total DNA in the tail and the distance between the centers of the mass of the head and tail regions (Mozaffarieh et al. 2008) :

$$OTM = (tail\ mean - head\ mean) \times \%\ of\ DNA\ in\ the\ tail$$

In order to obtain the best quantification of the assays 50 cells on each slide were chosen at random. Each experience had duplicates with two slides for each chemical. Therefore at least 200 cells were quantified enabling the correct measurement of the comets.

All the data was analyzed using a Kruskal-Wallis test (p value < 0.0001), and a Dunn's multiple comparison test to check if the results were statistical significant. Data presented in figure 10, show the results of each drug compared with the respective NC regarding each cell line to attest statistical significance. In order to test the data presented in figure 11 the results of each cell lines, for each drug, were tested *versus* the WT cell line as a way of determining statistical significance.

In figure 10, the response of individual cell lines to the agents can be observed. In figure 10A the response of the WT cell line for each drug can be observed. Results show that when we compare the mean values of NC with the mean values of the exposed drugs there is a significant variance between each pair. The statistical significance obtained for this results allowed us to understand that each drug *per se* influence the cell line response. Additionally, Figure 10B shows the response of APE1^{-} cell line after exposure to drugs under study. In this case there was no statistical significance between the pair NC/PAX, which means that there wasn't a significant

difference between NC and PAX measures. Figure 10C and 10D revealed the responses of the PARP1$^-$ and XRCC1$^-$ cell lines after drugs exposure. The results obtained indicate that when exposed to each drug, both cell lines have a significant increase in %DNA in tail when compared with the NC.

The overall response of the cell lines to each drug used is represented in Figure 11. The results demonstrate that doxorubicin originates more lesion that any other drug tested. However, the silenced lines show an increased sensitivity to the exposure of hydrogen peroxide when compared with the WT cell line. It is also perceivable that of the three chemotherapeutic agents tested, PAX is the one that causes less lesion. 5-FU although with a low percentage of DNA in tail has a slightly higher percentage than PAX, with the WT cell line showing an increased sensibility when exposed to the agents tested in this study. A slight variation is registered on the %DNA in tail when the cells are left untreated (NC). Fig 11C demonstrates that cell lines APE1$^-$ and PARP1$^-$ seem to have a statistical significance o when compared with the WT line.

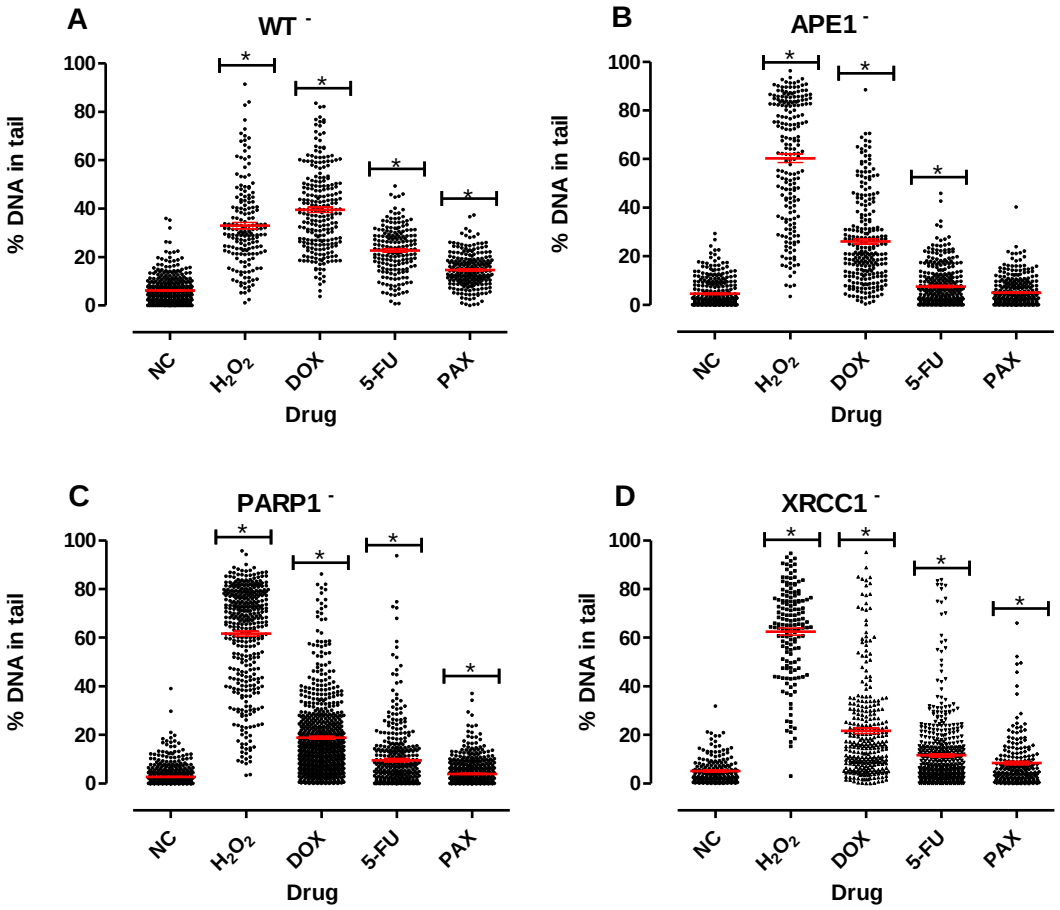

Figure 10: Response of individual cell lines to each compound in comet assay after 1-hour of exposure. %DNA in the tail was measured in each captured cells. 50 cells were randomly selected from 2 duplicate slides from 2 independent assays making it a total of 200 cells quantified. Lines represent median with SEM. p. value < 0.05 was considered significant according to non-parametric Dunn's multiple comparison test. All multiple comparisons of the drugs were made *versus* the NC. * p < 0.0001 according to non-parametric Kruskal-Wallis

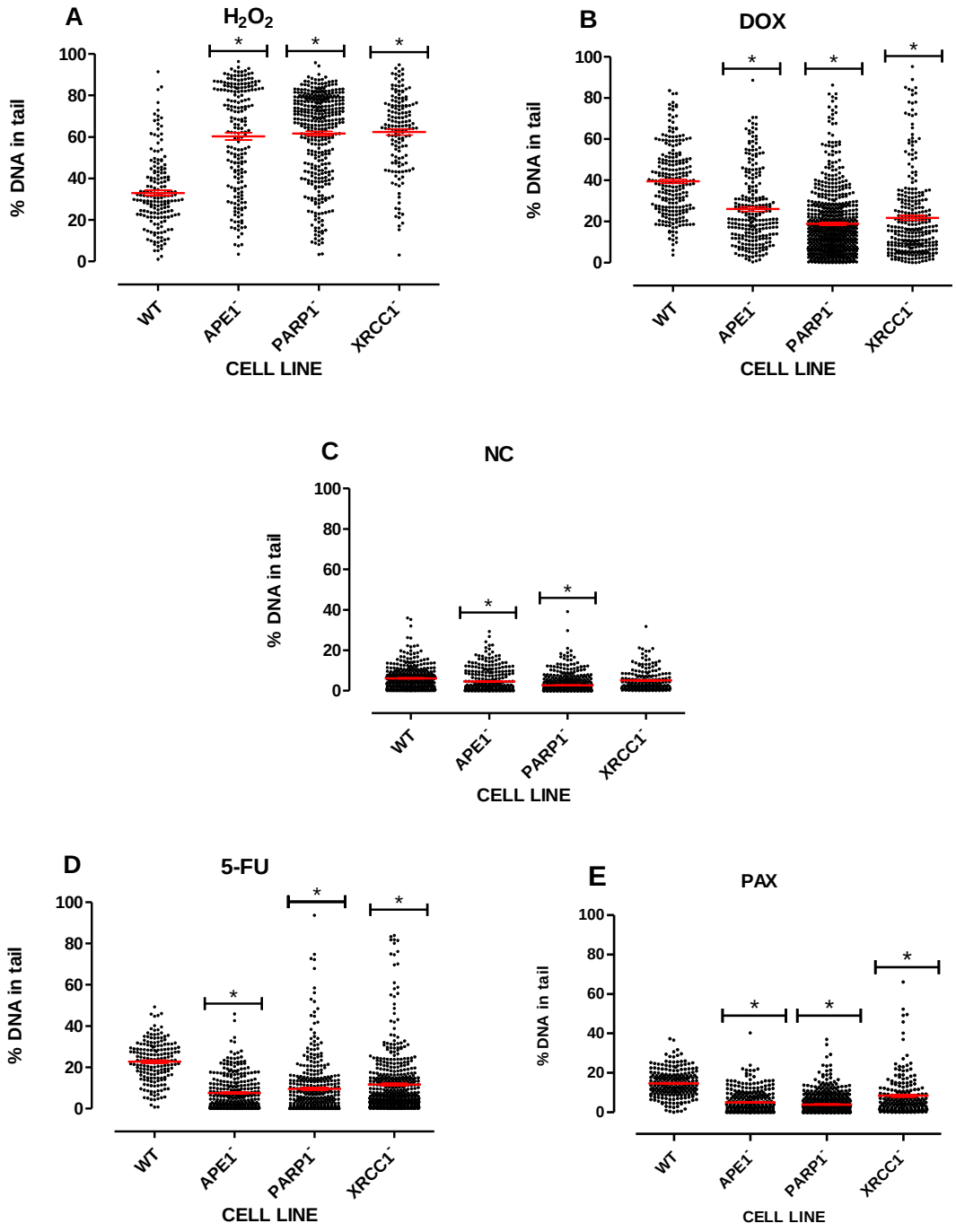

Figure 11: Variances in the %DNA in tail of each cell line after the comet assay with 1-hour exposure to each compound. 50 cells were randomly selected from 2 duplicate slides from 2 independent assays making it a total of 200 cells quantified. Lines represent median with SEM. p. value < 0.05 was considered significant according to non-parametric Dunn's multiple comparison test. All multiple comparisons of the silenced lines were made *versus* the WT line. * $p < 0.0001$ according to non-parametric Kruskal-Wallis.

4. Discussion

Base excision repair is a system used from bacteria to man to remove the vast multitude of endogenous DNA damages produced each day in every human cell (Wallace, Murphy, and Sweasy 2012). BER proteins are of extreme importance on a broad spectrum of DNA lesions due to their action on the damage caused resulting from endogenous and exogenous sources. BER pathway has an immense role in genome maintenance and is associated to chemotherapeutic response, neurodegeneration, cancer or aging. In this work four commercial cell lines were obtained. Three of them were silenced for a specific gene coding a protein that acts in the BER pathway. The cells were then exposed to three different chemotherapeutic agents used in the treatment of cancer (Tacar, Sriamornsak, and Dass 2013; Minotti et al. 2004; Longley, Harkin, and Johnston 2003; Khongkow et al. 2016), in order to evaluate the impact of each protein in the BER pathway. Therefore, a method for DNA damage detection was used with the purpose of assessing the susceptibility of each cell line to different compounds and to have a better understanding of the influence of the BER pathway in repairing SSBs induced by different agents.

4.1. Confirmation of silencing by Western Blot

In this work all cell lines were acquired via a commercial company. The HeLa SilenciX® (Tebu-Bio) cell line was used with the genes *APE1, PARP1* and *XRCC1* silenced by using a siRNA delivery system. This KD had an efficiency rating that varied accordingly to each silencing. In order to confirm the silencing of the cell lines a western blot analysis was performed (Figure 3). In Figure 3 we can observe that all the cell lines exhibit indeed a decrease in the production of the respective protein, although with some differences in the expression rate. We can also verify that the The HeLa SilenciX® for *APE1* was acquired with a 70% KD efficiency, and accordingly to the results obtained it is clear that the cell line is indeed silenced for the *APE1* gene. HeLa SilenciX® silenced for *PARP-1* and *XRCC1* were obtained with a 97% and 87% KD efficiency respectively. Observing Figure 3, it is clear that the KD of the genes in the cell lines was a success with almost no expression of the PARP-1 protein and slight expression of the APE1 and XRCC1 proteins.

4.2. Verification of the silencing rate by qRT-PCR

In order to further confirm the results of the western blot and to determine the silencing rate of the cell lines a qRT-PCR was performed. The rate was calculated with the mean relative expressions obtaining results of 77%, 95% and 85% for the $APE1^{-}$, $PARP1^{-}$ and $XRCC1^{-}$ cell lines respectively. This results correlate with the 70%, 97% and 87% KD efficiency advocated by Tebu-Bio. Therefore, corroborating the results of the western blot presented previously (section

3.1). This guarantees that the commercial cell lines didn't lose their silencing, avoiding any potential bias in our results.

4.3. MTT assays

As stated earlier the MTT assays were performed to encounter the optimal concentration in order to perform the comet assay without obtaining too much cell death. Therefore, the MTT assay enable the selection of one of the six possible concentrations (see table 2). The monotetrazolium salt MTT can only be reduced to its purple formazan form by NAD(P)H-dependent oxidoreductases in metabolic active cells (Berridge, Herst, and Tan 2005), consequently the formation of the formazans can only be achieved if the cells are active. If the cells are no longer viable no crystals will be formed and less purple the mean will become. As such the crystals were diluted in DMSO and read for absorbance at 595nm.

In Figure 5 we can observe the cell viability of each cell line to the six different concentrations. Sections A B C and D, shows the response of cell lines when exposed for 48h to doxorubicin. There is a clear reduction in cell viability when the exposed concentration rises from 100nM to 500nM of DOX, ranging from a percentages of 70% to 30% cell viability, respectively. This permitted the selection of 500nM as a concentration that would allow us to observe DNA lesions without resulting in the complete destruction of the cellular viability. Figure 6 demonstrates percentage cell viability of the four cell lines when exposed to 5-FU during 48h, and although the range of possible concentrations is larger, the concentration of 200µM was selected for being the only concentration that crossed the 50% cell viability mark in all cell lines, making it this way the ideal candidate to test in the comet assay. Figure 7 represents cell lines' response to 48h exposure of PAX, where it's visible that concentrations of 5 and 10nM cause an abrupt decrease in cell viability meaning that these concentrations could not be selected due to its high negative impact on the cells. A concentration associated with low cell viability will be also associated with high cell death in the comet assay, making it impossible to select either one of these concentrations. As such concentration of 1nM fulfilled all the requirements to be selected as a representative concentration and therefore the concentration of 1nM was selected due to being associated with slightly higher cell viability percentages.

4.4. Comet assay

The comet assay was performed under alkaline conditions in order to assess the formation of single strand breaks in the DNA of the cells when these are exposed to 1h of the chemotherapeutic agents (Rojas, Lopez, and Valverde 1999). Unlike the MTT assay, cells were exposed to the chemotherapeutic agents for 1 hour instead of the 48h. This was due to the fact that the mechanism of repair act simultaneously with the drugs effects, meaning that if cells were exposed for a period

of 48h there would be a high chance that either the cells was not viable or no DNA lesion would be observed due to the repair of the SSBs caused by the chemicals. After drug exposure cells were placed in a slide and stained with GelRed as described in section 2.6. It was very important to guarantee that the cells were placed in a homogeneous way to avoid clusters hence making it impossible to capture the comets (Rojas, Lopez, and Valverde 1999). Time of slides exposure to the fluorescence lamp was also an important experimental variable to carefully assess, since a high time of exposure could lead to an overexposure of the GelRed in cells and background of the image and compromising this way the data analysis of the captures.

5-FU is described as antimetabolite drug which will be misincorporated into macromolecules like DNA and RNA (Longley, Harkin, and Johnston 2003), and after analyzing the results we can infer about the time of exposure, meaning that 1 hour of exposure to 5-FU might not enough time for DNA lesions to be registered with a technique like comet assay. The negative control had little impact on the DNA with the possible DNA in the tail being caused by, for example, UV light from any source of light (Jackson and Bartek 2009). Hydrogen peroxide was used as a positive control and is a natural source of oxidative damage in cells. It causes a variety of DNA lesions including single and double strand breaks, by the production of the hydroxyl radical (˙OH) in the presence of transitional metals ions via the Fenton reaction (Benhusein et al. 2010). When exposed to H_2O_2 all the cell lines appear to suffer DNA lesions with an increase in DNA in the tail on the silenced cell lines displaying that the cell lines silenced for specific genes in the BER pathway present an increased sensitivity to the presence of hydrogen peroxide when compared to the WT line (figure 10). This can be explained with the dysfunction these cells present in one of the principal pathways responsible for the reparation of this kinds of lesions (Curtin 2012).

Doxorubicin is an anthracycline with an extensive clinical utilization in cancer cells. This drug is known to intercalate into DNA leading to the inhibition of macromolecules while generating free radicals leading to DNA damage. DOX can also interfere with DNA unwinding and inhibit the activity of topoisomerase, therefore making this one of the most potent chemotherapeutic agents approved by the Food and Drug Administration (FDA) (Tacar, Sriamornsak, and Dass 2013; Minotti et al. 2004). Once more all the cell lines showed an increase in %DNA in the tail but unlike H_2O_2 the silenced cells showed less DNA damage then the WT line, with DOX causing even more lesion then H_2O_2 (see Figure 10). One possible explanation to this is that, since the silenced lines are more fragile in the BER repair pathways it is possible that some other repair pathway, like NER for example, is more predisposed to act when some exogenous source of DNA lesion is detected (Curtin 2012).

When APE1$^-$ was exposed to the chemical agents (Figure 10B), no statistical significance was detected between the pair NC/PAX. This results emphasize the hypothesis that PAX also must be seen as a "negative control", since it didn't interfere with cell DNA or at least that its interference might not be measured by DNA breaks through % DNA in tail.

Once PARP1$^-$ and XRCC1$^-$ cell lines were exposed to drugs (Figures 10C and 10D), the results showed that when exposed to each drug, both cell lines have a significant increase in %DNA in tail when compared with the NC, except to PAX exposure as explained above. This can be explained since the mechanism of action of PAX does not involve a direct lesion against DNA, interfering at cell cycle level (Khongkow et al. 2016; Peng et al. 2014; Ganguly, Yang, and Cabral 2010; Jordan and Wilson 2004). In fact, observing each individual graph to each cell line, the PAX exposure in all of them didn't show a high % DNA in the tail.

The data for the overall response of the lines to each drug was tested as described in section 3.4. Figure 11A allowed us to observe that when exposed to H_2O_2 all the silenced lines show a statistical significance when compared to the WT line. This allowed us to conclude that when specific genes for proteins that act in the BER pathway are silenced, the cells are more predisposed to suffer DNA lesions from oxidative stress than a cell that was not silenced for any gene. Figure 11B displays the response of the cell lines when exposed to DOX. In this graph is visible variance can be observed between the silenced lines and the WT line. In this case, when exposed to DOX the silenced lines present less predisposition to suffer DNA lesions, even though %DNA in tail can be observed. This might be explained with the fact that due to the dysfunction of the BER pathway (in the silenced lines) there might be another repair pathway (like NER for example) overcompensating for the fact that BER is compromised. Although this explanation cannot be proven with the use of the comet assay.

The response of the cell lines when treated with no chemical is exposed (NC) is illustrated in Figure 11C. Results indicate that the XRCC1$^-$ line does not present any significant variance when compared with the results of the WT line. This was the expected result since no chemotherapeutic agent was exposed to the cells. However, cell lines APE1$^-$ and PARP1$^-$ seem to display a slight variance in their results. Although this small difference was not expected it can be associated with small errors or changes to the DNA of the cell during the cells metabolism (like replication) (Lodish et al. 2000; Minotti et al. 2004). Figures 10D and 10E show that like DOX (figure 11B), the silenced cell lines show less %DNA in tail when compared with the WT line, and can be correlated with the dysfunction in the BER pathway. Nevertheless, and although the silenced lines display less DNA damage, only H_2O_2 and DOX seemed to show a higher %DNA in the tail, with the chemotherapeutic agents 5-FU and PAX presenting a low percentage in tail DNA when compared with the other remaining drugs. One explanation to this can be the fact that

according to literature the mechanism of action of DOX can lead to the formation of ROS like H_2O_2, as such it is possible that the DNA lesions associated with 1 hour of exposure to DOX might derivate from the production of this ROS and not from intercalating with the DNA (Tacar, Sriamornsak, and Dass 2013; Minotti et al. 2004). This conclusion cannot be proved with the comet assay since this technique only allows to detect DNA lesions and not types of lesions (Azqueta et al. 2014; Gunasekarana, Raj, and Chand 2015).

Figure 10 shows that all the cell lines present some extent of DNA lesion when exposed to 5-FU. A similar response was observed by the silenced lines when exposed to the drug, with the silenced lines showing less DNA lesion than the WT line (see Figure 11). Since the comet assay only measures the DNA lesions it is not clear if there is in fact another DNA repair pathway being activated due to the silencing of the BER pathways (Olive and Banáth 2006). Paclitaxel was used in this study with the assumption that the comet assay would not show any DNA lesion. This is due to the mechanism of action of PAX, that acts by disrupting the microtubules (MT) dynamic thereby interrupting the process of cell division at mitoses (Khongkow et al. 2016). Consequently, no damage in the DNA is suffered from the exposure to PAX, which can be verified in figure 10 where paclitaxel causes near to no DNA lesions when compared with the other 4 drugs.

Variances in the DNA lesion of each cell line can be observed in Figure 10. After 1 hour, of exposition H_2O_2 was the compound that caused more DNA lesion followed by DOX, 5-FU and PAX. As stated no DNA lesion was expected to be seen when the cells were exposed to PAX, however it would be expected to observe DNA lesion when the cells were exposed to the 5-FU agent. This was not the case leading to believe that although 5-FU is an intercalating agent, 1 hour of exposure was not enough for 5-FU to be converted and misincorporated in the macromolecules of the cells. This way little DNA damage would be recorded in the %DNA in tail which seemed to be the case. Although DOX is also an intercalating agent it is described that DOX can also generate reactive oxygen species (ROS) like superoxide anion and hydrogen peroxide. As observed earlier, H_2O_2 can cause immediate DNA lesions and as such it is possible that the lesions observed in the comet assay regarding 1 hour of exposure to DOX might be associated with the production of ROS and not with the intercalating mechanism associated with the action of this chemotherapeutic agent.

Overall, this study showed us that the silencing of individual genes of the BER pathway is enough to cause an increase in cell sensibility to some drugs that induce DNA damage. The study also allowed us to infer that other pathways mays exert concomitantly action with BER pathway. Further studies need to be performed to reinforce the findings, including recover assays in order to evaluate the response of the cells when left to recuperate from the lesions cause by the

chemotherapeutic agents. Further, apoptosis assays should also be performed to better understand the apoptosis pathway followed by the cell when they accumulate high levels of DNA lesions.

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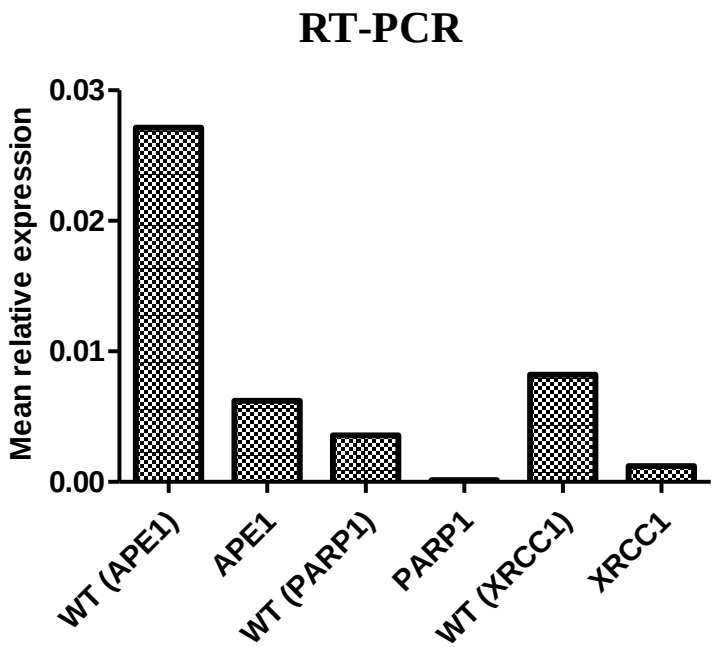
6. Appendix

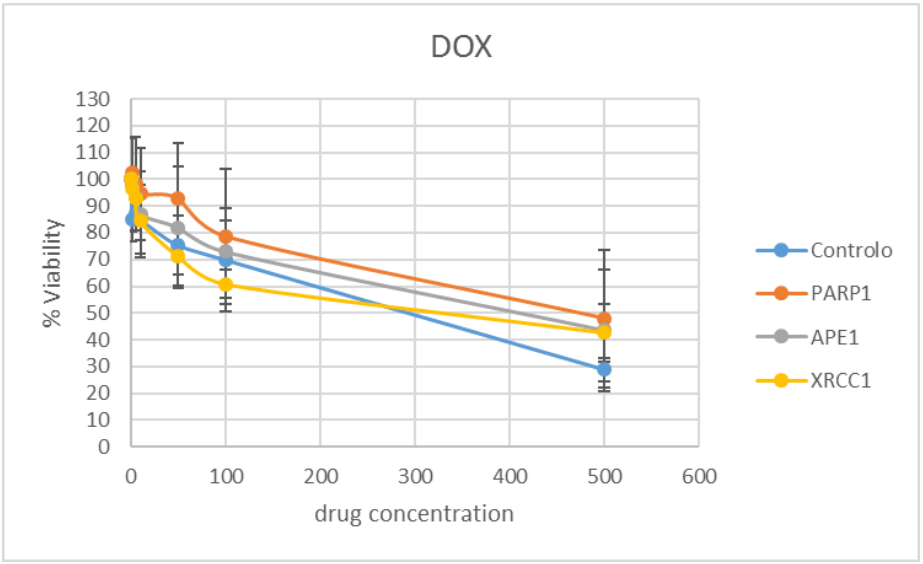
6.1. Appendix A

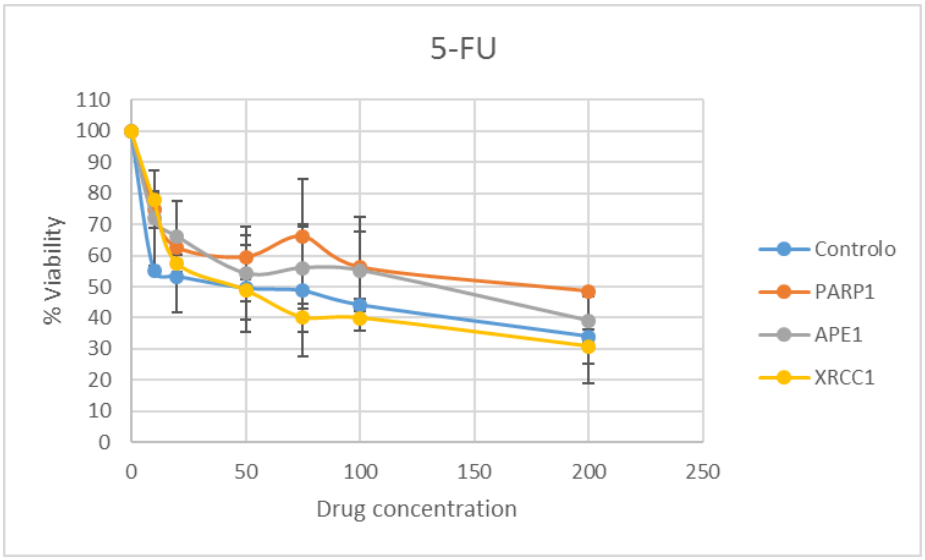
Mean relative expressions of the HeLa SilenciX® genes.

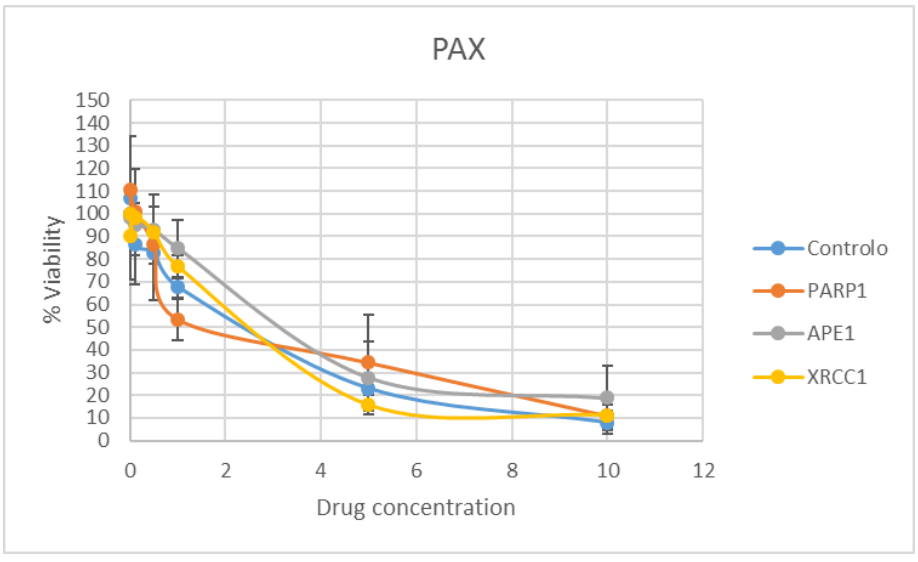


6.2. Appendix B

MTT assays preformed for the three chemotherapeutic agents (DOX; 5-FU; PAX)







6.3. Appendix C

Calibration line of the Bradford assays