Toxicity assessment on HeLa and HEK 293 cells after treatment with Acetaminophen and 5-fluorouracil

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Context: Acetaminophen is a commonly used pain killer, which is available without prescription. 5-fluorouracil is an anticancer molecule which is a heavy treatment with a strict regulatory access. These drugs are known to induce apoptosis in some cells type. For instance, their cytotoxicity and mechanism of action has been deciphered in HeLa cell, a well characterized cell line. However, other used cell lines as HEK 293, an embryonic kidney cell-derived cell line very used in cell biology researches, lack some insights about the effect of acetaminophen compared to an anti-cancer drug, 5-fluorouracil, on cell death.

Methods: Cytotoxicity of acetaminophen and 5-fluorouracil on the human embryonic kidney cell line HEK 293 was assessed by carrying out XTT viability assay. In a second time, the cell death mechanism, apoptosis or necrosis, was investigated by performing an AO/BE and Hoechst staining. Finally, a western-blot analysis was done to evaluate the extrinsic or intrinsic character of the apoptosis. HeLa cell line was used as a control, as this cell line is already well known, both in terms of cellular mechanisms and of toxicity.

Results: Hoechst and XTT viability assay demonstrated both 5-fluorouracil and acetaminophen treatment induced cell death by both apoptosis or necrosis for the two cell lines.
However, HEK 293 displayed a better relative viability compared to HeLa. The 5-fluorouracil toxicity increases with incubation time and is maximal at 72h for 10^{-5}M and 10^{-4}M respectively.
Results suggest that acetaminophen maximal effective concentration was reached, as the results were not different between the two different chosen concentrations.

Keywords: acetaminophen, 5-fluorouracil, toxicity, HeLa cells, HEK 293 cells

I - Introduction

We focused on the toxicity of two distinct molecules:

• The first one is an anticancer molecule, the 5-fluorouracil, which is commonly used as an anti-cancer treatment.

5-fluorouracil is used for colon, pancreas, esophagus, stomach or breast cancer [1] and came into medical use in 1962. This treatment is listed on the World Health Organization's List of Essential Medicines, which are the most effective and safe medicines used in a health system. Its mode of action is based on the incorporation into RNA under UTP form and the blocking of the thymidylate synthase. This drug is often among the most used cures in cancer treatment, so we aimed at studying its impact on cells during a treatment.

• The second one is the acetaminophen, which is a pain-killer. This drug is easy to access and can be used by anyone.

Acetaminophen is the most commonly used medication for pain in the United States and Europe, and is normally safe at recommended doses [7]. However, at too high doses, it can result in liver failure [9]. Its effect on liver is well known and apoptosis is one of the cellular mechanism resulting from a potential acetaminophen overdose. [3]

The goal here was to study the toxicological impact of these two drugs *in vitro*, in order to evaluate the mechanism induced by an easy-to-access medicine. 5-fluorouracil is taken as a control treatment, as it is a common anti-cancer treatment and the chosen cell lines are both cancerous [5]. The cell death mechanisms are assessed in HEK 293 cell line and compared to HeLa cell line, which metabolism is already well known [8].

The HEK 293 cell line has been chosen because of its ease to maintain in culture and well described [12]. However, they are not as documented as HeLa cells, and some cellular responses as apoptosis under cytotoxic drugs remain unclear [6].

II - Material and Methods

a. Chemicals and Reagents

Acetaminophen was purchased as “Doliprane” (SANOFI, FRANCE) tablet. Tablets were reduced to powder, and solubilized in DMEM medium. 5-fluorouracil, DMEM/F12 (HEPES, L-Glutamine), streptomycin, penicillin, acridine orange, ethidium bromide, TGS buffer (with SDS and ethanol 10%), RIPA buffer and Bradford reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). XTT kit, Hoechst 33342 (Trihydrochloride), DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride) and DAB staining kit were purchased from ThermoFisher (Waltham, Massachusetts, United States).
Rabbit anti-human cleaved caspase 8, rabbit anti-human caspase 8, rabbit anti-human cleaved caspase 9 and rabbit anti-human caspase 9 were purchased from Cell Signaling Technology (Danvers, Massachusetts, United States).

b. Cell lines and treatments

The human cervical cancer cell line HeLa and human embryonic kidney cell line (HEK 293) were obtained from CEA (Commissariat à l'Energie Atomique et aux énergies alternatives). The cells were cultured in B10 Petri dishes or 96-wells microplate (Dominique Dutscher, France) containing DMEM supplemented with 10% of FBS, 1% of amino acids, 1% of pyruvate sodmium, 100 U/ml of penicillin and 100 U/ml of streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. For XTT viability test, 5.10^3 HeLa cells/well or $2,5.10^3$ HEK 293 cells/well were seeded. Cells were plated 24h before treatments for adhering. Then cells were treated for the time indicated on the figures in the presence or absence of

different acetaminophen and 5-fluorouracil concentrations (acetaminophen: 4mg/ml, 8mg/ml [4], 5-fluorouracil: 5.10^{-5} M, 10^{-4} M [5]).

c. XTT viability test

The cytotoxic effect was measured by using ThermoFisher XTT Cell Viability Assay kit X6493. Cells were plated into 96-wells plates and cultured in a humidified 5% CO2-containing atmosphere at 37˚C for 24h. 10 µL of PMS solution (10 mM) were added to the XTT solution immediately before labeling cells. 50 µl of XTT/PMS solution (4 mg/ml) was added to each well, and the plates were incubated for 2h at 37˚C. The absorbance at 450 nm was measured using a Multiskan GO microplate reader from ThermoFisher Scientific.

d. Acridine Orange/BET assay

This staining is used to detect cells apoptosis and necrosis. A 1:1 dye mix was prepared with acridine orange (ThermoFisher) and ethidium bromide (ThermoFisher). Cells were trypsinized and transferred to a 96-wells plate before performing the staining. Cells were counted with a ZOE microscope.

e. Hoechst staining

This staining is used to detect cells apoptosis. Cells were incubated 24h and 30h with test solutions in a 96-wells plate in a humidified 5% CO2-containing atmosphere at 37°C. After incubation, the supernatant was removed and the staining solution was added in each well and incubated 10 minutes with protection against light. After this treatment the staining solution was removed and wells washed 3 times with PBS before imaging the cells with an excitation wavelength at 350 nm and a reading wavelength at 461 nm.

f. Western-blot

HeLa and HEK 293 cells proteins were extracted using RIPA buffer with centrifugation at 14 000g for 15 min at 4°C. Proteins samples were loaded in a 10% SDS-PAGE electrophoresis. A Coomassie blue staining was realized to confirm the presence of protein on the electrophoresis gel, then they were transferred on a nitrocellulose membrane at 25V at 4°C overnight. After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with cleaved caspase 8 (1:1000), caspase 8 (1:1000), cleaved caspase 9 (1:1000), caspase 9 (1:1000) antibodies for 1 hour at room temperature. The membranes were then washed with the TBS three times for 5 min each time. The membranes were then incubated with anti-rabbit (1:1000) antibody for 1 h at room temperature. The membranes were revealed using 10mg of DAB reagent in 10ml of PBS with 10µl of H2O2.

g. Statistical analysis

Cellular viability by XTT and Hoechst assay results were analyzed with GraphPad (GraphPad Prism Software). Two-way analysis of variance (Anova) was used for statistical comparison.
The data presented are the mean ± standard deviation of a duplicate.
$P<0.05$ was considered to indicate a statistically significant result.

III - Results

Acetaminophen and 5-fluorouracil induce cell death on HEK 293 and HeLa cells lines.

XTT analysis demonstrated significant reduced cell viability of HeLa cells treated with acetaminophen at 24h ($P<0.001$, Fig. 1A) but no effect with 5-fluorouracil. HEK 293 were not affected by any treatment at 24h. After 30h of treatment, a significant cell viability decrease was observed for both treatments on HeLa cells ($P<0.05$, Fig. 1B) but no significant change in

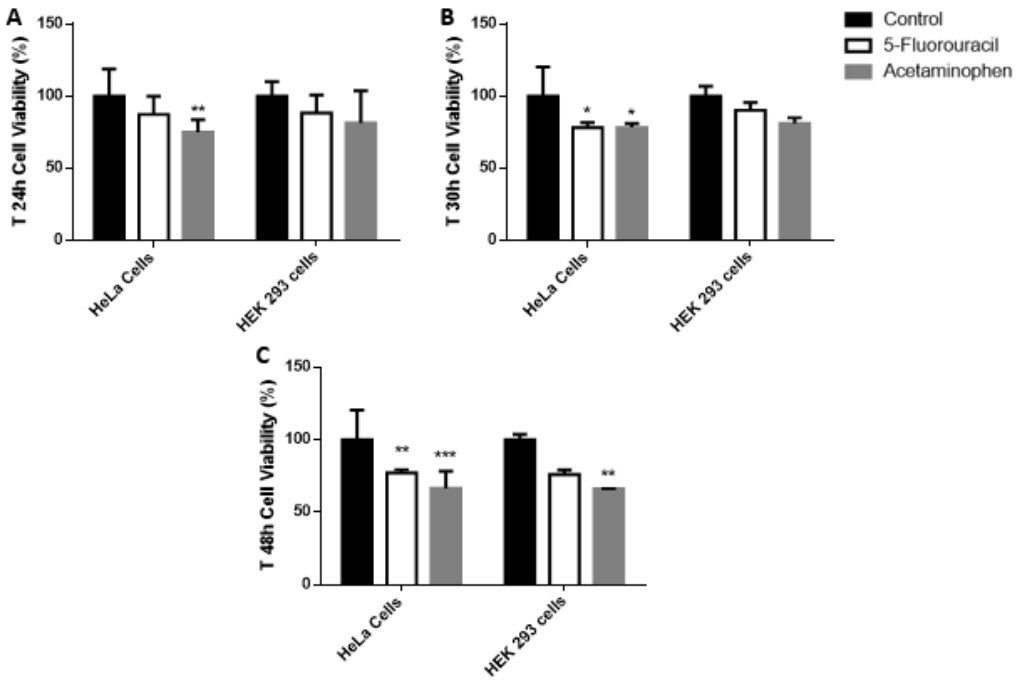

Figure 1: Cell viability of HeLa and HEK 293 cells lines treated with acetaminophen or 5-fluorouracil decreases with incubation time. (A) Cell viability analysis for 24h of incubation. (B) Cell viability analysis for 30h of incubation. (C) Cell viability analysis for 48h incubation time. *P<0.05, ** P<0.01, *** P<0.001 compared with the control group.

HEK 293 cells compared to the control group. After 48h of treatments, cell death was also observed on HeLa cells (P<0.01, Fig. 1C) at similar level that for the 30h incubation results. HEK 293 cells viability was significantly affected by the acetaminophen treatment. HEK 293 seemed to be affected by the 5-fluorourcil but the results obtained were not significant (P<0.01, Fig. 1C). Therefore, Hela cells are more sensitive than HEK 293 cells for both, 5 fluorouracil and acetaminophen treatments, at all observed times. In addition, drugs-induced cell viability reduction is faster in HeLa cell line compared to HEK 293 cells.

Acetaminophen induce necrosis and 5-fluorouracil induce apoptosis of HeLa cells.

Acridine orange permeates all cells and stains the nuclei in green. Ethidium bromide is taken in cells when membrane integrity is lost and stains nucleus red by dominating the green staining. Consequently, the living cells present a normal green nucleus. The early apoptotic cells have a bright green nucleus because of pyknosis: the chromatin condense in the nucleus. The late apoptotic cells present a bright orange chromatin due to loss of membrane integrity. Finally, an orange nucleus is observed for necrotic cells. Necrotic cells and late apoptotic cells are both stained in orange but can be differentiated because of their nuclei shapes. Indeed, necrotic cells keep a normal spheric nucleus shape whereas late apoptotic cells have a condensed and fragmented nucleus shape.

Acridine Orange/BET assay seemed to show a necrotic death for HeLa cells after 24h of treatment with acetaminophen (Fig. 2A). On the opposite, results for 5-florouracil (Fig. 2B) tend to show an apoptotic cell death.

Acetaminophen and 5-fluorouracil induce a greater apoptosis rate on Hela cells than on HEK 293 cells.

The Hoechst 33342 staining (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl] 2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA stain that is

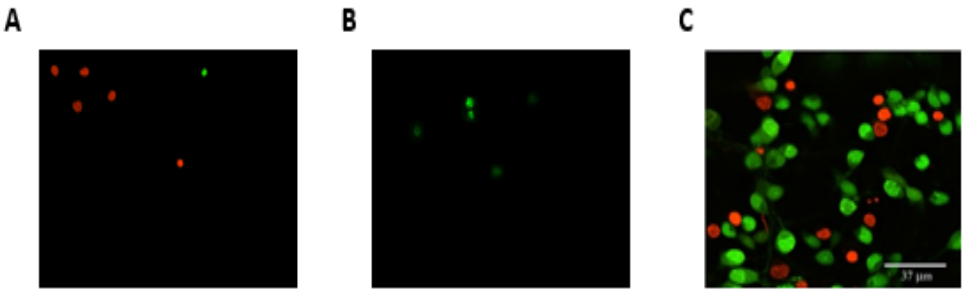

Figure 2: Acridine Orange/BET assay results compared to expected results. (A) Acridine Orange/BET assay on HeLa cells treated with acetaminophen after 24h. (B) Acridine Orange/BET assay on HeLa cells treated with 5-fluorouracil after 24h (C) Expected results [2].

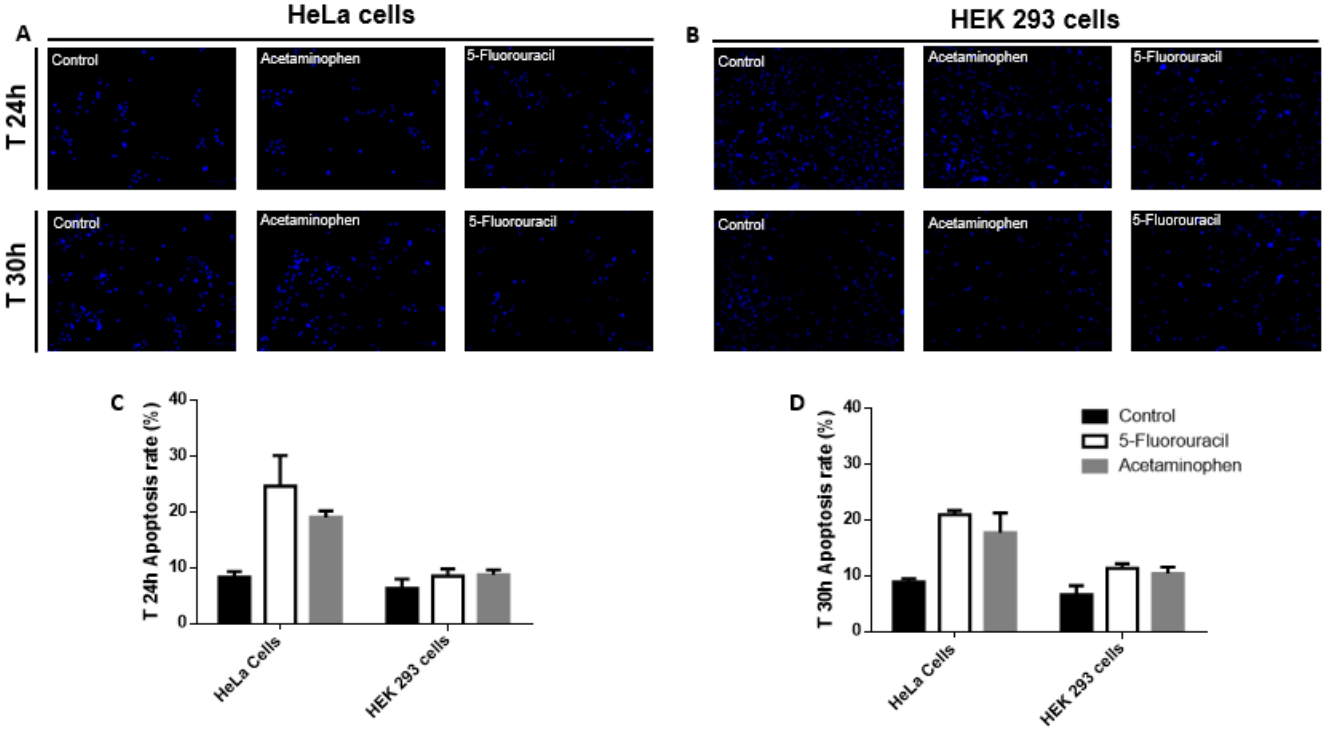

Figure 3: Apoptosis rate in HEK 293 cell lines. (A) Hoechst staining in HeLa cells with acetaminophen and 5-fluorouracil after 24h and 30h. (B) Hoechst staining in HEK 293 cells with acetaminophen and 5-fluorouracil after 24h and 30h. (C) Apoptosis rate after 24h incubation time. (D) Apoptosis rate after 30h incubation time.

excited by ultraviolet light and emits blue fluorescence at 460-490 nm. It binds preferentially to adenine-thymine (A-T) regions of double stranded DNA. The Hoechst 33342 dye was used to distinguish condensed pyknotic nuclei in apoptotic cells (Fig. 3A, 3B). Results show a greater apoptosis rate for Acetaminophen and 5-fluorouracil of HeLa cells than for HEK 293 cells after 24h of treatments (Fig. 3C). This result is confirmed after 30h of incubation with the two different treatments, the apoptosis rate of HeLa cells is still higher than for HEK 293 cells (Fig. 3D). Those observations are consistent with the viability assay.

A table containing the failed experiments and the explanations has been made and placed in appendix.

IV - Discussion

Preliminary results seemed to indicate that HEK 293 and Hela cells have different reactions according to the two treatments.

These results could have been interpreted as the fact that HEK 293 cell line have a better resistance than the Hela one. We hypothesized that it was probably due to a specific renal metabolism related to HEK 293. Another hypothesis is that the growth rate of HEK 293 cells, higher than the growth rate of HeLa cells, leads to an artificial resistance with higher survival rate of HEK 293 cells. Moreover, the statistical analysis of the results tends to confirm this hypothesis.

Also, the death mechanism was determined by Hoechst staining, according to each treatment: acetaminophen seems to induce necrosis rather than apoptosis in both cell lines, showing that this drug presents uncontrolled side-effects. However, 5-fluorouracil provokes more apoptosis than necrosis, which is its primary function. Indeed, an anti-cancer drug is supposed to induce apoptosis in cancerous cells [14]. Acridine Orange/BET assay did not show any accurate results to confirm this observation. Increasing the well's cell density might improve the staining. The extrinsic or intrinsic pathways of apoptosis might be assessed by western blot, with 4 types of antibodies: caspase 8, cleaved caspase 8, caspase 9 and cleaved caspase 9. As the caspase 8 is related to membrane death-mediated apoptotic pathway (extrinsic) and that caspase 9 is related to mitochondrial apoptotic pathway (intrinsic), then the detection of cleaved (activated) caspases would have indicated a predominance of one way rather than other one.

Nevertheless, the Western Blot did not show any conclusive result. The electrophoresis step was let overnight at 25V, which made the proteins migrate over the membrane as any of them was observable. The Coomassie Blue still indicated the presence of proteins as a control. Therefore, further investigations on apoptotic pathway in HEK 293 is needed, both in acetaminophen-mediated and 5-fluorouracil-mediated cell death.

A previous study deciphered that HeLa acetaminophen-mediated cell death went through caspase-3 activation and release of cytochrome c. [16] As cytochrome c is a major compound in the formation of apoptosome (Apaf-1/caspase-9), their results seem to indicate that HeLa underwent mitochondrial apoptosis (intrinsic) rather than extrinsic [16]. Indeed, extrinsic pathway is characterized by the activation of caspase 8 through Fas receptor while intrinsic pathway is characterized by the formation of apoptosome (Fig. 4). Also, they demonstrated that another major factor of acetaminophen-mediated apoptosis in different cultured cells (as HeLa cells) is a deoxyribonuclease 1-like 3 (DNAS1L3) [16]. HeLa cells are DNAS1L3-deficient, and this endonuclease has been transfected in cells in the study, hypothetically enabling them to undergo this way of apoptosis. It is therefore possible that HeLa cells in our own study showed more necrosis than apoptosis, unfortunately, the experiment did not produce any valuable data. Indeed, they were not transfected with DNAS1L3 and so could not undergo an efficient apoptosis. HEK 293 are also DNAS1L3 deficient, so the same hypothesis could be applied. In order to really compare acetaminophen toxicity with 5-fluorouracil, cell lines transfected with DNAS1L3 caspase 3-dependent are needed to assess apoptosis.

Also, another previous study [15] demonstrated that 5-fluorouracil induced both extrinsic, in Apo-1/CD95 fashion, and intrinsic pathway, via the cytochrome C release. The predominance of either way however has not been deciphered. Therefore, upregulation of genes/proteins implied in intrinsic or extrinsic pathway could be expected in HEK 293 5-fluorouracilmediated cell death. However, determining dominance of one pathway would require a successful quantification of cleaved caspase 8 and cleaved

caspase 9. The study also showed that HeLa 5-fluorouracil-mediated cell death implied activation of pro-apoptotic factor (as p53) but also reduction of viral oncogenic genes expression in cells, like E6/E7 genes of the HPV virus. As the HEK 293 are embryonic kidney cells transfected with adenovirus, study of the influence of 5-fluorouracil on viral genes in HEK 293 could give some insights about its mechanism of action and targets for viral-mediated cancer.

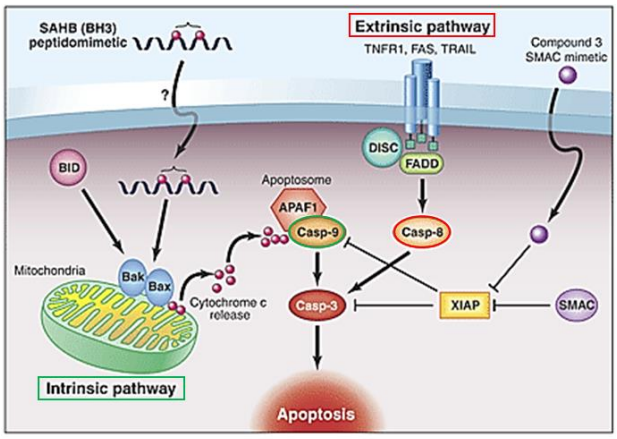

Figure 4: caspase 9 is involved in intrinsic pathway and caspase 8 in extrinsic pathway [13].

V – Conclusion

To conclude this study, several observations have been made: acetaminophen and 5-fluorouracil induce different cell death rate on HEK 293 and HeLa cells lines, suggesting a better resistance of the HEK 293 cells in both cases. This may be an advantage concerning the acetaminophen but a possible drawback for the 5-fluorouracil. Indeed, if cells show a resistance to the treatment, this would slow the recovery. As there are two different cell types, cervix cells for the HeLa and renal cells for the HEK 293, the cell type might be a determinant factor. Further investigations need to be conducted in order to assess the significance of the comparison. It is already well-known that acetaminophen is toxic for the liver cells [10] and the results showed here that acetaminophen is also toxic for both HeLa and HEK 293 cell lines and so cervix and renal cells. However, our results on acetaminophen-induced cell death might have been disrupted as the HeLa cell line used in this study did not display some factors that seem to be involved in acetaminophen-induced apoptosis and then, they rather undergo necrosis. A better characterization of apoptosis/necrosis ratio in each test could be reassessed with an Acridine Orange/BET assay. Then, the involved ways of apoptosis could be investigated by successfully carrying out a caspase-antibody assay. This can be done both by achieving protein quantification (other techniques than Bradford assay could be more efficient) and Western Blot. To go further, the evaluation of apoptosis ways could be coupled with semi-quantitative RT-PCR analysis of candidate genes and also by using caspase-3/DNAS1L3-transfected cell lines.

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Appendix

Failed experiment	Explanation
XTT test	On the first week of experiment, we obtained good results with this method. During the second week, the previous solution was used, and no cell death was detected. Two hypothesis were made: - The first is that the XTT solution had to be prepared extemporaneously - The second is that the XTT solution was badly prepared.
Acridine Orange Ethidium Bromide test	The acridine orange/ethidium bromide staining was prepared using a standard protocol but very few cells were observed. The cells needed to be concentrated in the plate in order to have significant results. However, that was not the case here, due to a lack of material. The major part of the cells was floating, and we observed very few adherent cells. A centrifugation of the 96-wells plate is necessary to have accurate results for this experiment.
Bradford assay	Bradford technique was performed to determine the protein concentration in the samples. The absorbance was the same for all samples. The used buffer for the bradford assay was the same than the sample buffer, RIPA buffer. The RIPA buffer contains SDS, which interacted with the BSA and denatured our standard and sample.
Western-Blot	The running of western-blot was made at 80V for one hour, but some problems were encountered with the migration as the proteins did not migrate. Another equipment was used to perform western-blot technique. The voltage was increased at 90V for an hour. Again, the sample did not migrate. The explanation is that the connexion was serial and not in derivation. The running was finally done at 25V overnight, but no protein was observable. An explanation can be that the SDS present in the buffer associated with overnight running, the proteins went over the membrane and were not observable.