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Study of mechanisms by which ponatinib induces vascular occlusive events in the treatment of Philadelphia chromosome-positive leukemia

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STUDY OF MECHANISMS BY WHICH PONATINIB INDUCES VASCULAR OCCLUSIVE EVENTS IN THE TREATMENT OF PHILADELPHIA CHROMOSOME-POSITIVE LEUKEMIA

Mémoire présenté pour l'obtention

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Study of mechanisms by which ponatinib induces vascular occlusive events in the treatment of Philadelphia chromosome-positive leukemia

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Summary

Philadelphia chromosome-positive leukemia is characterized by the production of the BCR-ABL tyrosine kinase in leukemia cells. This fusion protein induces the excessive proliferation of leukemia cells, preventing the normal production of blood cells. Tyrosine kinase inhibitors (TKIs) were developed to inhibit the anarchic proliferation of leukemia cells in bone marrow by preventing the BCR-ABL target proteins phosphorylation. Ponatinib is the latest commercialized TKI and has the highest antileukemia activity among its drug family. Therefore, it represents the last treatment option for patients suffering from Philadelphia chromosome-positive leukemia in case of resistance and/or intolerance to other TKIs. However, this medication is often related to thrombotic events development in patients, leading to its temporary withdrawal from the market.
This master thesis aims at better understanding the mechanisms by which ponatinib induces vascular occlusive events in order to enhance the management and prophylaxis of this adverse drug reaction.
Several mechanisms could explain the thrombosis development following ponatinib therapy. During this master thesis, two assumptions were investigated: a potential impact of ponatinib on platelet aggregation and on endothelial cells. Firstly, the impact of this TKI on primary hemostasis was assessed. This allowed to highlight that ponatinib should not induce thrombosis by increasing platelet aggregation. Secondly, the impact of ponatinib on vascular endothelium was investigated by studying its effect on the hemostatic balance and on the metabolism of endothelial cells. Ponatinib seems to slightly activate endothelial cells and decrease their metabolism which could partly explain the increased risk of vascular occlusive events development in ponatinib-treated patients.

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Etude des mécanismes par lesquels le ponatinib induit des évènements occlusifs vasculaires dans le traitement des leucémies associées au chromosome de Philadelphie

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Résumé

Les leucémies associées au chromosome de Philadelphie sont caractérisées par la synthèse de la tyrosine kinase BCR-ABL dans les cellules leucémiques. Cette protéine de fusion entraîne la prolifération excessive des cellules leucémiques, empêchant la production normale des cellules sanguines. Des inhibiteurs de tyrosine kinase (TKIs) ont été développés afin d'inhiber la prolifération anarchique des cellules leucémiques dans la moelle osseuse en empêchant la phosphorylation des protéines cibles de BCR-ABL. Le ponatinib est le dernier TKI mis sur le marché et possède la plus grande activité anti-leucémique de sa classe de médicament. Par conséquent, il représente la dernière option de traitement pour les patients atteints d'une leucémie associée au chromosome de Philadelphie en cas de résistance et/ou d'intolérance aux autres TKIs. Cependant, la prise de ce médicament est souvent associée au développement d'évènements thrombotiques chez les patients, ce qui a valu sa suspension temporaire du marché.
Ce mémoire vise à mieux comprendre les mécanismes par lesquels le ponatinib induit des évènements occlusifs vasculaires afin d'améliorer la prise en charge et la prophylaxie de cet effet secondaire.
Plusieurs mécanismes pourraient expliquer le développement de thromboses suite à la prise de ponatinib. Au cours de ce mémoire, deux hypothèses ont été étudiées: un impact potentiel du ponatinib sur l'agrégation plaquettaire et sur les cellules endothéliales. Dans un premier temps, l'impact de ce TKI sur l'hémostase primaire a été évalué. Ceci a permis de montrer que le ponatinib ne devrait pas induire de thromboses en augmentant l'agrégation plaquettaire. Dans un second temps, l'impact du ponatinib sur l'endothélium vasculaire a été analysé en étudiant son effet sur la balance hémostatique et sur le métabolisme des cellules endothéliales. Le ponatinib semble légèrement activer les cellules endothéliales et diminuer leur métabolisme ce qui pourrait partiellement expliquer le risque augmenté d'accidents vasculaires chez les patients traités au ponatinib.

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D'emblée, je vous souhaite à tous une bonne lecture.

List of abbreviations

ABL:	Abelson murine leukemia viral oncogene homolog
ADP:	Adenosine diphosphate
ADRs:	Adverse drug reactions
ALL:	Acute lymphoblastic leukemia
aPC:	Activated protein C
AT:	Antithrombin
ATP:	Adenosine triphosphate
BBE:	Bovine brain extract
BCR:	Breakpoint cluster region
CML:	Chronic myeloid leukemia
CML-AP:	Accelerated phase of chronic myeloid leukemia
CML-BC:	Blast crisis in chronic myeloid leukemia
CML-CP:	Chronic phase of chronic myeloid leukemia
DMEM:	Dulbecco's modified eagle's medium
DMSO:	Dimethyl sulfoxide
EBM:	Endothelial cell basal medium
EGM:	Endothelial cell growth medium
EPCR:	Endothelial protein C receptor
EVs:	Extracellular vesicles
FVIIa:	Activated factor VII
FXa:	Activated factor X
FXII:	Factor XII
FBS:	Fetal bovine serum
FDA:	Food and drug administration
GPIb/IX:	Glycoprotein Ib/IX
HCT:	Hematopoietic cell transplantation
HSCT:	Hematopoietic stem cell transplantation
HUVECs:	Human umbilical vein endothelial cells
ICAM-1:	Intercellular adhesion molecule-1
LDH:	Lactate dehydrogenase
NPP:	Normal pooled plasma
PACE:	Ponatinib Ph-positive acute lymphoblastic leukemia and CML evaluation
PAF:	Platelet-activating factor
PAI-1:	Plasminogen activator inhibitor-1
PBS:	Phosphate buffer saline
PDGFR:	Platelet-derived growth factor receptor
PE:	Phosphatidylethanolamine
PEG:	Polyethylene glycol
PGI_2:	Prostacyclin
Ph:	Philadelphia chromosome
PL:	Phospholipid
PPP:	Platelet-poor plasma
PRP:	Platelet-rich plasma
PS:	Phosphatidylserine
rhEGF:	Recombinant human epidermal growth factor
rIFNα:	Recombinant interferon α
SEM:	Standard error of the mean

SFKs:	Src-family kinases
TF:	Tissue factor
TFPI:	Tissue factor pathway inhibitor
TKIs:	Tyrosine kinase inhibitors
TM:	Thrombomodulin
TNF-α:	Tumor necrosis factor-α
t-PA:	Tissue-plasminogen activator
VAEs:	Vascular adverse events
VEGF:	Vascular endothelial growth factor
VEGFR:	Vascular endothelial growth factor receptor
VOEs:	Vascular occlusive events
VTE:	Venous thromboembolism event
vWF:	Von Willebrand factor

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I. Introduction

I. Introduction

I.1 Philadelphia chromosome positive leukemia

I.1.1 The pathology

Leukemia is a neoplastic disorder which originates from a genetic change in a type of hematopoietic stem cell. This genetic change induces the uncontrolled proliferation of this blood stem cell. Philadelphia chromosome-positive (Ph-positive) leukemia is characterized by the presence of Philadelphia chromosome, which is not found in healthy people (An et al., 2010). This genetic abnormality is encountered in chronic myeloid leukemia (CML) and in a subset of acute lymphoblastic leukemia (ALL) (Cortes et al., 2012a). CML affects myeloid progenitor cells while ALL affects lymphoblastic progenitor cells. The worldwide incidence of CML is ranging from 0.6 to 2.0 cases per 100000 inhabitants, making this disorder the most prevalent Ph-positive leukemia (Rohrbacher and Hasford, 2009). The median age of patients suffering from CML is around 60 years old with a low incidence in patients under 20 years old (Cortes, 2004). The worldwide annual incidence of adult ALL is about 1 case per 100000 inhabitants (Abbasi et al., 2013). Philadelphia chromosome is the most frequent cytogenetic abnormality observed in ALL adult patients (Foa et al., 2011). Indeed, the prevalence of Ph-positive ALL increases with age and the patients suffering from this acute leukemia are mostly over 60 years old (Brissot et al., 2015).

I.1.2 Philadelphia chromosome and its oncogenic product, BCR-ABL

The Philadelphia chromosome was described for the first time in 1960 by Peter Nowell and David Hungerford in Philadelphia (Koretzky, 2007). Philadelphia chromosome is encountered in more than 90% of CML patients and in 25 to 30% of adult ALL patients (Tanguy-Schmidt et al., 2013). This chromosome results from a translocation between the chromosomes 9 and 22, which induces the fusion between the abl and bcr genes (Figure I.1) (An et al., 2010).

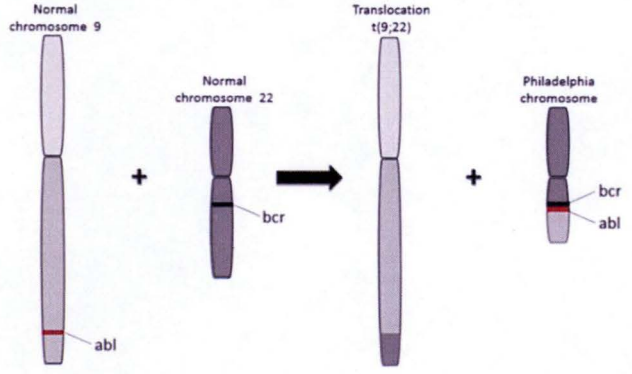

Figure I.1: **Origin of Philadelphia chromosome.** This chromosome is the result of a translocation between the chromosomes 9 and 22. This genetic abnormality involves a fusion between the abl (normally on the chromosome 9) and bcr (normally on the chromosome 22) genes. The protein product of the bcr-abl fusion gene is the tyrosine kinase BCR-ABL which is not present in healthy people.

The breakpoint cluster region protein (BCR) has a serine/threonine kinase activity while the Abelson murine leukemia viral oncogene homolog (ABL) gene encodes a cytoplasmic and nuclear tyrosine kinase implicated in cell differentiation, division, adhesion and stress response (An et al., 2010). The bcr-abl oncogene encodes a constitutively active protein with a molecular weight ranging from 185 to 230 kDa (210 kDa in most cases), depending on the site of the breakpoint in the BCR gene (Deininger et al., 2000).

BCR-ABL, as all kinases, transfers a phosphate from adenosine triphosphate (ATP) to tyrosine residues of target molecules. The excessive proliferation encountered in Ph-positive leukemia is induced by the interaction of BCR-ABL with multiple downstream signaling partners (An et al., 2010). In brief, $p210^{BCR/ABL}$ activates signal transduction pathways such as RAS/MAPK, PI-3 kinase/AKT and JAK-STAT pathways (Figure I.2) (Sawyers, 1997). Overall, the activation of these pathways by BCR-ABL leads to the altered cellular adhesion, the activation of mitogenic signaling and the inhibition of apoptosis in BCR-ABL-expressing cells. These mechanisms lead to an abnormal proliferation of mutated BCR-ABL hematopoietic stem cells (An et al., 2010).

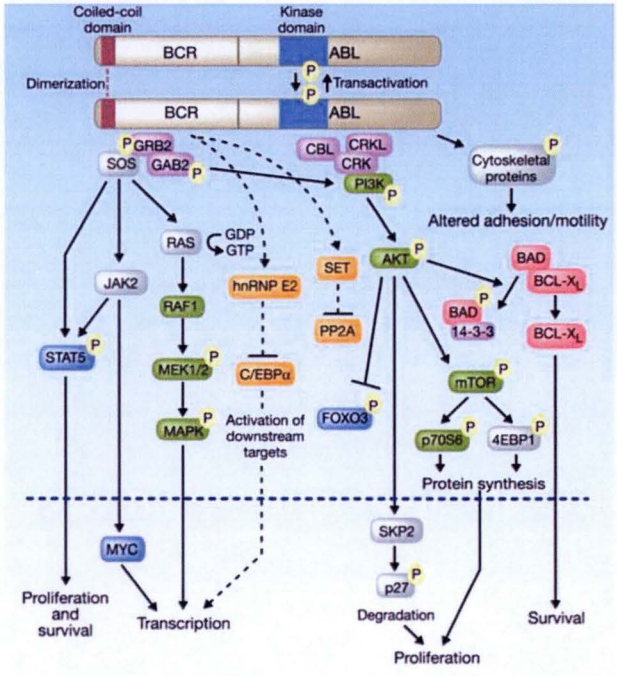

Figure I.2: **BCR-ABL-downstream signaling pathways.** The BCR-ABL tyrosine kinase transfers a phosphate from ATP to tyrosine residues of target molecules, activating them. RAS/MAPK, PI3K/AKT and JAK-STAT pathways are especially activated. These signaling pathways lead to the altered cellular adhesion, inhibition of apoptosis and survival, resulting in the abnormal proliferation of mutated BCR-ABL hematopoietic stem cells. Therefore, the excessive proliferation encountered in Ph-positive leukemia is induced by the interaction of BCR-ABL with multiple downstream signaling partners. Adapted from (O'Hare et al., 2011).

Immature progenitors of blood cells are released into circulation because of the adherence impairment of progenitors to the marrow stroma (Loren et al., 2015). Signaling pathways mediated by BCR-ABL also induce a defecting DNA repair, leading to an accumulation of mutations in cells and partly explaining the disease progression and the aggressive nature of advanced CML (An et al., 2010).

I.1.3 Chronic myeloid leukemia

The CML patients suffer from thrombocytopenia and anemia due to a lack of normal blood cells caused by the invasion of blast cells in the bone marrow. The most common clinical manifestations are splenomegaly, weakness, fatigue and bleeding (Sawyers, 1999). Three distinct phases can be observed in CML: (i) the chronic phase (CML-CP) is primarily asymptomatic and is characterized by an increase in granulocytes. CML is usually diagnosed in this phase. (ii) When the CML-CP progresses, the disease reaches the accelerated phase (CML-AP) which is characterized by a rapid expansion of granulocytes. (iii) The ultimate phase is characterized by a blast crisis (CML-BC), resembling to acute leukemia and leading to organ failure, metastasis and death (An et al., 2010).

I.2 Treatments of Philadelphia chromosome positive leukemia

I.2.1 The first therapies

The Ph-positive ALL patients were commonly treated with allogeneic hematopoietic stem cell transplantations (HSCT) (Byun et al., 2015). The first therapies used to treat CML patients were cytotoxic drugs (such as busulfan and hydroxyurea) (Bolin et al., 1982), a recombinant interferon α (rIFN-α) monotherapy (Authors not communicated 1997) and allogeneic hematopoietic cell transplantations (HCT) (Silver et al., 1999). All these therapies had drawbacks. Indeed, the use of cytotoxic drugs did not prevent the disease progression, rIFN-α induced many adverse events and HCT were associated with a significant morbidity and mortality rate besides the need of a compatible donor (An et al., 2010). Therefore, research has focused on compounds that selectively inhibit the BCR-ABL tyrosine kinase. This resulted in the development of a new drug class called tyrosine kinase inhibitors (TKIs).

I.2.2 Tyrosine kinase inhibitors

I.2.2.1 The action mechanism of tyrosine kinase inhibitors

The action mechanism of TKIs is the competitive inhibition of the ATP binding site of BCR-ABL. Indeed, TKIs bind to the inactive conformation of BCR-ABL, block the binding of ATP to the tyrosine kinase and prevent its conformational switch to the active form, resulting in the inhibition of the ATPase catalytic activity of BCR-ABL (Figure I.3) (An et al., 2010). The TKIs-induced inactivation of pathways activated by BCR-ABL significantly decreases the excessive myeloid cell proliferation in CML (Kantarjian et al., 2007).

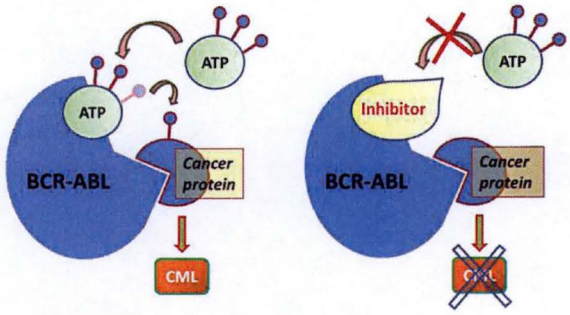

Figure I.3: **The TKIs action mechanism.** BCR-ABL tyrosine kinase transfers a phosphate from ATP to target proteins implicated in several signaling pathways. TKIs competitively inhibits the ATP binding site of BCR-ABL. Indeed, these inhibitors bind to the inactive conformation of BCR-ABL, block the binding of ATP and prevent its conformational switch to the active form. Therefore, the ATPase catalytic activity of BCR-ABL is inhibited by TKIs. (Zaharieva et al., 2013).

I.2.2.2 The different generations of tyrosine kinase inhibitors

Currently, five TKIs are approved by the Food and Drug Administration (FDA) and are classified in three generations. Their year of approval in the USA and their indications in CML can be found in the Table I.1 (Sweet et al., 2013).

Table I.1: **The different FDA-approved TKIs, their year of approval in the USA and their indications in CML.** (Sweet et al., 2013).

Name	Year approved	Indications in CML
Imatinib	2001	CP, AP, or BC after failure of interferon therapy
	2003	Newly diagnosed CP
Dasatinib	2006	CP, AP, or BC after resistance to or intolerance of imatinib
	2010	Newly diagnosed CP
Nilotinib	2007	CP or AP after resistance to or intolerance of imatinib
	2010	Newly diagnosed CP
Bosutinib	2012	CP, AP, or BC after resistance to or intolerance of prior therapy
Ponatinib	2012	CP, AP, or BC after resistance to or intolerance of prior TKI therapy

Abbreviations used: *AP* accelerated phase, *BC* blast crisis, *CP* chronic phase

a) The first generation of tyrosine kinase inhibitors

The first TKI approved by the FDA in 2001 was imatinib mesylate (Sweet et al., 2013). The introduction of this TKI has revolutionized the management of Ph-positive CML patients because the first-line imatinib treatment allows to achieve high response rates in CML patients (An et al., 2010). Therefore, imatinib is the gold standard for the treatment of newly diagnosed Ph-positive leukemia patients (Valent et al., 2015). The recommended dose of imatinib is 400 mg daily *per os* (An et al., 2010). However, patients may develop resistance or intolerance to the imatinib therapy. Indeed, up to 40% of patients have a failure of the imatinib therapy because of resistance or development of unacceptable side events (Hochhaus et al., 2009).

b) The second generation of tyrosine kinase inhibitors

A second generation of TKIs was developed to treat patients who have discontinued the imatinib therapy due to intolerance or resistance (Cortes et al., 2013). Indeed, more potent TKIs have been developed to treat these patients and are successfully used in daily practice (Valent et al., 2015). The second treatment generation includes three molecules: nilotinib, dasatinib and bosutinib. Dasatinib and nilotinib were approved by FDA in 2006 and 2007, respectively (Sweet et al., 2013) and have a high potency against BCR-ABL wild type and against the most imatinib-resistant BCR-ABL point mutants (An et al., 2010). Moreover, compared to imatinib, these two TKIs induce higher response rates in patients (Hochhaus et al., 2008). Bosutinib is the last second-generation TKI approved by FDA and also inhibits a large amount of BCR-ABL mutants. These three second-generation TKIs are inactive against the common T315I mutant of BCR-ABL (Cortes et al., 2012b).

c) The third generation of tyrosine kinase inhibitors

The approving of ponatinib by the FDA in 2012 has allowed the inhibition of proliferation of the most mutated BCR-ABL leukemia cells, particularly those with the common T315I mutation (Cortes et al., 2013). Indeed, unlike other TKIs, ponatinib is able to place itself correctly in the ATP-binding side of the T315I mutant of BCR-ABL thanks to its triple-bond linkage (Figure I.4 and I.5) (Cortes et al., 2012a).

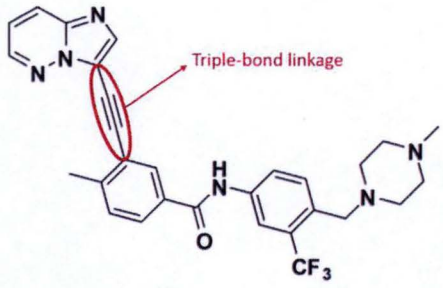

Figure I.4: **The chemical structure of ponatinib.** Ponatinib, unlike the TKIs of the first and second generations, has a triple-bound linkage which allows its correct positioning in the ATP-binding side of the most mutated BCR-ABL. Adapted from (O'Hare et al., 2009).

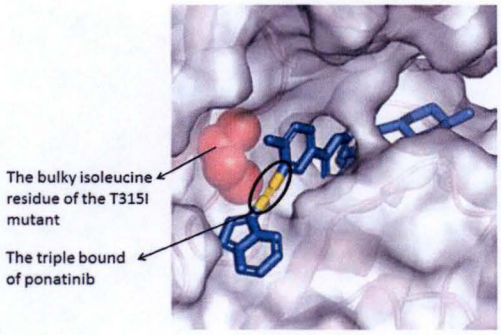

Figure I.5: **Ponatinib in the active site of the T315I mutant of BCR-ABL.** Isoleucine, compared with threonine, is a bulky residue so that it blocks the TKIs access to the ATP binding site of BCR-ABL. Ponatinib has a triple-bound linkage which allows its correct positioning in the ATP-binding side of BCR-ABL despite the presence of the isoleucine residue. Adapted from (Cortes et al., 2012a).

Beside BCR-ABL, ponatinib also inhibits a subset of other tyrosine kinases including the platelet-derived growth factor receptor-α (PDGFR-α), the vascular endothelial growth factor receptor 2 ($VEGFR_2$) and the Src-family kinases (SFKs) (O'Hare et al., 2009).

Ponatinib has a high antileukemic activity and allows to reach high rates of molecular, cytogenetic and hematologic responses in Ph-positive leukemia patients compared to the other TKIs. Ponatinib is also highly active in heavily pretreated patients and in patients suffering from all the CML phases, including the advanced stages (Hochhaus et al., 2013). This superior potency of ponatinib can be explained by its effect on a wide range of BCR-ABL mutants as well as a stronger inhibition of BCR-ABL and the downregulation of further targets (Valent et al., 2015). Moreover, unlike the other TKIs, ponatinib does not seem to induce any single BCR-ABL mutation emergence (Cortes et al., 2013).

Currently, ponatinib is indicated for the CML and ALL patients resistant or intolerant to the imatinib or dasatinib or nilotinib therapy or for patients with the T315I mutation (Jülicher, 27/11/2013). The recommended starting dose of ponatinib is 45 mg/day *per os*. Indeed, this dose is optimum for safety, pharmacokinetic and pharmacodynamics (Cortes et al., 2012a).

I.2.2.3 The resistance associated to tyrosine kinase inhibitors

a) The mechanism of resistance

The failure of the TKIs therapy in patients with a Ph-positive leukemia is mainly due to a TKIs resistance. One of the major causes of TKIs resistance is the emergence of point mutations in the kinase active domain of BCR-ABL. Indeed, point mutations induce amino acid substitutions in the kinase domain of BCR-ABL (An et al., 2010). Moreover, amino acid substitutions induce a decrease of contact points between TKI and the BCR-ABL active site, disrupting the interaction between BCR-ABL and the drug which lead to TKIs resistance (Branford et al., 2003).

b) The T315I mutation

The T315I mutation is one of the most frequent BCR-ABL point mutations. Indeed, up to 20% of patients with a TKI-resistant disease present this mutation (Quintas-Cardama et al., 2007). The T315I mutation is a substitution of a threonine by an isoleucine at the amino acid position 315. The isoleucine residue is bulky and blocks the TKIs access to the ATP binding site of BCR-ABL, conferring resistance to the first and second generations of TKIs. Indeed, imatinib, nilotinib, dasatinib and bosutinib need to form an hydrogen bond with the threonine residue. Only ponatinib has a triple-bond linkage, enabling it to place itself correctly in the ATP-binding side of the T315I mutant of BCR-ABL (Cortes et al., 2012a).

I.2.2.4 Adverse drug reactions associated with the tyrosine kinase inhibitor therapy

a) Adverse drug reactions associated with the first and second tyrosine kinase inhibitor generations

Although they belong to the same drug family, each TKI generates a unique pattern of adverse drug reactions (ADRs) because they interact with different target molecules. Indeed, the chemical structures of the different TKIs are slightly different to differently interact with BCR-ABL and to inhibit the proliferation of increasingly BCR-ABL mutants (Valent et al., 2015).

Imatinib is a relatively safe TKI with well-documented associated adverse reactions. A low amount of vascular adverse events (VAEs) occurs in patients receiving imatinib. In contrast to patients receiving imatinib, patients treated with a second-generation TKI may develop several types of VAEs (Valent et al., 2015). The nilotinib-treated patients can suffer from cardiovascular events, peripheral arterial occlusive disease and cerebral ischemia (Aichberger et al., 2011) while the patients receiving dasatinib often develop pulmonary hypertension (Montani et al., 2012). Moreover, dasatinib is known to induce platelet dysfunction leading to bleeding in treated patients (Neelakantan et al., 2012). Finally, bosutinib-treated patients have a slightly increased risk of arterial hypertension and VAEs (Cortes et al., 2014a).

b) Adverse drug reactions associated with the ponatinib therapy

The ponatinib therapy is associated with multiple ADRs which can be classified in three categories: (i) the non-hematological ADRs, (ii) the hematological ADRs and (iii) the vascular adverse events.

- *Non-hematological adverse drug reactions induced by ponatinib*

As ponatinib inhibits a wider range of BCR-ABL mutants than the previous TKI generations, it is associated with more ADRs because of its inhibition of additional targets. Therefore, non-hematological ADRs may occur in patients receiving this TKI due to an inhibition of several targets which are expressed in non-hematopoietic cells (Valent et al., 2015). In the Ponatinib Ph-positive Acute lymphoblastic leukemia and CML Evaluation (PACE) clinical trial, the most common non-hematologic side events were rash, dry skin and abdominal pain (Cortes et al., 2013). Constitutional symptoms like arthralgia, fatigue and nausea are also encountered in the most common non-hematological adverse reactions related to ponatinib. Other adverse effects such as increased lipase or amylase levels and pancreatitis are encountered with an increased dose of ponatinib (Cortes et al., 2012a).

- *Hematological adverse drug reactions induced by ponatinib*

The most common ponatinib-related hematologic ADRs are thrombocytopenia, anemia and neutropenia (Cortes et al., 2013).

- *Vascular adverse events*

A large number of VAEs, including venous and arterial thromboembolic events, are reported in ponatinib-treated patients (Hochhaus et al., 2013). The PACE clinical trial highlighted that 8.9 % of patients receiving ponatinib underwent VAEs after 11 months and 17.1 % after 24 months (Valent et al., 2015). After a long-term follow-up of ponatinib safety, the rates of venous and arterial thrombotic events were 37% and 24% in the phase 1 and 2 clinical trials, respectively (Talpaz et al., 2014; Cortes et al., 2014b).

The thrombotic vascular events caused by ponatinib led to its temporary suspension by the FDA in late 2013 but it was reintroduced in January 2014 following the introduction of new security measures (Sanford et al., 2015).

To decrease the risk of VAEs development in ponatinib-treated patients, the potential benefits of this treatment must exceed the risk factors for VAEs development in each patient starting a ponatinib therapy. Currently, ponatinib must be discontinued in patients developing intolerable vascular events (Valent et al., 2015). However, ponatinib has the highest antileukemic activity of the TKIs family and represents the last treatment option for patients suffering from a Ph-positive leukemia. Moreover, as mentioned above, ponatinib is the only TKI potent against the common T315I mutation. Therefore, the discovery of mechanisms by which ponatinib induces VAEs development in Ph-positive leukemia patients could significantly increase the survival of patients treated with this TKI by a better management and prophylaxis of this ADR. Finally, a decrease of the VAEs risk in ponatinib-treated patients could lead to a wider use of this drug which is the most potent of its family.

I.3 The potential underlying mechanisms for the development of a prothrombotic state in ponatinib-treated patients

The underlying mechanisms of the VAEs development following the ponatinib therapy are poorly understood but, considering the physiopathology of thrombosis in cancer patients and researches performed on other chemotherapic agents which increase the thrombotic risk, several assumptions can be highlighted. Some of these potential mechanisms will be developed but other valid hypotheses will not be further described.

I.3.1 Cancer: a prothrombotic disease

Thrombotic events are frequent in cancer patients and cancer is often referred as a prothrombotic disease (Blann, 2012). Among patients suffering from an hematological cancer, 2 to 12% develop vascular occlusive events (VOEs) (Elice and Rodeghiero, 2012). After the disease itself, venous thromboembolism events (VTE) are one of the main causes of mortality in cancer patients (Esposito et al., 2015). This procoagulant state observed in cancer patient is due to abnormalities in one or more components of Virchow's triad i.e. an hypercoagulability, an endothelial dysfunction and an hemodynamic change (Blann, 2012).

The physiopathology of thrombosis in cancer is not well understood but multiple factors seem to be involved in the mechanism which leads to the increased thrombotic risk of cancer patients. The most likely assumption is that both cancer cells and activated vascular cells exhibit molecules and release extracellular vesicles (EVs) which induce thrombin generation, leading to the prothrombotic phenotype (Blann, 2012).

Overall, the risk for cancer patients to develop VTE is linked to factors related to (i) the individual characteristics of patients (such as the age, the presence of comorbidities and the previous VTE events), (ii) the type and the extent of cancer (e.g. the hematological malignancies are associated with a higher risk of VTE (Thodiyil and Kakkar, 2002)) and (iii) the used anti-cancer treatment. Indeed, the use of some chemotherapeutic agents still increases the risk of cancer patients to develop thrombotic complications (Falanga and Marchetti, 2012b).

I.3.2 Potential direct impact of ponatinib on the coagulation cascade

Ponatinib could impact one or more factors of the coagulation cascade. This cascade is divided in two pathways: (i) the intrinsic pathway which takes place within blood and is triggered when factor XII (FXII) comes in contact with negatively charged surfaces and (ii) the extrinsic pathway which is triggered when activated factor VII (FVIIa) comes in contact with tissue factor (TF) bearing by cells. These two pathways lead to the formation of activated factor X (FXa). The following steps form the common pathway which results in the cleavage of fibrinogen into fibrin by thrombin (Figure I.6) (Smith, 2009). A potential direct impact of ponatinib on the coagulation cascade could partly explain the prothrombotic state of treated patients.

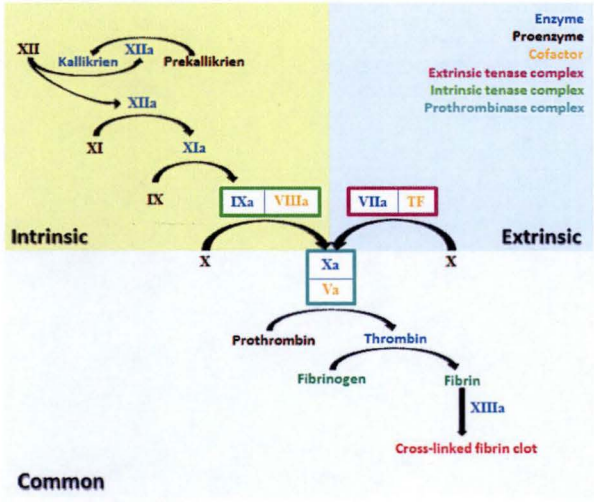

Figure I.6: **The cascade model of fibrin formation.** This model consists of a succession of enzymatic reactions in which a proenzyme is converted into an active enzyme by the previous activated enzyme of the cascade. This cascade is divided in two pathways: (i) the intrinsic pathway and (ii) the extrinsic pathway. These two pathways lead to the formation of FXa. The following steps form the common pathway which results in the cleavage of fibrinogen into fibrin by thrombin. The soluble fibrin forms aggregates and polymerizes into fibrin strands so that an insoluble cross-linked clot is generated. Inspired by (Smith, 2009).

I.3.3 Potential impact of ponatinib on vascular endothelium

I.3.3.1 The endothelial cells in resting conditions

The vascular endothelium can both trigger and inhibit the coagulation cascade (Geenen et al., 2012). Under normal physiological conditions, the vascular endothelium prevents the initiation and progression of the coagulation steps by exhibiting a non-thrombogenic surface to the bloodstream (Fu et al., 2010). The anticoagulant properties of the vascular endothelium are maintained by three mechanisms: (i) endothelial cells exhibit heparin-like proteoglycans on their membrane which allow antithrombin (AT) to better bind and inhibit its ligand, thrombin; (de Agostini et al., 1990) (ii) tissue factor pathway inhibitor (TFPI) is present on the surface of endothelial cells (Ameri et al., 1992) and (iii) the protein C pathway takes place on vascular endothelium thanks to the presence of thrombomodulin (TM) (Esmon and Owen, 1981) (Figure I.7).

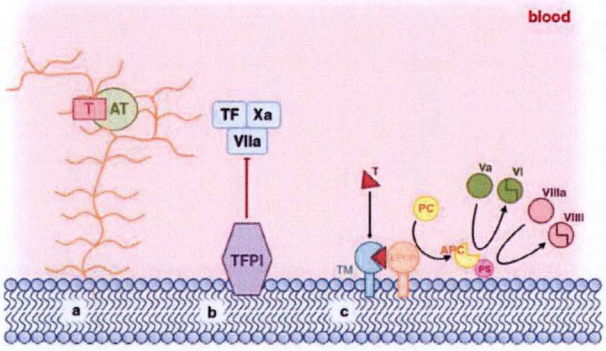

Figure I.7: **Three mechanisms maintain the anticoagulant properties of the vascular endothelium.** (a) Heparin-like proteoglycans are present on the endothelial cells membrane. AT binds to and is activated by these heparin-like molecules and is the main inhibitor of thrombin and FXa. (b) TFPI is the main inhibitor of TF and is exhibited on the resting endothelial cell surface. TFPI inhibits the TF-FVIIa-FXa complex. (c) Thrombin is implicated in a negative feedback pathway. Indeed, when thrombin binds to TM, the endothelial protein C receptor (EPCR) converts protein C into activated protein C (aPC). aPC and its protein S cofactor inactive FVa and FVIIIa. (Bouvy et al., 2014).

TFPI forms a complex with TF, FVIIa and FXa, preventing their further involvement in coagulation reactions (Smith, 2009). In the protein C pathway, the binding of thrombin to TM allows the protein C receptor to convert protein C into aPC. Then, aPC is able to interact with protein S and this complex inhibits FVa and FVIIIa, preventing their further involvement in the coagulation cascade (Coll et al., 2013). This system allows a negative feedback if thrombin is generated beyond the damaged areas (Verhamme and Hoylaerts, 2006).

Moreover, the resting vascular endothelium does not support coagulation cascade steps due to a presence of neutral phospholipids (PL) on the external leaflet of cell membranes. It was also highlighted that resting endothelial cells do not exhibit TF on their surface (Geenen et al., 2012). Then, resting endothelial cells secrete molecules such as prostacyclin (PGI_2), which can inhibit the primary hemostasis (Blann, 2012). Finally, endothelium exhibits fibrinolytic properties in physiological conditions, synthetizing and releasing tissue-plasminogen activator (t-PA) (Verhamme and Hoylaerts, 2006).

I.3.3.2 The potential activation of endothelial cells by ponatinib

Ponatinib could induce adverse effects on the integrity of vascular endothelium and deregulate the hemostatic balance of endothelial cells. Indeed, vascular endothelium is one of the major players of the hemostatic balance regulation (Geenen et al., 2012). On one hand, endothelial cells triggers the clot formation when a vascular injury happens and, on the other hand, they downregulates the hemostatic pathways in resting conditions. If this system is deregulated, the balance between pro- and anticoagulant pathways is also impacted and that may lead to thrombosis (Figure I.8) (Verhamme and Hoylaerts, 2006).

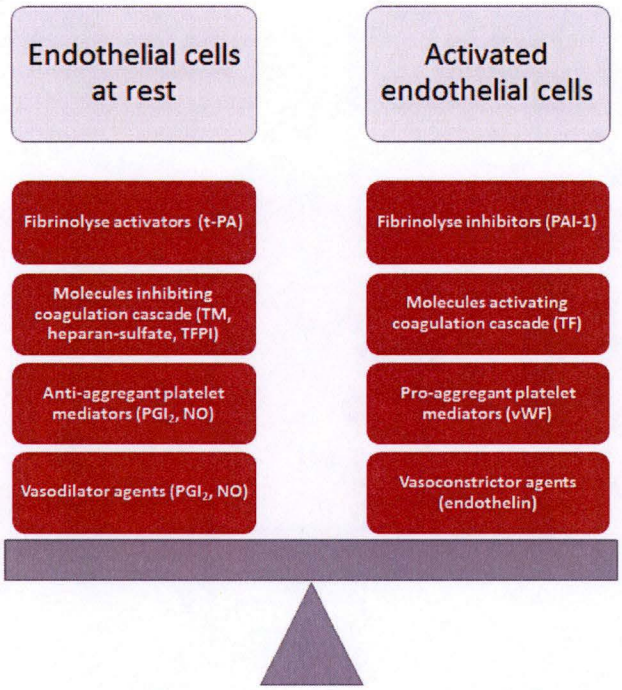

Figure I.8: **The hemostatic balance of endothelial cells.** At rest, endothelial cells express molecules inhibiting the coagulation cascade like TM, heparin-sulfate and TFPI and release anti-aggregant mediators (PGI_2 and NO), vasodilator agents (PGI_2 and NO) and fibrinolysis activators (t-PA). The activation of endothelial cells is associated with an overexpression of coagulation cascade triggers like TF as well as an oversecretion of platelet aggregation mediators (vWF), vasoconstrictor agents (endothelin) and fibrinolysis inhibitors (PAI-1).

Ponatinib could deregulate the hemostatic balance of endothelial cells by activating endothelial cells which then become procoagulant and proadherant. On one hand, ponatinib could activate endothelial cells by downregulating the expression of one or more of the three main anticoagulant pathways which take place on their surface i.e. the AT, TFPI and protein C pathways. On the other hand, the overexpression of several procoagulant or proaggregant molecules by ponatinib could also lead to the activation of endothelial cells. Indeed, activated endothelial cells increase their expression of platelet adhesion molecules, allowing platelet adhesion which is a necessary step of primary hemostasis. Then, the endothelial cells activation leads to an up-regulation of membrane bound TF and to the secretion of various soluble factors like the von Willebrand factor (vWF), the platelet-activating factor (PAF) and the plasminogen activator inhibitor-1 (PAI-1). Activated endothelial cells also release procoagulant EVs (Figure I.9) (Verhamme and Hoylaerts, 2006).

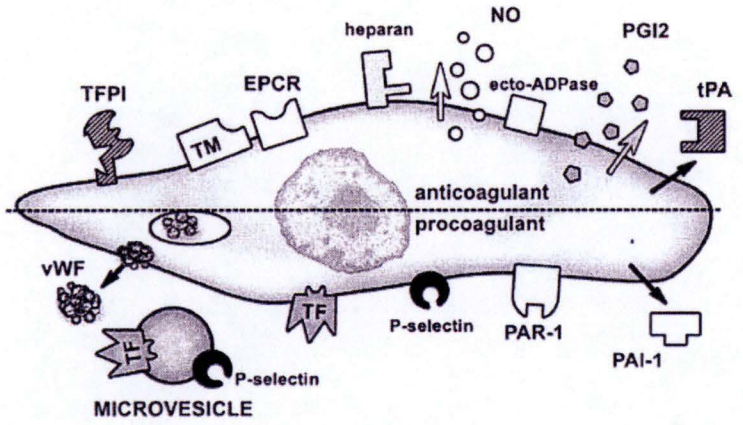

Figure I.9: **The endothelial cells may shift between an anticoagulant and a procoagulant phenotype according to their local environment.** On one hand, activated endothelial cells exhibit a procoagulant phenotype induced by the exhibition and/or release of molecules which trigger the coagulation cascade (TF), the platelet adhesion (vWF), the platelet activation and adhesion (P-selectin) and the inhibition of fibrinolysis (PAI-1). The endothelial cells also produce EVs which bear similar molecules to those exhibited by the originating cell. On the other hand, resting endothelial cells exhibit an anticoagulant phenotype induced by the exhibition and/or release of molecules which inhibit the platelet adhesion and activation and have vasodilatory properties (PGI_2 and NO), active fibrinolysis (t-PA) and inhibits the coagulation cascade by different ways (heparin, the EPCR, TM and TFPI). (Verhamme and Hoylaerts, 2006).

Ponatinib could also disrupt the hemostatic balance of endothelial cells by inducing a phosphatidylserine (PS) exposure on the cell surface. Indeed, the composition of the lipid membrane bilayers differs depending on the activation state of the cell. In the membrane of inactive resting cells, PS and phosphatidylethanolamine (PE) are rather found in the inner layer. However, the composition of these two layers is changed in damaged or activated cells, PS and PE being found on the external part of the membrane (Fu et al., 2010). This PS exposure leads to a change of the coagulation phenotype of membranes, most of the coagulation cascade steps being supported by membranes containing PS. Indeed, PS is negatively charged so that it can bind with high affinity and in a calcium-dependent manner the different factors involved in the coagulation cascade (Spronk et al., 2014). As PS-rich membranes bind these factors, the speed of reactions is markedly increase because all the coagulation players are located near each other which helps their properly alignment. Consequently, a PS-rich membrane provides a catalytic surface for the coagulation cascade reactions (Smith, 2009).

In summary, ponatinib could induce an increased risk of VAEs development in treated patients by activating endothelial cells. The activation of endothelial cells by ponatinib could affect their hemostatic balance by inducing an exposure of PS, an increase of the endothelial expression and release of procoagulant and proaggregant molecules and an inhibition of the physiological anticoagulant pathways.

I.3.4 Potential impact of ponatinib on the extracellular vesicles release

Ponatinib could increase the release of procoagulant EVs into the bloodstream, activating the coagulation cascade and disturbing the physiological anticoagulant properties of endothelial cells. Indeed, elevated level of TF- and PS-bearing EVs could help to explain the increased risk of thrombosis in ponatinib-treated patients.

The size of EVs varies from 0.03 to 1 µm and they are produced by a great number of cells. By interacting with target cells, EVs take part in the intercellular communication (Anderson et al., 2010). Both surface lipids and proteins exhibited by EVs and the molecules contained in these vesicles come from their originating cells and can impact the expression and phenotype of their target cells (Ratajczak et al., 2006). In resting conditions, EVs are mainly derived from endothelial cells, platelets and monocytes but cancer cells also release large amounts of EVs (Smith, 2009).

EVs may exhibit pro- or anticoagulant properties depending on the molecules they bear and contain. It is likely that the hemostatic balance of their producing cells influences the coagulation properties of the EVs released. The EVs produced by leukemic cells and activated endothelial cells can exhibit TF and PS on their surface which can directly trigger the coagulation cascade in the bloodstream (Gheldof et al., 2014). A decrease in TFPI-positive EVs has also been highlighted in cancer patients' blood (Aharon and Brenner, 2009).

In addition to directly trigger the coagulation cascade in the bloodstream, the procoagulant EVs could target and activate endothelial cells by disrupting their hemostatic balance (Bouvy et al., 2014). Several mechanisms could explain the procoagulant phenotype of endothelial cells targeted by EVs. First of all, after a treatment with EVs, endothelial cells could begin to overexpress platelet adhesion molecules which enhance the platelet adhesion to endothelium (Figure I.10 b). An overexpression of vWF on the endothelial cell surface triggered by EVs has also been highlighted (Terrisse et al., 2010). Then, the endothelial cells targeted by EVs could present an increased TF expression (Figure I.10 C). Moreover, a down-expression of the main anticoagulant molecules exhibited on the endothelial cell surface like TM and TFPI could also occur (Figure I.10 C). Finally, the EVs produced in cancer could induce apoptosis of the endothelial cells which lead to a change of the membrane composition with an exposure of PS and to a production of apoptotic EVs (Figure I.10 D) (Aharon et al., 2008) (Bouvy et al., 2014).

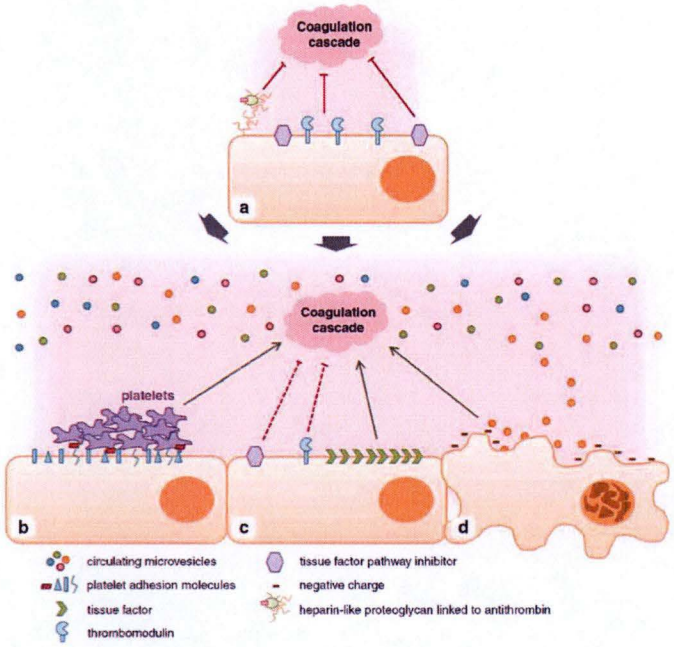

Figure I.10: **The anticoagulant properties of resting endothelial cells are impacted by EVs.** (a) The endothelial cells exhibit several anticoagulant molecules in normal conditions which allow to maintain an antithrombotic phenotype of these cells. (b) In cancer, released EVs could induce the endothelial cells expression of adhesion molecules. These molecules can support the adhesion of platelets which is an important step of the primary hemostasis. (c) EVs could also induce a procoagulant phenotype of endothelial cells by the upregulation of TF expression and the downregulation of TFPI and TM on the cell surface. (d) The apoptosis of endothelial cells targeted by EVs induces an exposition of negatively charged PL which support the binding of most of the coagulation cascade factors. Apoptotic cells also shed procoagulant EVs. (Bouvy et al., 2014).

I.3.5 Potential impact of ponatinib on platelet aggregation

The increased risk of VAEs development in ponatinib-treated patients could be partly induced by an increase of platelet aggregation. Indeed, ponatinib could enhance primary hemostasis by downregulating platelet aggregation inhibitors such as PGI_2 and/or by overexpressing platelet agonists like P-selectin and vWF (Verhamme and Hoylaerts, 2006). P-selectin enhances the thrombus stability and is released from alpha granules of platelets during platelet activation and aggregation (Merten and Thiagarajan, 2000). vWF is released from Weibel-Palade bodies of endothelial cells and allows platelet adhesion by acting as a bridge between the glycoprotein Ib/IX (GPIb/IX) receptor of platelets and collagen of damaged tissue (Verhamme and Hoylaerts, 2006).

I.3.6 Potential impact of ponatinib on fibrinolysis

A decrease of fibrinolysis is also a valid assumption. Indeed, a dysfunction of the normal fibrinolysis pathways may be involved in the occurrence of ponatinib-related coagulopathy in Ph-positive leukemia. Indeed, a downregulation of t-PA and/or an overregulation of PAI-1 could lead to an inhibition of the clot lysis (Zhang et al., 2010).

I.3.7 Potential impact of ponatinib on cancer cells

Ponatinib could induce thrombotic events in treated patients by acting on cancer cells. First of all, ponatinib could increase the TF expression of cancer cells on their membrane which triggers the coagulation cascade. Then, by inducing the apoptosis of BCR-ABL-positive cells, ponatinib could induce an exposure of PS on the cancer cells membrane, allowing the exhibition of a favorable surface for the coagulation factors association. By damaging cancer cells, ponatinib could also induce an overproduction of proinflammatory cytokines and angiogenic factors (like the vascular endothelium growth factor (VEGF)) and an increase of procoagulant EVs release by cancer cells. These molecules and EVs could target endothelial cells and induce a more procoagulant phenotype of these cells by an overregulation of the procoagulant and a downregulation of the anticoagulant properties of endothelial cells (Figure I.11) (Falanga and Marchetti, 2012a).

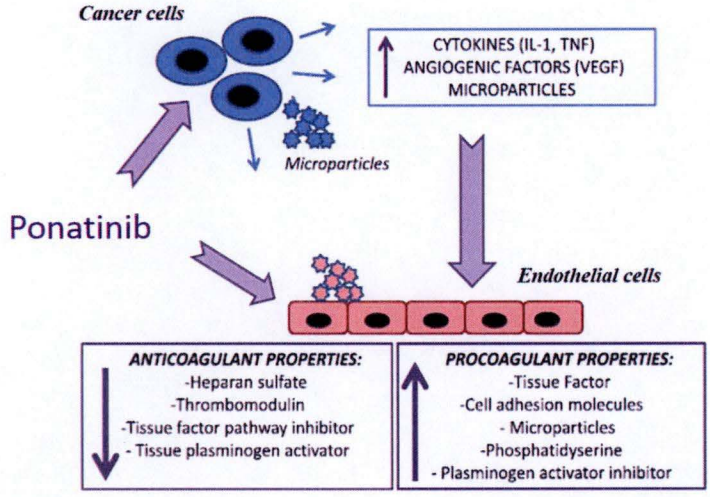

Figure I.11: **Ponatinib could impact the coagulation phenotype of cancer and endothelial cells.** Ponatinib could induce a shift from an anticoagulant to a more procoagulant phenotype of endothelial cells by two ways: (i) it could directly impact the coagulant properties of endothelial cells. Therefore, these cells begin to express TF and adhesion molecules on their surface, shed procoagulant EVs, shuffle the PL of their membrane to expose PS on the external leaflet and synthesize PAI-1. All these mechanisms lead to an increase of the procoagulant properties of endothelial cells. In parallel, ponatinib could induce a decrease of the endothelial cells synthesis and expression of molecules which inhibit hemostasis and thrombus formation such as heparin-like proteoglycans, TM, TFPI and t-PA. (ii) Ponatinib could damage BCR-ABL-positive cells, inducing a release of cytokines and angiogenic factors by these cells. Cancer cells can also shed EVs. Cytokines, angiogenic factors and EVs can induce a prothrombotic phenotype of endothelial cells by the upregulation of their procoagulant properties and the downregulation of their anticoagulant properties. Adapted from (Falanga and Marchetti, 2012a).

I.3.8 Impact of ponatinib on various tyrosine kinases targets

The VAEs associated with ponatinib could also be initiated by the targeting of other tyrosine kinases than BCR-ABL. The VEGFR could be particularly affected, inducing an endothelial dysfunction. Indeed, the VEGF pathway inhibition is known to induce a decreased capability of the endothelial cells regeneration (Esposito et al., 2015).

II. Objectives

II. Objectives

A large number of VOEs are reported in ponatinib-treated patients, leading to the discontinuation of this therapy in patients developing intolerable vascular events. However, ponatinib has the highest antileukemic activity of the TKIs family and it represents the last treatment option for patients suffering from a Ph-positive leukemia. Moreover, ponatinib is the only TKI potent against the common T315I mutation. The discovery of mechanisms by which ponatinib induces VOEs development in Ph-positive leukemia patients could significantly increase the survival of patients treated with this TKI by a better management and prophylaxis of this ADR. Moreover, a decrease of the VAEs risk in ponatinib-treated patients could lead to a wider use of this drug.

This master thesis aims at better understanding the mechanisms by which ponatinib induces thrombotic events in Ph-positive leukemia patients. Several mechanisms could explain the induction of this prothrombotic phenotype. This study was focused on two of these underlying mechanisms: the potential impact of ponatinib on endothelial cells and on platelet aggregation.

On one hand, ponatinib could have an impact on the hemostatic balance of endothelial cells by inducing a change of endothelial cells expression and/or a release of procoagulant EVs. This drug could also have a toxic effect on endothelial cells by inducing metabolic changes and/or cell death. Overall, the impact of ponatinib on endothelial cells could explain the increased thrombotic risk of treated patients.

On the other hand, ponatinib could induce an increase of platelet aggregation by impacting the expression of pro- and/or anti-aggregant molecules. This second assumption could also partly explain the increased clot formation in ponatinib-treated patients.

III. Material and methods

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III.1 Platelet aggregation

III.1.1 Platelet aggregation assay by turbidimetry

Platelet aggregation assay by turbidimetry was used to assess the impact of ponatinib and imatinib on human platelet aggregation.

a) Principle

This test is based on the variation of optical density in a glass cuvette containing plasma. Before aggregation, platelet-rich plasma is turbid and, when a beam of light is emitted, the transmittance trough the cuvette containing plasma is low. When a platelet agonist is added, platelet-rich plasma becomes less turbid due to the clumping of platelets so that the light transmittance is higher (Figure III.1).

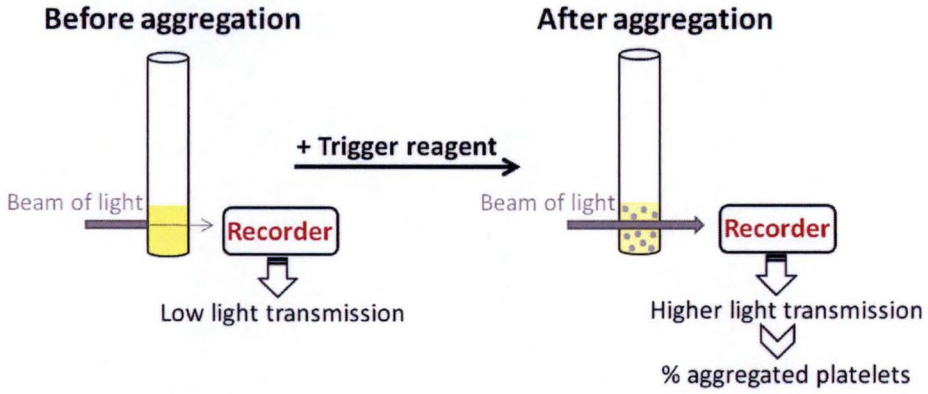

Figure III.1: **Principle of platelet aggregation assay by turbidimetry.** Before aggregation, the transmittance trough a cuvette containing platelet-rich plasma is low. When a platelet agonist is added, platelet-rich plasma becomes less turbid due to the clumping of platelets so that the beam of light can better pass through the cuvette. The percentage of aggregated platelets is calculated from the light transmission.

The percentage of aggregated platelets is calculated from the light transmission. A compound is considered as anti-aggregant when it decreases the percentage of aggregated platelets relative to control while a compound is pro-aggregant when it increases the percentage of aggregated platelets relative to control.

b) Material

- Citrated blood tubes (Venosafe, Terumo Europe N.V., Leuven, Belgium)
- Glass cuvette (Chrono-Log Corporation, Kordia Life Sciences, Havertown, USA)
- Aggregometer (Chrono-Log Corporation, Kordia Life Sciences, Havertown, USA)
- Platelet-rich plasma (PRP)
- Platelet-poor plasma (PPP)
- Centrifuge 5702 (Eppendorf AG, Hamburg, Germany)
- Stir bar (Chrono-Log Corporation, Kordia Life Sciences, Havertown, USA)

- Trigger reagents:
  - Arachidonic acid (5 mg/ml; Hart Biologicals, Hartlepool, UK)
  - Adenosine diphosphate (ADP) (200 µM; Hart Biologicals, Hartlepool, UK)
  - Collagen (100 µg/ml; Hart Biologicals, Hartlepool, UK)
- Ponatinib (Selleck Chemicals, Bio-Connect, Huissen, The Netherlands) (three concentrations tested: 100 nM, 150 nM and 200 nM) or imatinib (Sigma-Aldrich, Diegen, Belgium) (three concentrations tested: 0.5 µM, 1 µM and 1.5 µM)

c) Method

The platelet-rich plasma (PRP) and the platelet-poor plasma (PPP) were obtained from the supernatant fraction of citrated blood tubes after a centrifugation of 10 minutes at 200 g at room temperature for PRP and after a second centrifugation of 10 minutes at 2000 g at room temperature for PPP. As reference, 255 µl of PPP and 45 µl of distilled water were placed in a glass cuvette with a stir bar. For each assay, 255 µl of PRP, 15 µl of a specific ponatinib or imatinib concentration and a stir bar were placed in a glass cuvette. This last was incubated at 37°C in the aggregometer for 3 or 20 minutes after which 30 µl of trigger reagent were added. Three different triggers of platelet aggregation were tested: (i) arachidonic acid (ii) ADP and (iii) collagen. The tests were performed with two different concentrations of each platelet agonist: (i) with the concentrations recommended by the supplier (5 mg/ml, 200 µM and 100 µg/ml for arachidonic acid, ADP and collagen, respectively) or (ii) with three-quarters of the recommended concentrations (3.75 mg/ml, 150 µM and 75 µg/ml for arachidonic acid, ADP and collagen, respectively). Each assay was performed in duplicate. To check the platelet reactivity, a test with distilled water instead of the ponatinib or imatinib sample was carried out before and after any experiment. The percentage of platelet aggregation was calculated from the light transmittance through the cuvette at a given time and was graphically shown by the Aggrolink software.

III.1.2 Multiplate® assay

The Multiplate® assay was used to assess platelet aggregation in whole blood samples containing ponatinib.

a) Principle

The Multiplate® system is based on the measurement of the electric current variation between two electrodes placed into whole blood samples where platelet aggregation has been induced. Indeed, platelet aggregation on the electrode surface enhances the electrical resistance between the two sensor wires. After the beginning of the test, platelets contained in the whole blood will gradually stick to the electrodes which lead to a decrease of measured electric current (Figure III.2). The electric conductivity curve is then converted in a platelet aggregation curve.

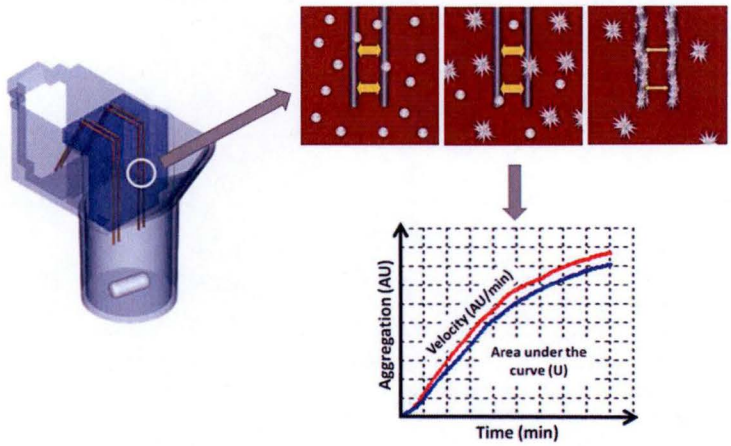

Figure III.2: **Principle of Multiplate® assay.** The measurements are conducted in a disposable single use test cuvette containing two independent electrode pairs (at the left of this figure). Whole plasma and the tested compound are placed in a cuvette. After addition of a platelet agonist, platelets contained in the whole blood will gradually stick to the electrodes which lead to a decrease of measured electric current. The electric conductivity curve is then converted in a platelet aggregation curve. Three parameters of the platelet aggregation curves were studied: (i) the velocity which is the maximal slope of the curve, (ii) the area under the curve (see on the graph at the bottom of this figure) and (iii) the maximal aggregation reached. Adapted from http://www.cobas.com/home/product/hemostasis-testing/multiplate-analyzer.html.

b) Material

- Hirudin blood tubes (Roche Diagnostics International AG, Rotkreuz, Switzerland)
- Multiplate® system (Roche Diagnostics International AG, Rotkreuz, Switzerland)
- Disposable single use test cuvettes (Roche Diagnostics International AG, Rotkreuz, Switzerland)
- Whole blood
- Multiplate® ADPtest reagent (Roche Diagnostics International AG, Rotkreuz, Switzerland)
- Ponatinib (Selleck Chemicals, Bio-Connect, Huissen, The Netherlands) (three concentrations tested: 100, 150 and 200 nM)
- NaCl 0.9%
- Dimethyl sulfoxide (DMSO) 0.02%

c) Method

Disposable single use test cuvettes containing two independent electrode pairs and a stir bar are placed in the Multiplate® device. Before the beginning of the test, 300 µl of ponatinib diluted in NaCl 0.9% and 300 µl of whole blood were placed in each cuvette using an automatic pipette. This sample was incubated during 20 minutes at 37°C. Then, 20 µl of the platelet agonist ADP were added and the electric current variation was recorded. For a same test condition, two platelet aggregation curves were obtained, each derived from one of the two electrode pairs. Then, each test was performed in duplicate. Two parameters of the platelet aggregation curves were studied: (i) the velocity which is the maximal slope of the curve and (ii) the area under the curve (Figure III.2). A test with the maximal DMSO concentration was used as negative control.

III.2 Cell culture

III.2.1 EAhy926 endothelial cells

EAhy926 is an immortalized human endothelial cell line which was established by fusing primary human umbilical vein cells with a thioguanine-resistant clone of A549 by exposure to polyethylene glycol (PEG). Certified EAhy926 endothelial cells (Figure III.3 A and B for their morphology) were kindly provided by the Research Unit in Cellular Biology (URBC) of the University of Namur.

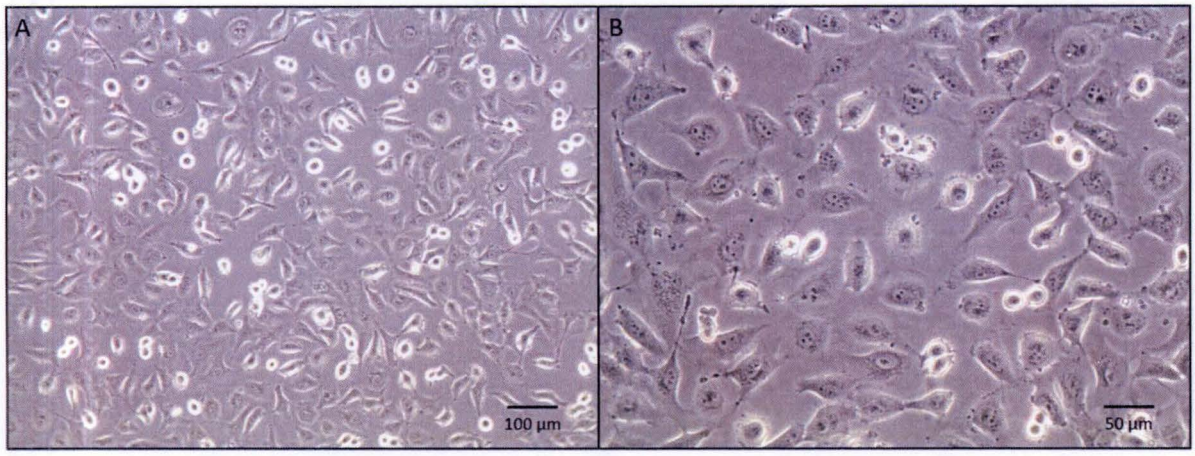

Figure III.3: **EAhy926 cells morphology.** These pictures were taken by phase contrast microscopy with a magnification of 100x (A) or with a magnification of 200x (B).

III.2.1.1 Maintenance

a) Material

- Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Verviers, Belgium) containing:
  - 10% Fetal Bovine Serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel)
  - 4.5 g/L glucose
  - L-glutamine
  - 1% penicillin/streptomycin
- Phosphate Buffered Saline (PBS) with 0.0067 M PO_4 and without Ca and Mg (Lonza, Verviers, Belgium)
- Trypsin 0.25% (Lonza, Verviers, Belgium)

b) Method

This line of immortalized endothelial cells was grown in complete DMEM containing 10% FBS. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. PBS was used to rinse cells and trypsin to remove cells from the plate.

III.2.1.2 Tests

a) Material

- DMEM (Lonza, Verviers, Belgium) containing:
  - 1% FBS (Biological Industries, Kibbutz Beit Haemek, Israel)
  - 4.5 g/L glucose
  - L-glutamine
  - 1% penicillin/streptomycin
- 96-well flat bottom plate treated for cell culture (Costar, Corning Incorporated, USA)
- 24-well flat bottom plate treated for cell culture (Cellstar, Greiner bio-one, Kremsmünster, Austria)
- Imatinib (Sigma-Aldrich, Diegen, Belgium)
- Ponatinib (Selleck Chemicals, Bio-Connect, Huissen, The Netherlands)
- Tumor necrosis factor-α (TNF-α) (R&D Systems, Minneapolis, USA)

b) Method

For all the tests, the last passage of cells before the experiment was performed in complete DMEM containing 1% FBS. EAhy926 cells were grown in a 96-well flat bottom plate with 20 000 cells per well for the thrombin generation assays on a cell monolayer and for the lactate dehydrogenase (LDH) and MTS assays. For the thrombin generation tests with cell supernatant, EAhy926 cells were grown in a 24-well flat bottom plate with 110 000 cells per well. After an overnight incubation in DMEM 1% FBS, cells were treated with 0.5 µM, 1 µM or 1.5 µM of imatinib or with 100 nM, 150 nM or 200 nM of ponatinib or with 0.1 ng/ml or 1 ng/ml of TNF-α. All the tests were also performed on untreated EAhy926 cells. For the phase contrast microscopy photographs, the cell monolayers were only treated with the maximal concentration of each treatment i.e. 1.5 µM imatinib, 200 nM ponatinib or 10 ng/ml TNF-α.

III.2.2 Human umbilical vein endothelial cells

Human Umbilical Vein Endothelial Cells (HUVECs) are primary cells isolated from a vein of the umbilical cord. Certified pooled HUVECs (Lonza, Verviers, Belgium) were used. The morphology of these cells can be seen on the Figure III.4 A and B.

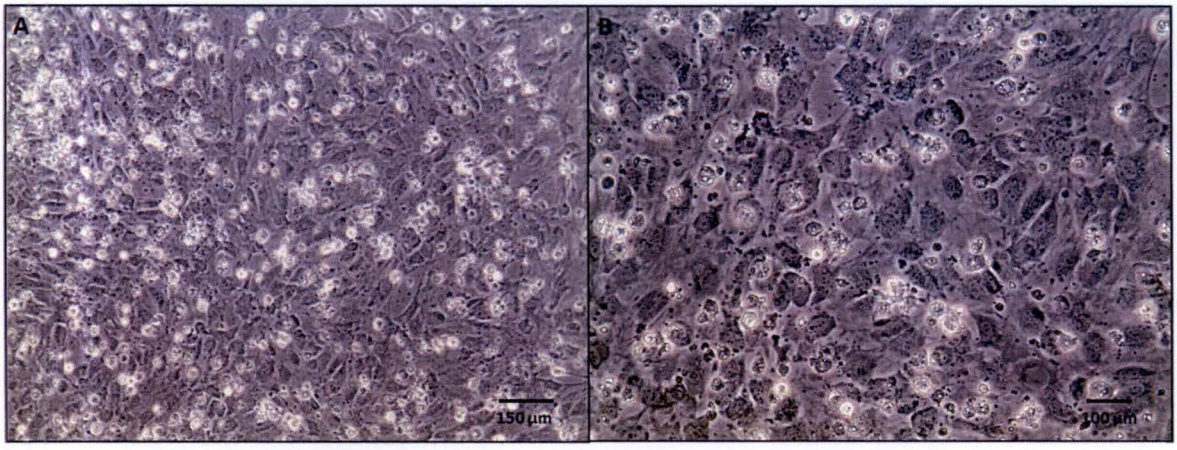

Figure III.4: **HUVECs cells morphology.** These pictures were taken by phase contrast microscopy with a magnification of 100x (A) or with a magnification of 200x (B).

III.2.2.1 Maintenance

a) Material

- Endothelial Cell Growth Medium (EGM; Lonza, Verviers, Belgium) containing:
  - 2% FBS
  - 0.4% Bovine Brain Extract (BBE) with heparin
  - 0.1% recombinant human Epidermal Growth Factor (rhEGF)
  - 0.1% hydrocortisone
  - 0.1% ascorbic acid
  - 0.1% gentamicin sulfate amphotericin-B (GA-1000)
- PBS with 0.0067 M PO_4 and without Ca and Mg (Lonza, Verviers, Belgium)
- Trypsin 0.25% (Lonza, Verviers, Belgium)

b) Method

HUVECs were grown in complete EGM containing 2% FBS. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. PBS was used to rinse cells and trypsin to remove cells from the plate.

III.2.2.2 Tests

a) Material

- EGM (Lonza, Verviers, Belgium) containing:
  - 2% FBS
  - 0.4% BBE with heparin
  - 0.1% rhEGF
  - 0.1% hydrocortisone
  - 0.1% ascorbic acid
  - 0.1% GA-1000
- Endothelial Cell Basal Medium (EBM; Lonza, Verviers, Belgium)
- 96-well flat bottom plate treated for cell culture (Costar, Corning Incorporated, USA)
- 24-well flat bottom plate treated for cell culture (Cellstar, Greiner bio-one, Kremsmünster, Austria)
- Imatinib (Sigma-Aldrich, Diegen, Belgium)
- Ponatinib (Selleck Chemicals, Bio-Connect, Huissen, The Netherlands)
- TNF-α (R&D Systems, Minneapolis, USA)

b) Method

HUVECs cells were grown in a 96-well flat bottom plate with a concentration of 1600 cells per well for the thrombin generation assays on a cell monolayer and for the LDH and MTS assays. For the thrombin generation tests with cell supernatant, HUVECs cells were grown in

a 24-well flat bottom plate with a concentration of 8400 cells per well. Until the cells reach confluence, a complete medium (EGM) was used and regularly changed. The day they reached confluence, cells were deprived in growth factor and treated with 0.5 µM, 1 µM or 1.5 µM of imatinib or with 100 nM, 150 nM or 200 nM of ponatinib or with 1 ng/ml or 10 ng/ml of TNF-α. These compounds were diluted in a basal culture medium (EBM). The tests were also performed on untreated HUVECs cells. For the phase contrast microscopy photographs, the cell monolayers were only treated with the maximal concentration of each treatment i.e. 1.5 µM imatinib, 200 nM ponatinib or 10 ng/ml TNF-α.

III.2.3 Phase contrast microscopy photographs

Pictures of the EAhy926 and HUVECs cells monolayers were taken by a phase contrast microscope equipped with a camera to assess cellular morphology and death rate after their treatment with imatinib, ponatinib or TNF-α.

a) Material

- Phase contrast microscope equipped with a camera (Leica Microsystems DFC290, Leitz, Wetzlar, Germany)
- Leica FireCam software (Leica Microsystems, Leitz, Wetzlar, Germany)

b) Method

The EAhy926 cells monolayers pictures were taken after 24 and 48 hours of treatment and the HUVECs monolayers pictures were taken only after 24 hours of treatment. The pictures were taken with the magnification 100x or 200x.

III.3 Thrombin generation assay

Thrombin generation assay is used to assess the overall tendency of a plasma sample to form thrombin after initiation of coagulation.

III.3.1 General principle

Thrombin generation assay is based on the cleavage of a fluororogenic substrate by active thrombin. In this test, a trigger reagent is used to trigger the coagulation cascade in plasma by the intrinsic and/or extrinsic pathway in the measurement wells. After addition of FluCa containing calcium chloride and the fluorogenic substrate, the citrated plasma is recalcified so that the reaction starts. The initiated coagulation cascade leads to the production of thrombin which cleaves the fluorogenic substrate so that this substrate becomes fluorescent. Therefore, the fluorescence produced over time is proportional to the quantity of produced thrombin (Figure III.5).

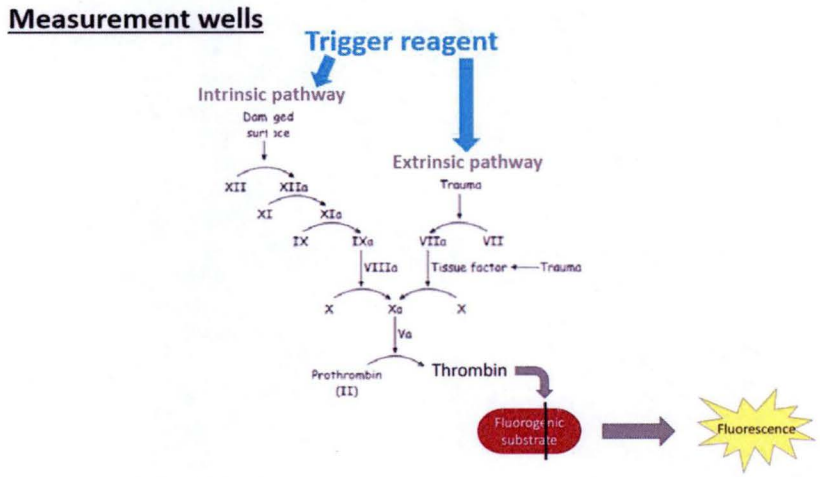

Figure III.5: **The trigger reagent triggers the coagulation cascade by the intrinsic and/or extrinsic pathway in the measurement wells.** That leads to a conversion of prothrombin into thrombin. The fluorogenic substrate is cleaved by the produced thrombin so that this substrate becomes fluorescent.

Before being converted into thrombin concentration, the fluorescence signal derived from the measurement wells is corrected for inner-filter effects and substrate consumption thanks to the fluorescence signal derived from the calibration wells running in the same condition (Figure III.6).

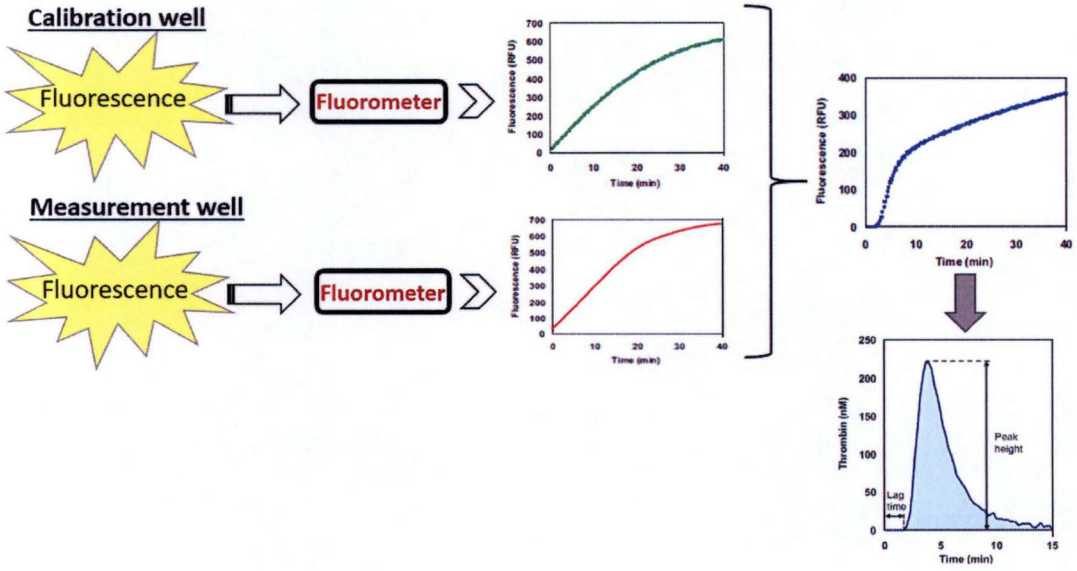

Figure III.6: **Transformation of the raw fluorescence data.** The fluorescence data obtained from the measurement wells (the curve in red) are corrected for inner-filter effects thanks to the fluorescence signal derived from the calibration wells (the curve in green) running in the same condition. The fluorescence curve (in blue) is then converted into a thrombin concentration curve (the curve at the bottom right). Two parameters of these curves were studied: (i) the lagtime which is the initiation phase of coagulation and (ii) the peak height (Cmax) which is the concentration reached at the peak. Adapted from (Castoldi and Rosing, 2011).

The principle of the calibration wells is based on the addition of a Thrombin Calibrator to plasma without activating coagulation. The Thrombin Calibrator is thrombin bound to α2-macroglobulin which cannot be inhibited by plasma protease inhibitors. Thereby, the

Thrombin Calibrator splits the fluorogenic substrate with a constant rate pre-encoded into the Thrombinoscope software. Thrombin generation assay allows to explore the different phases of coagulation: (i) the initiation phase where a few amount of thrombin is generated, (ii) the amplification phases where a massive amount of thrombin is generated and (iii) the termination phase where thrombin activity is inhibited by coagulation cascade inhibitors (Figure III.7).

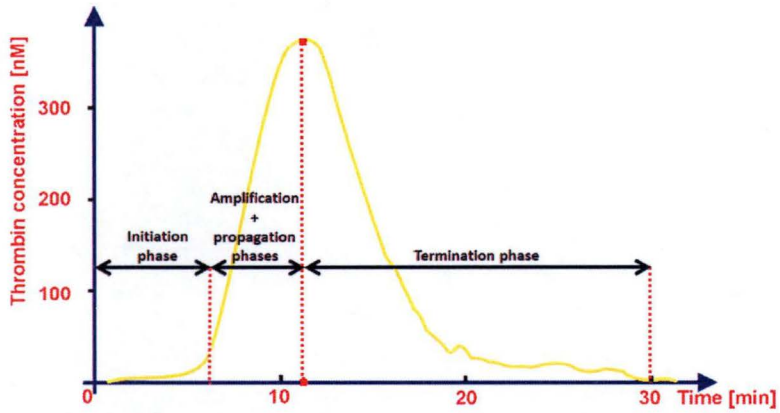

Figure III.7: **Thrombin generation assay allows to explore the different coagulation phases.** Unlike the classical clotting assays which only probe the initiation phase, thrombin generation assays also probe the amplification and the termination phases. Adapted from (Stępień, 2011).

All the reagents used for a thrombin generation assay and their usefulness are summarized in the Table III.1.

Table III.1: **The different reagents used for thrombin generation test and their usefulness.** Plasma provides the factors required for the coagulation cascade which is triggered by TF and/or PL contained in a trigger reagent. Plasma is recalcified by calcium present in FluCa. Thrombin cleaves the fluorogenic substrate contained in FluCa and the fluorescence released by the cleft substrate is measured. In the calibration wells, the Thrombin Calibrator splits the fluorogenic substrate with a constant rate which allows to correct the fluorescence curve released from the measurement wells for inner-filter effects.

Reagents used for thrombin generation assay	Usefulness
Plasma	Provides the factors required for coagulation cascade
FluCa	Provides fluorogenic substrate and calcium
Trigger reagent	Triggers coagulation cascade with TF and/or PL
Thrombin calibrator	Cleaves fluorogenic substrate with a constant rate

III.3.2 General method

Measurements are conducted in 20µl of trigger reagent and 80 µl of normal pooled plasma (NPP) at which 20µl of FluCa are added (Figure III.8). The fluorescence released by the cleft fluorogenic substrate is measured by a Fluoroskan Ascent FL reader. In the calibration wells, 20 µl of thrombin calibrator is added instead of the trigger reagent (Figure III.8).

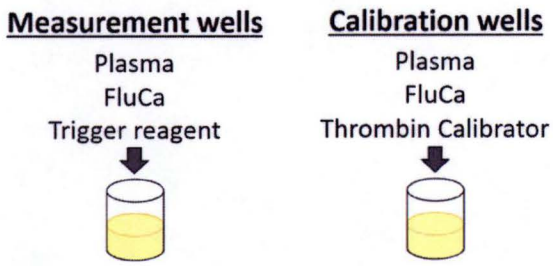

Figure III.8: **The different reagents placed in the measurement wells and in the calibration wells for thrombin generation assay.** In the measurement wells, plasma, FluCa and a trigger reagent are placed while in the calibration wells, thrombin calibrator is added instead of the trigger reagent.

For each condition, the test is run in triplicate. Two parameters of the thrombin generation curves were studied: (i) the lagtime which is the initiation phase of coagulation and (ii) the peak height (Cmax) which is the concentration reached at the peak (Figure III.6).

III.3.3 Thrombin generation assay on a cell monolayer treated with ponatinib, imatinib or TNF-α

Thrombin generation assay on a monolayer of cells treated with ponatinib, imatinib or TNF-α was used to assess the impact of these three molecules on the hemostatic balance of endothelial cells.

a) Material

- NPP
- MP reagent (Thrombinoscope bv, Stago Group Company, Maastricht, The Netherlands)
- Thrombin Calibrator (Thrombinoscope BV, Stago Group Company, Maastricht, The Netherlands)
- FluCa (Thrombinoscope BV, Stago Group Company, Maastricht, The Netherlands)
- Fluoroskan Ascent FL reader (Thermo Electron Corporation, Waltham, Massachusetts, USA)

b) Method

This test was performed after 24 and 48 hours of treatment for the tests carried out on an EAhy926 cells monolayer and only after a 24-hour treatment for the HUVECs cells. The trigger reagent used was MP Reagent (containing 4 µM PL) (Figure III.9). During a test, all the test conditions were performed in triplicate.

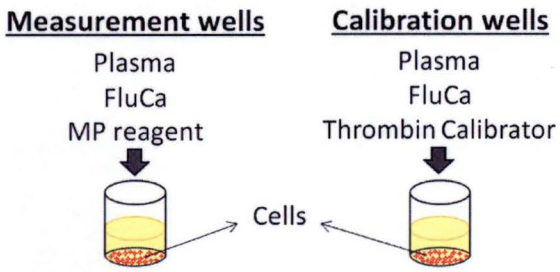

Figure III.9: **The different reagents placed in the measurement wells and in the calibration wells for thrombin generation assay on a cell monolayer treated with ponatinib, imatinib or TNF-α.** In the measurement wells, plasma, FluCa and MP reagent are placed on the endothelial cells monolayer while in the calibration wells, thrombin calibrator is added instead of MP reagent.

III.3.4 Thrombin generation assay on the supernatant of cells treated with ponatinib, imatinib or TNF-α

Thrombin generation assay on cell supernatant was used to assess the global coagulation profile of EVs and proteins released in the supernatant of endothelial cells treated with ponatinib, imatinib and TNF-α.

a) Material

- NPP
- MP reagent (Thrombinoscope bv, Stago Group Company, Maastricht, The Netherlands)
- Thrombin Calibrator (Thrombinoscope BV, Stago Group Company, Maastricht, The Netherlands)
- FluCa (Thrombinoscope BV, Stago Group Company, Maastricht, The Netherlands)
- Fluoroskan Ascent FL reader (Thermo Electron Corporation, Waltham, Massachusetts, USA)
- Centrifuge Biofuge pico (Heraeus Instruments, Osterode, Germany)
- 96-well round bottom microtiter plate (Immulon 2HB, Thermo Scientific, Waltham, Massachusetts, USA)
- DMEM (Lonza, Verviers, Belgium) 1% FBS or EBM (Lonza, Verviers, Belgium)

b) Method

This test was performed after 24 and 48 hours of treatment for the tests carried out with the supernatant of EAhy926 cells and only after a 24-hour treatment for HUVECs cells. The milliliter of treated cells supernatant contained in a 24-well plate was removed and placed in an Eppendorf just before the thrombin generation test. After a centrifugation of 15 minutes at 2500 g to remove potential remaining cells, 20 µl of the supernatant of each condition were placed in three measurement wells of a 96-well round bottom plate. For the calibration wells, 20 µl of DMEM 1% FBS (for the tests with EAhy926) or 20 µl of EBM (for the tests with HUVECs) were placed in each well instead of the 20 µl of supernatant (Figure III.10). The trigger reagent used was MP Reagent. During a test, all test conditions were performed in triplicate.

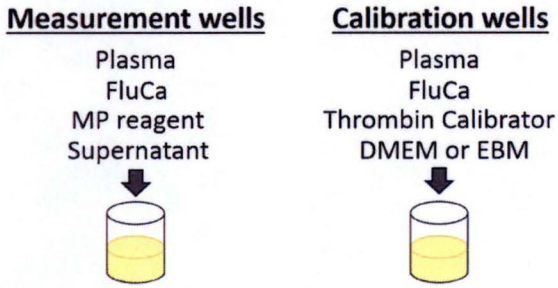

Figure III.10: **The different reagents placed in the measurement wells and in the calibration wells for thrombin generation assay on supernatant of cells treated with ponatinib, imatinib or TNF-α.** Plasma, FluCa, MP reagent and the supernatant of treated cells are placed in the measurement wells while thrombin calibrator and DMEM 1% FBS or EBM are added in the calibration wells.

III.4 Cytotoxicity assay

III.4.1 MTS assay on endothelial cells treated with ponatinib, imatinib or TNF-α

A MTS assay was used to assess the cytotoxicity of ponatinib, imatinib and TNF-α on endothelial cells.

a) Principle

The MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is reduced into a colored formazan product in metabolically active cells. This conversion is catalyzed by NADH and NADPH produced by dehydrogenase enzymes in living cells (Figure III.11). The quantity of produced formazan is measured by a reading of absorbance at 490nm and is directly proportional to the number of viable cells.

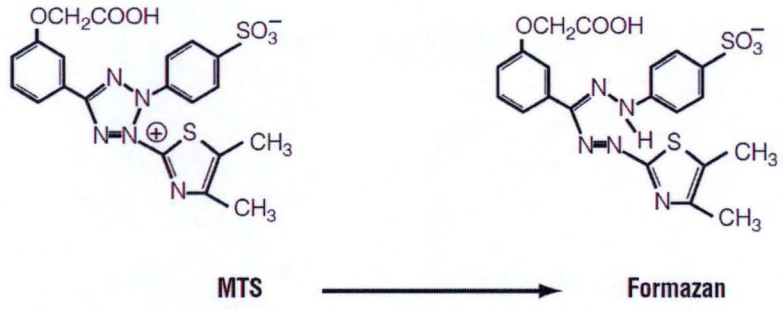

Figure III.11: **Principle of MTS assay.** The MTS reagent is converted into colored formazan thanks to NADH and NADPH produced by dehydrogenase enzymes in the metabolically active cells. (Promega, 2012).

b) Material

- CellTiter 96® AQueous One Solution Reagent (Promega, Madison, USA)
- Microplate Absorbance Spectrophotometer (xMark, Bio-Rad, Hercules, California, USA)
- Triton 21%

c) Method

MTS assay was performed after 24 and 48 hours of treatment for the tests carried out on an EAhy926 cells monolayer and only after a 24-hour treatment for HUVECs cells. For the test, 20 µl of CellTiter 96® AQueous One Solution Reagent were added to 100 µl of culture medium in wells containing adherent cells. The read of absorbance was performed one hour after the beginning of reaction by a spectrophotometer. For the positive control, 10 µl of triton 21% were placed in the cell medium just before the test. For each assay, the same test condition was performed in triplicate.

III.4.2 LDH assay on endothelial cells treated with ponatinib, imatinib or TNF-α

This colorimetric assay was used to assess the cytotoxicity of ponatinib, imatinib and TNF-α on endothelial cells.

a) Principle

LDH assay is based on the measurement of the activity of LDH released from damaged cells. When the integrity of cytoplasmic membrane is compromised, intracytoplasmic enzymes are released from the cytosol of damaged cells to the supernatant. In this test, the LDH activity is quantified in the supernatant by adding a tetrazolium salt. Indeed, the conversion of lactate in pyruvate is catalyzed by LDH and induces the reduction of NAD^+ into NADH. The hydrogen of NADH is transferred on the tetrazolium salt which is converted into formazan (Figure III.12). The quantity of produced formazan is measured by a read of absorbance at 490nm and reflects the integrity of cytoplasmic membrane.

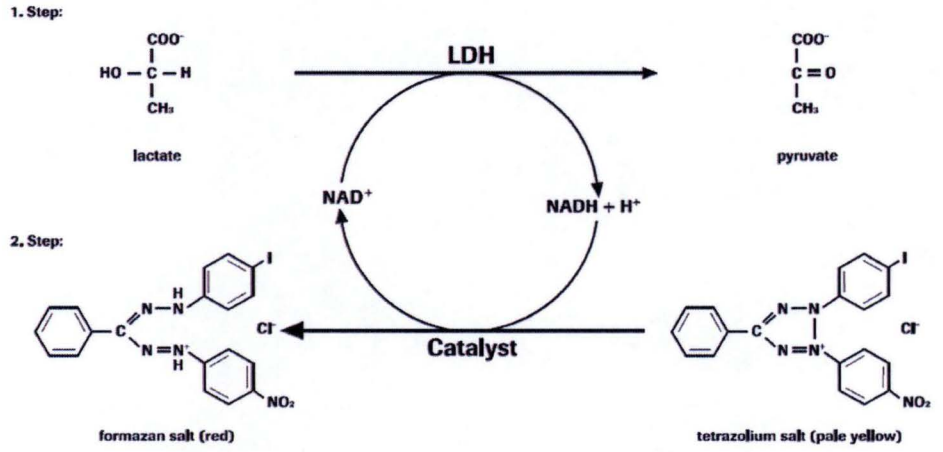

Figure III.12: **Principle of LDH assay.** LDH released in the supernatant of damaged cells catalyzes the conversion of lactate in pyruvate with a production of NADH. In this test, the LDH activity is quantified in the supernatant by adding a tetrazolium salt on which the hydrogen of NADH is transferred. The tetrazolium salt is then converted in colored formazan which absorbs at 490nm. (Roche, 2011).

b) Material

- Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Indianapolis, USA) containing:
  - Dye solution
  - Catalyst
- Microplate Absorbance Spectrophotometer (xMark, Bio-Rad, Hercules, California, USA)
- Triton 21%

c) Method

This test was performed after 24 and 48 hours of treatment for the tests carried out on EAhy926 cells and only after a 24-hour treatment for HUVECs cells. Before the test, the dye solution and the catalyst were mixed. For the test, 100 µl of this reaction mixture was added to 100 µl of cell-free supernatant. The absorbance reading was performed 10 minutes after the beginning of the reaction by a spectrophotometer. For the positive control, 10 µl of triton 21% were placed in the cell medium just before removing the cell-free supernatant. A same test condition was performed in triplicate during a same test.

III.5 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 software package. Results are expressed as means ± standard error of the mean (SEM). Statistical significance was assessed using Wilcoxon tests. Statistical significance represented a P value lower than 0.05.

IV. Results

IV. Results

IV.1 Impact of ponatinib on platelet aggregation

The impact of ponatinib on platelet aggregation was assessed by light transmission aggregometry assay. These assays were performed with three different platelet aggregation inducers: arachidonic acid, ADP or collagen. The first tests were carried out with the concentrations of platelet agonist recommended by the supplier (5 mg/ml, 200 µM and 100 µg/ml for arachidonic acid, ADP and collagen, respectively) and with three different concentrations of ponatinib and imatinib. A 3-minute incubation period with the two TKIs was used. In these conditions, ponatinib and imatinib did not change the percentage of aggregated platelets relative to control, whatever the platelet aggregation inducer and the TKIs concentration used (Figure IV.1).

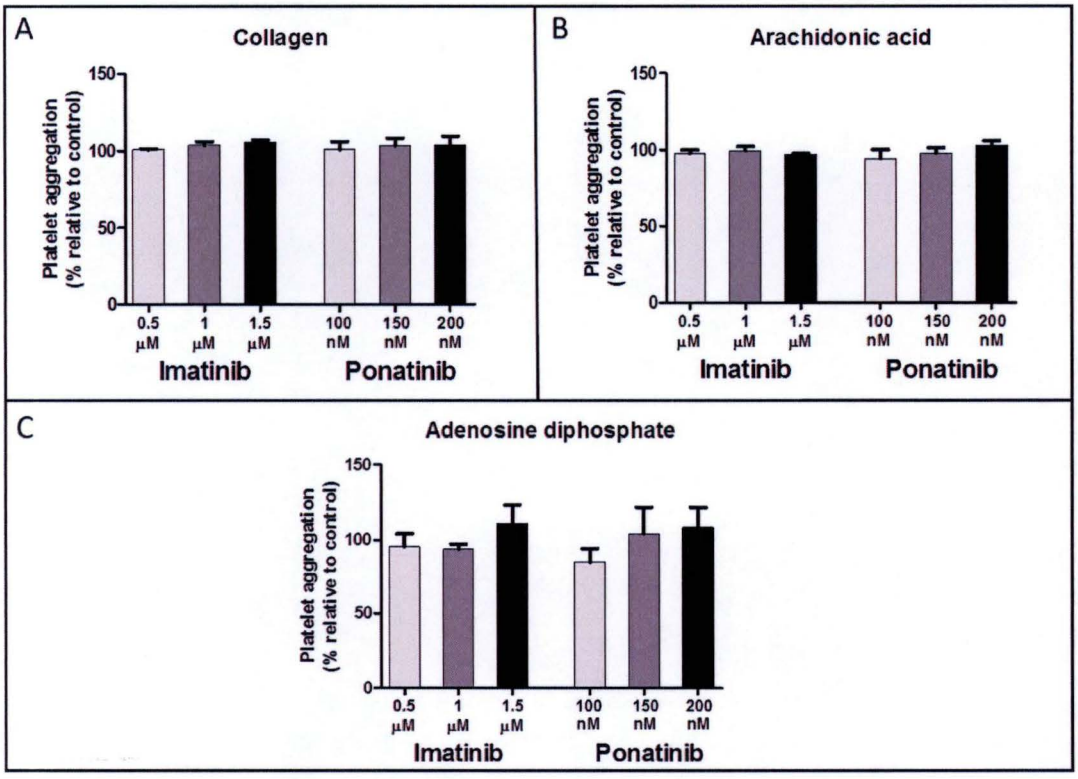

Figure IV.1: **Impact of ponatinib and imatinib on platelet aggregation triggered by collagen (A), arachidonic acid (B) or ADP (C).** The results are expressed as means of n=2 ± SEM of platelet aggregation percentages normalized on PRP with distilled water considered as control. The concentrations of platelet agonist recommended by the supplier (5 mg/ml, 200 µM and 100 µg/ml for arachidonic acid, ADP and collagen, respectively) were used. The three ponatinib concentrations tested were 100, 150 and 200 nM and the three concentrations of imatinib were 0.5, 1 and 1.5 µM.

Then, a reduced concentration of platelet aggregation inducer was used to increase the sensitivity of the tests. The highest concentration of imatinib (1.5 µM) and ponatinib (200 nM) was tested with three-quarters of the recommended concentration of arachidonic acid (3.75 mg/ml) or ADP (150 µM). Despite this decreased inducer concentration, the percentages of aggregated platelets obtained with imatinib and ponatinib did not seem to change relative to the control (Figure IV.2).

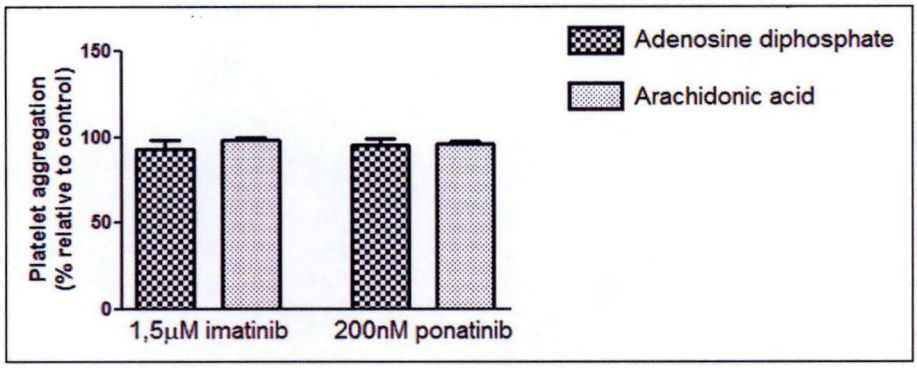

Figure IV.2: **Impact of 200 nM ponatinib and 1.5 µM imatinib on platelet aggregation triggered by 3.75 mg/ml arachidonic acid or 150 µM ADP.** A 3-minute incubation period with the two TKIs was performed. The results are expressed as means of n=2 ± SEM of platelet aggregation percentages normalized on PRP with distilled water considered as control.

The incubation period with ponatinib and imatinib was then increased to 20 minutes. The recommended or reduced concentrations of ADP or arachidonic acid were used to trigger platelet aggregation. When the recommended inducer concentration was used, ponatinib and imatinib did not seem to change the percentage of aggregated platelets relative to control despite the prolongation of the incubation period with the two TKIs (Figure IV.3 A and B). However, when 150 µM ADP was used, a decreased platelet aggregation was obtained for 1.5 µM imatinib and 200 nM ponatinib. This effect was observed on the PRP of one donor but when the same test conditions were repeated with another donor, this decrease of platelet aggregation was no more observed (Figure IV.3 A).

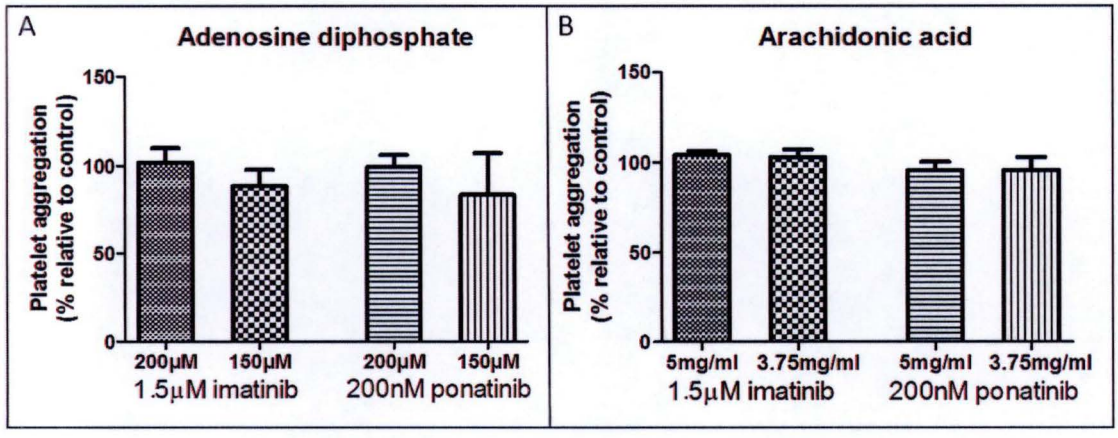

Figure IV.3: **Impact of 200 nM ponatinib and 1.5 µM imatinib on platelet aggregation triggered after a 20-minute incubation period.** The platelet aggregation was triggered by (A) ADP (200 or 150 µM) or (B) arachidonic acid (5 or 3.75 mg/ml). The results are expressed as means of (A) n=4 ± SEM or (B) n=2 ± SEM of platelet aggregation percentages normalized on PRP with distilled water considered as control.

Multiplate® assays were also performed to assess the impact of ponatinib on platelet aggregation. A 20-minute incubation period with ponatinib and imatinib and the reduced concentration of ADP (150 µM) were used for these assays. These tests were only carried out in duplicates so that no statistical analysis was achieved. For a ponatinib concentration of 100 nM, it seemed that the area under the platelet aggregation curve and the maximal achieved aggregation were approximately the same than those obtained with the control. The velocity of the platelet aggregation curve was slightly increased for this ponatinib concentration

relative to the control. The ponatinib concentration of 150 nM seemed to increase the area under the curve, the maximal aggregation and the velocity. Conversely, these three parameters seemed to be decreased by 200 nM of ponatinib relative to the control (Figure IV.4 A, B and C).

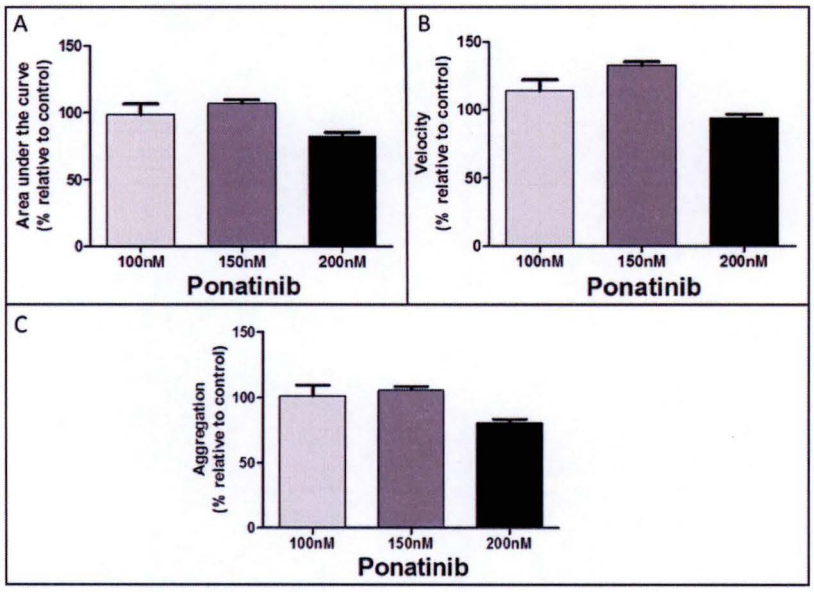

Figure IV.4: **Impact of ponatinib on platelet aggregation assessed by Multiplate® assay.** Three parameters of the platelet aggregation curves were studied: (A) the area under the curve, (B) the velocity, and (C) the maximal achieved aggregation. The platelet aggregation was triggered by three-quarters of the concentration of ADP recommended by the supplier (150 µM) after a 20-minute incubation period with ponatinib. A test with the maximal concentration of DMSO was considered as control. The results were normalized on this control and reported as means of n=2 ± SEM.

IV.2 Impact of ponatinib on endothelial cells

The impact of ponatinib on the hemostatic balance of endothelial cells was assessed by thrombin generation assays. Two endothelial cell lines were used during these tests: the EAhy926 and HUVECs cells. Thrombin generation assays were performed on a monolayer of treated cells and on their supernatant. Then, the potential toxic effect of ponatinib on endothelial cells was assessed by LDH and MTS assays.

IV.2.1 Impact of ponatinib on the hemostatic balance of endothelial cells

IV.2.1.1 The coagulation profile of endothelial cells treated with ponatinib, imatinib or TNF-α

For the thrombin generation assays performed on a EAhy926 cells monolayer after 24-hour treatments, the mean lagtime obtained for 200 nM ponatinib was significantly decreased relative to untreated cells. Indeed, the mean lagtime obtained was reduced of 22.9% relative to untreated cells (Figure IV.5 D). The peak heights obtained with 150 nM of ponatinib and 1 ng/ml of TNF-α were significantly increased relative to untreated cells (Figure IV.5 A, C and E).

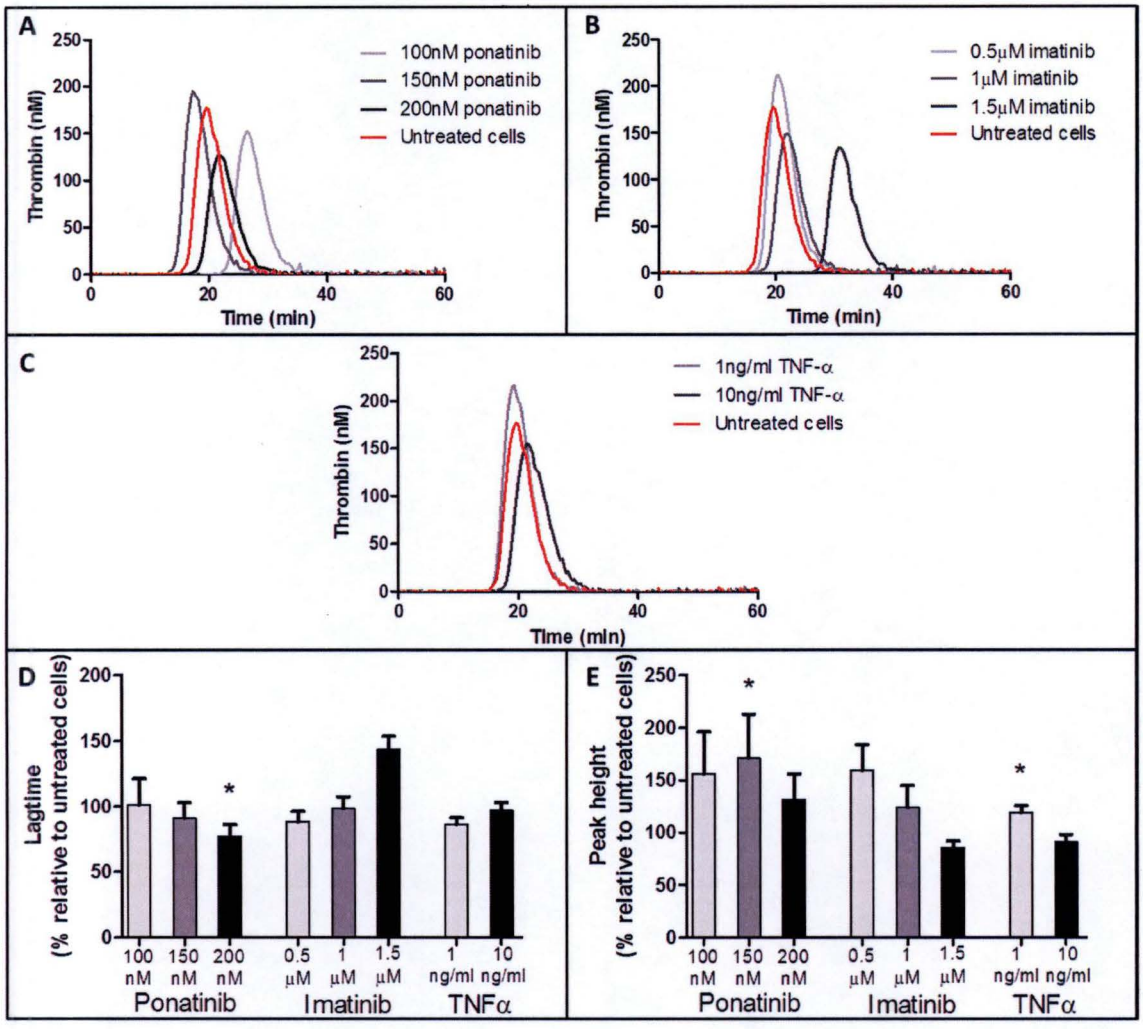

Figure IV.5: **Impact of ponatinib, imatinib and TNF-α on the coagulation profile of EAhy926 cells treated during 24 hours.** The thrombin generation curves were obtained for the tests performed on EAhy926 cells treated with (A) 100, 150 or 200 nM of ponatinib, (B) 0.5, 1 or 1.5 µM of imatinib or (C) 1 or 10 ng/ml of TNF-α. Each thrombin generation curve represents a mean of n=3. Two parameters of the thrombin generation curves were studied: (D) the lagtime and (E) the peak height. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) on these graphs.

After a 48-hour treatment of EAhy926 cells, there was no significant difference of lagtime and peak height relative to untreated cells, whatever the treatment and the concentration used (Figure IV.6).

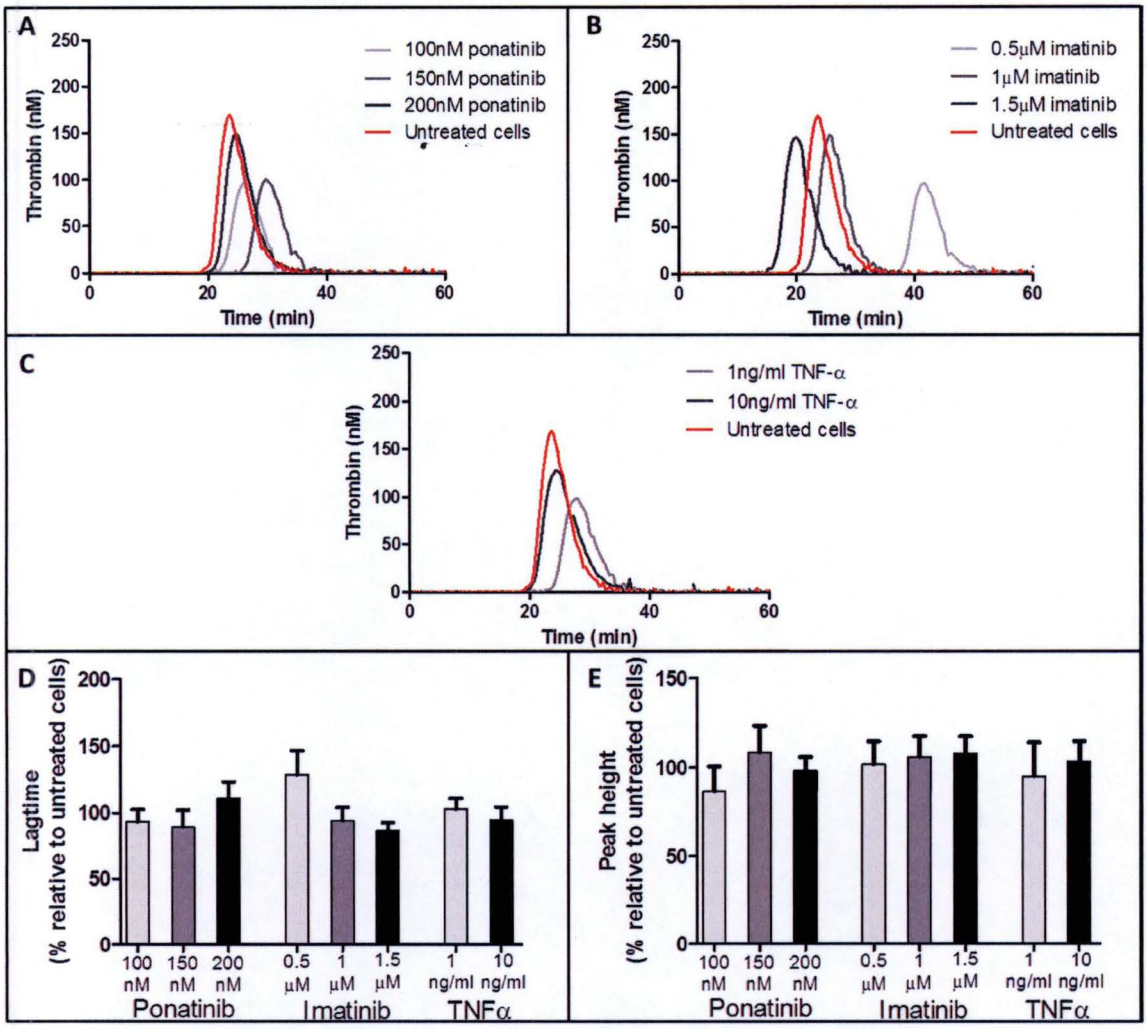

Figure IV.6: **Impact of ponatinib, imatinib and TNF-α on the coagulation profile of EAhy926 cells treated during 48 hours.** The thrombin generation curves were obtained for the tests performed on EAhy926 cells treated with (A) 100, 150 or 200 nM of ponatinib, (B) 0.5, 1 or 1.5 µM of imatinib or (C) 1 or 10 ng/ml of TNF-α. Each thrombin generation curve represents a mean of n=3. Two parameters of the thrombin generation curves were studied: (D) the lagtime and (E) the peak height. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM.

For the thrombin generation assays performed on HUVECs cells monolayers treated during 24 hours, the mean lagtimes obtained for the two tested concentrations of TNF-α were significantly decreased relative to untreated cells (Figure IV.7 C and D). For ponatinib and imatinib, there was no significant difference of lagtime, whatever the TKIs concentration tested (Figure IV.7 A, B and D).

Furthermore, the mean peak heights obtained for 100 nM and 150 nM of ponatinib were significantly increased compared to untreated cells (Figure IV.7 A and E). For the two tested concentrations of TNF-α, a significant increase of the mean peak heights could also be observed (Figure IV.7 C and E). Conversely, for the three concentrations of imatinib, there was no significant difference of peak height relative to untreated cells (Figure IV.7 B and E).

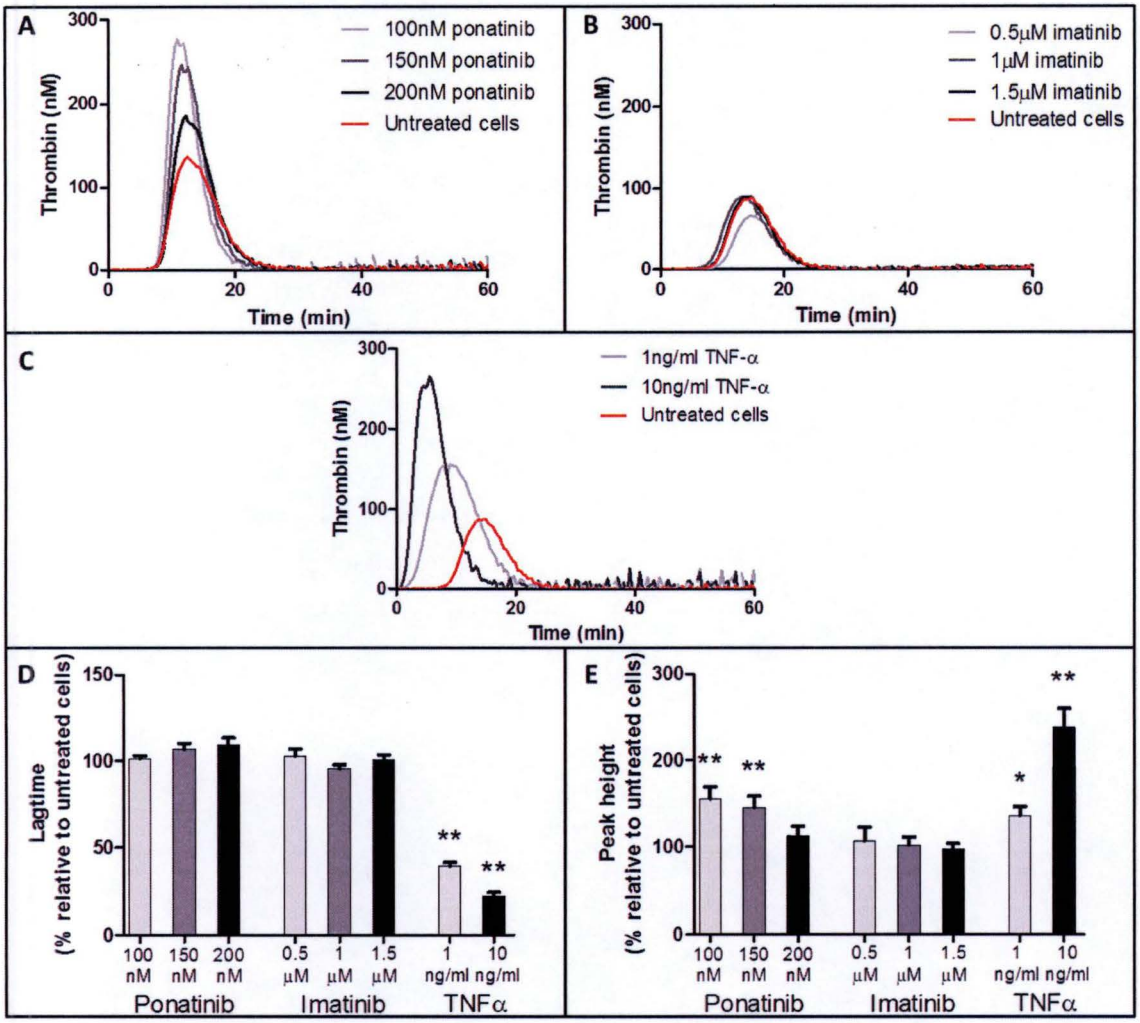

Figure IV.7: **Impact of ponatinib, imatinib and TNF-α on the coagulation profile of HUVECs cells treated during 24 hours.** The thrombin generation curves were obtained for the tests performed on HUVECs cells treated with (A) 100, 150 or 200 nM of ponatinib, (B) 0.5, 1 or 1.5 µM of imatinib or (C) 1 or 10 ng/ml of TNF-α. Each thrombin generation curve represents a mean of n=3. Two parameters of the thrombin generation curves were studied: (D) the lagtime and (E) the peak height. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) and lower than 0.01 are represented by two asterisks (**) on these graphs.

IV.2.1.2 The coagulation profile of the supernatant of endothelial cells treated with ponatinib, imatinib or TNF-α

For the thrombin generation assays performed on the EAhy926 cells supernatant, the mean lagtimes obtained after a 24-hour treatment with 1.5 µM of imatinib and 10 ng/ml of TNF-α were significantly decreased relative to untreated cells (Figure IV.8 B, C and D). No significant difference of peak height could be highlighted in the tests performed on the supernatant of EAhy926 treated during 24 hours, whatever the treatment and concentration used (Figure IV.8 A, B, C and E).

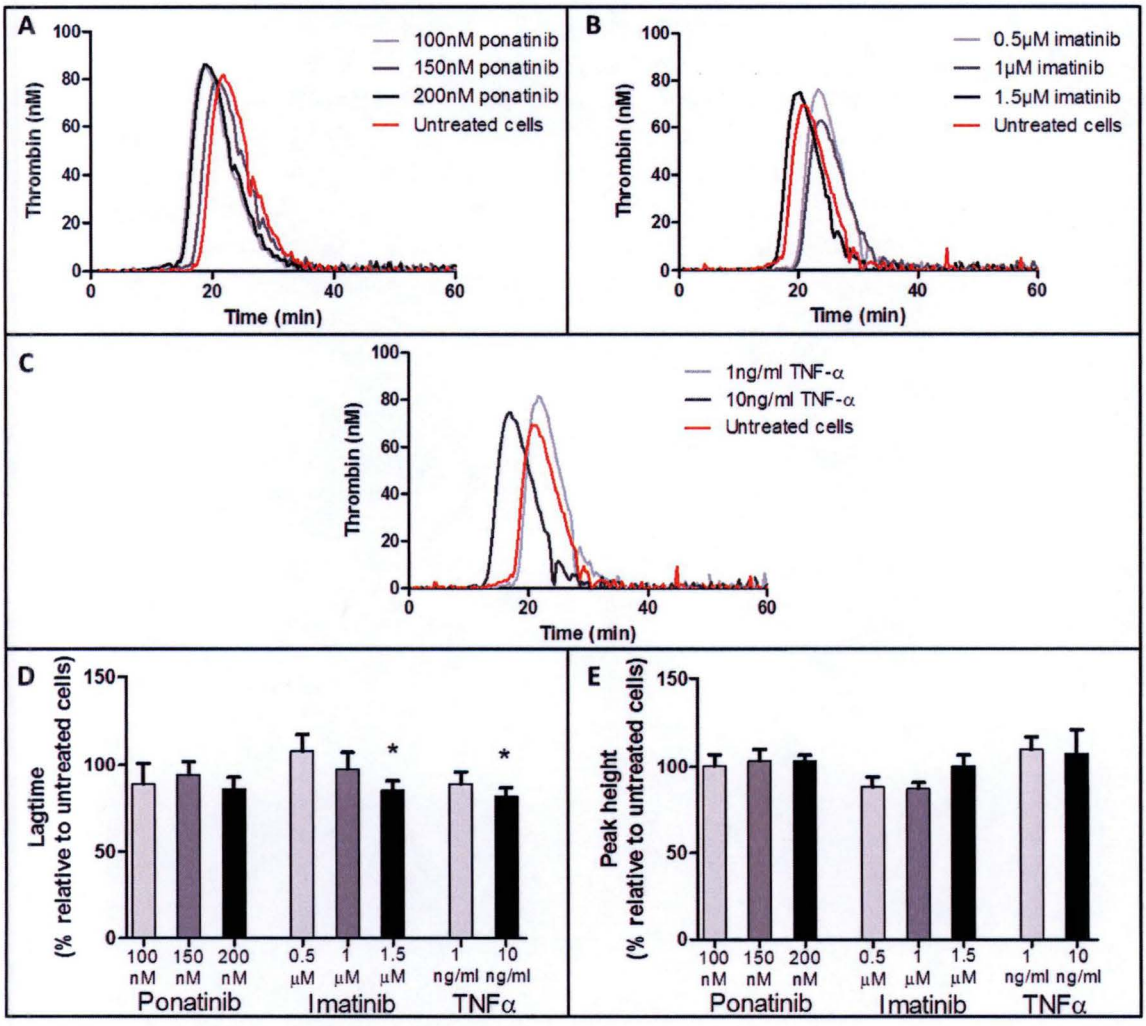

Figure IV.8: **Impact of ponatinib, imatinib and TNF-α on the coagulation profile of the supernatant of EAhy926 cells treated during 24 hours.** The thrombin generation curves were obtained for the tests performed on the supernatant of EAhy926 cells treated with (A) 100, 150 or 200 nM of ponatinib, (B) 0.5, 1 or 1.5 µM of imatinib or (C) 1 or 10 ng/ml of TNF-α. Each thrombin generation curve represents a mean of n=3. Two parameters of the thrombin generation curves were studied: (D) the lagtime and (E) the peak height. Tests performed on the supernatant of untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) on these graphs.

For the tests performed on the supernatant of EAhy926 treated during 48 hours, no significant difference of lagtimes and peak heights relative to untreated cells could be observed (Figure IV.9).

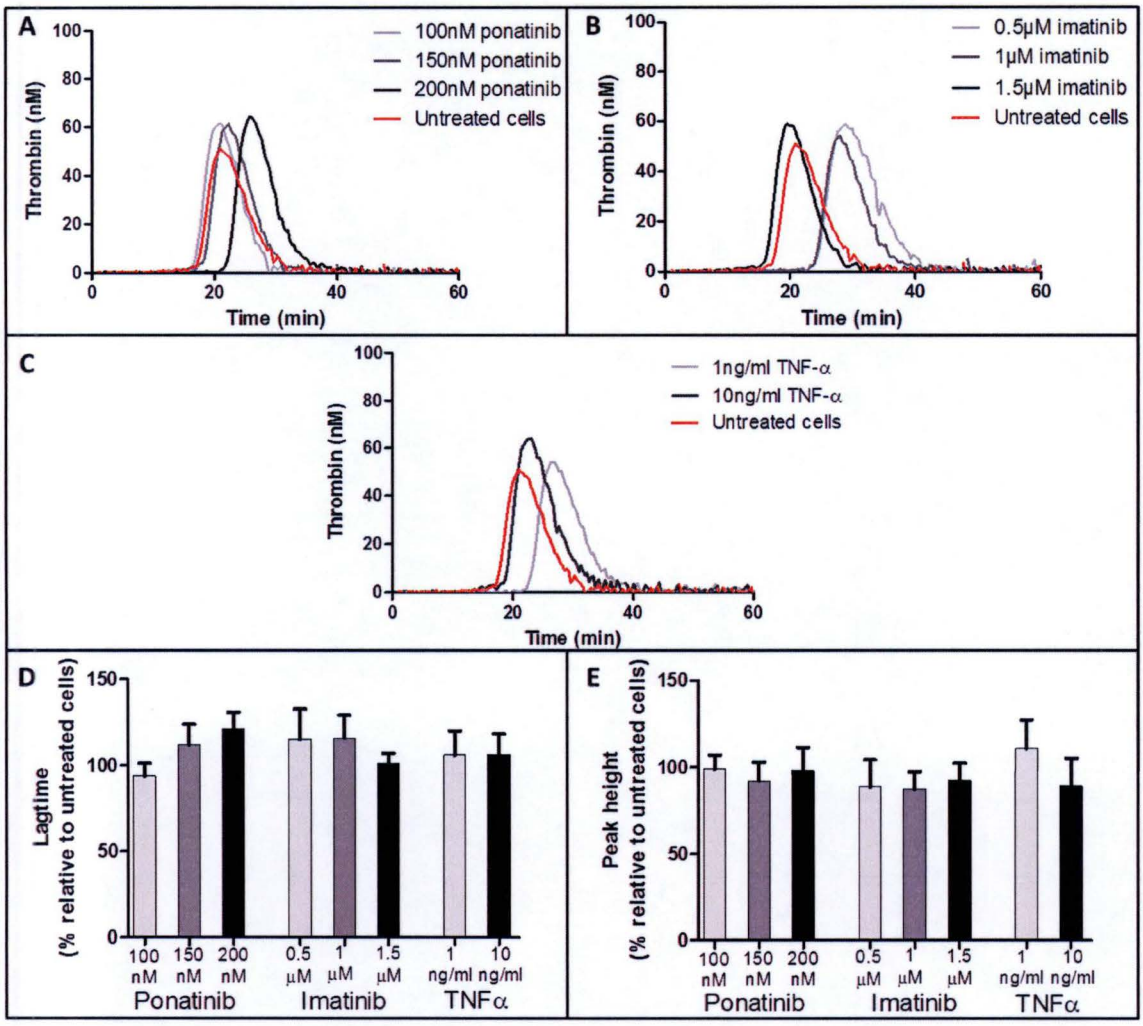

Figure IV.9: **Impact of ponatinib, imatinib and TNF-α on the coagulation profile of the supernatant of EAhy926 cells treated during 48 hours.** The thrombin generation curves were obtained for the tests performed on the supernatant of EAhy926 cells treated with (A) 100, 150 or 200 nM of ponatinib, (B) 0.5, 1 or 1.5 µM of imatinib or (C) 1 or 10 ng/ml of TNF-α. Each thrombin generation curve represents a mean of n=3. Two parameters of the thrombin generation curves were studied: (D) the lagtime and (E) the peak height. Tests performed on the supernatant of untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM.

There was a significant increase of the lagtime obtained with the supernatant of HUVECs cells treated with 100 nM of ponatinib (Figure IV.10 A and D). Moreover, the lagtime observed with the supernatant of HUVECs cells treated with 10 ng/ml of TNF-α was significantly decreased relative to untreated cells (Figure IV.10 C and D). There was no significant difference of peak height obtained for the HUVECs supernatant, whatever the treatment used (Figure IV.10 A, B, C and E).

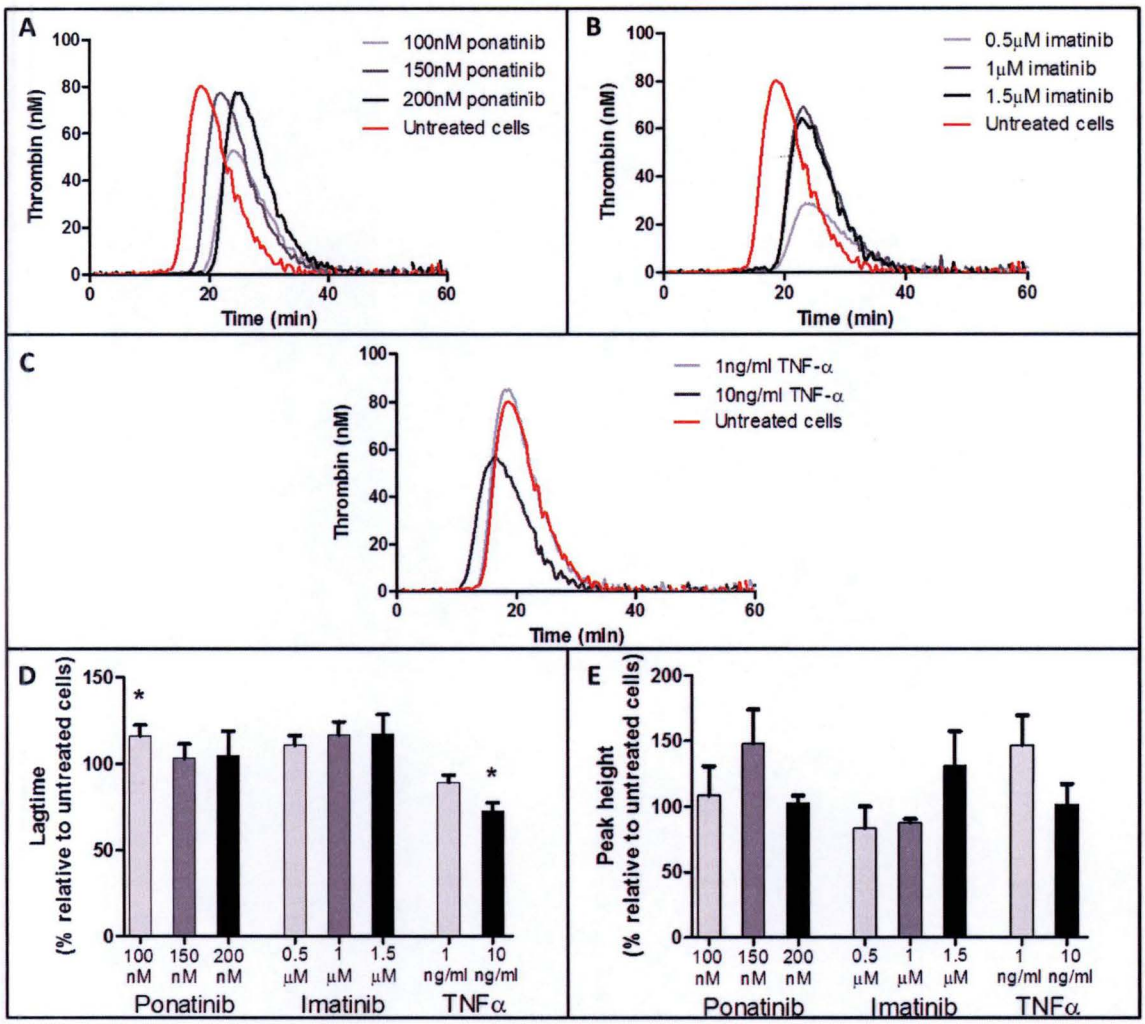

Figure IV.10: **Impact of ponatinib, imatinib and TNF-α on the coagulation profile of the supernatant of HUVECs cells treated during 24 hours.** The thrombin generation curves were obtained for the tests performed on the supernatant of HUVECs cells treated with (A) 100, 150 or 200 nM of ponatinib, (B) 0.5, 1 or 1.5 µM of imatinib or (C) 1 or 10 ng/ml of TNF-α. Each thrombin generation curve represents a mean of n=3. Two parameters of the thrombin generation curves were studied: (D) the lagtime and (E) the peak height. Tests performed on the supernatant of untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) on these graphs.

IV.2.1.3 Reproducibility of the results obtained by thrombin generation assay

For the thrombin generation assays performed on EAhy926 cells monolayers, the thrombin generation curves obtained for the same condition (same treatment and concentration) were quite different from one test to another. For example, during a first test performed on ponatinib-treated cells after a 24-hour treatment, the mean lagtime obtained for 150 nM of ponatinib was 36.4 minutes (Figure IV.11 A) and during another experiment, it was 14.49 minutes (Figure IV.11 B). Moreover, the tendencies obtained for each concentration were different from one test to another. Indeed, during an experiment, the mean lagtimes obtained with ponatinib were lower for 200 nM, equal for 100 nM and higher for 150 nM than the

lagtime obtained with untreated cells (Figure IV.11 A). Conversely, during another experiment, the lagtimes obtained relative to untreated cells were lower for 150 nM and higher for 200 nM and 100 nM of ponatinib (Figure IV.11 B). For the tests performed after 48 hours of treatment, the lagtimes and peak heights obtained were also rather different from one replicate to another.

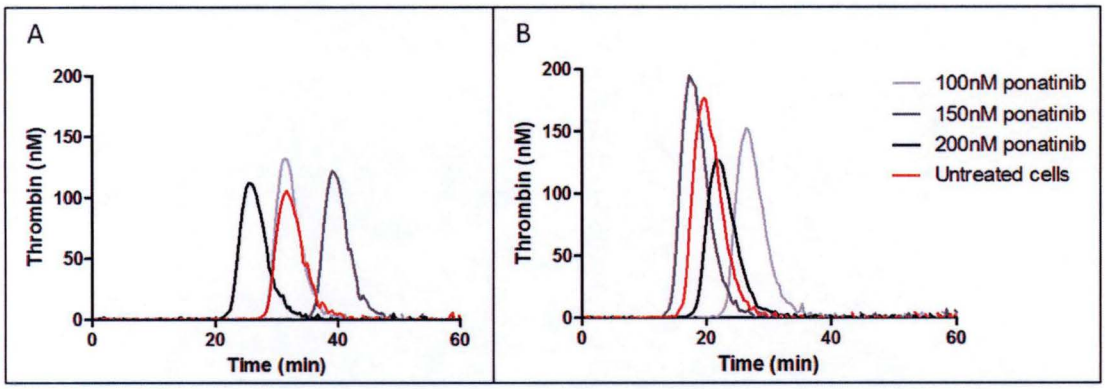

Figure IV.11: **Reproducibility of the thrombin generation assays performed on a EAhy926 cells monolayer.** The thrombin generation curves obtained for tests performed on cells treated with the three concentrations of ponatinib during a first test (A) and during another test (B) are represented on these graphs. Tests performed with untreated cells were used as reference (curves in red) and each thrombin generation curve represents a mean of n=3.

The lagtimes and peak heights of the thrombin generation curves obtained with a monolayer of HUVECs cells were similar for the different replicates of a same test condition (Figure IV.12).

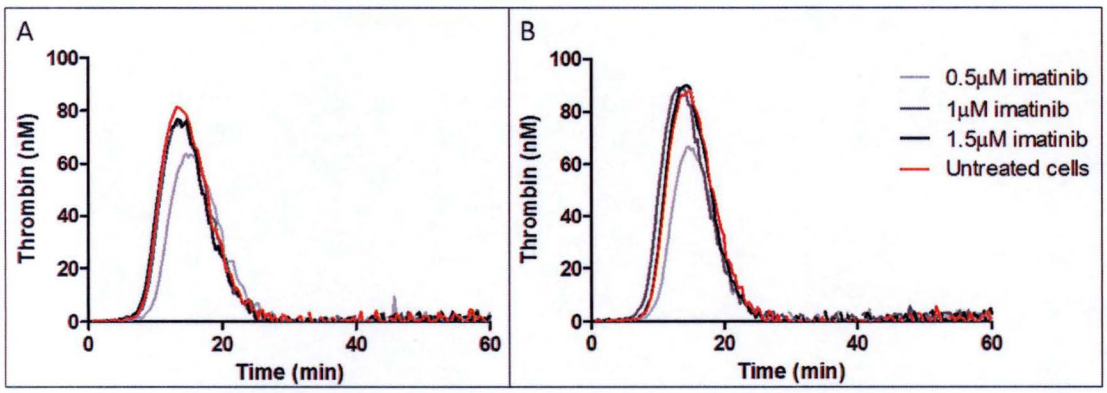

Figure IV.12: **Reproducibility of the thrombin generation assays performed on a HUVECs cells monolayer.** The thrombin generation curves obtained for tests performed on cells treated with the three concentrations of imatinib during a first test (A) and during another test (B) are represented on these graphs. Tests performed with untreated cells were used as reference (curves in red) and each thrombin generation curve represents a mean of n=3.

The different lagtimes and peak heights obtained for a same test condition during the thrombin generation assays performed with the EAhy926 cell supernatant were relatively variable from one test to another. This was observable for the results obtained after 24 and 48 hours of treatment. For example, the coefficient of variation of the main lagtime obtained with the supernatant of cells treated with 150 nM of ponatinib during 48 hours was 26.58%. An example of the variability of the results is shown in Figure IV.13 for the tests performed with the supernatant of EAhy926 cells treated with TNF-α during 48 hours.

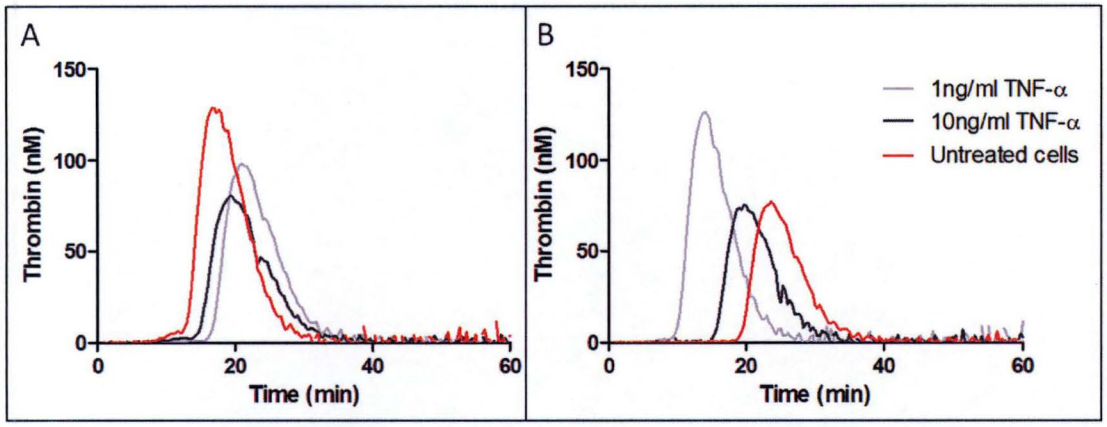

Figure IV.13: **Reproducibility of the thrombin generation assays performed on the EAhy926 cells supernatant.** The thrombin generation curves obtained for tests performed on the cell supernatant treated with the two concentrations of TNF-α during a first test (A) and during another test (B) are represented on these graphs. Tests performed with untreated cells were used as reference (curves in red) and each thrombin generation curve represents a mean of n=3.

Finally, the thrombin generation curves obtained for the tests with the supernatant of treated HUVECs cells were also quite different for the same test condition from one test to another (Figure IV.14).

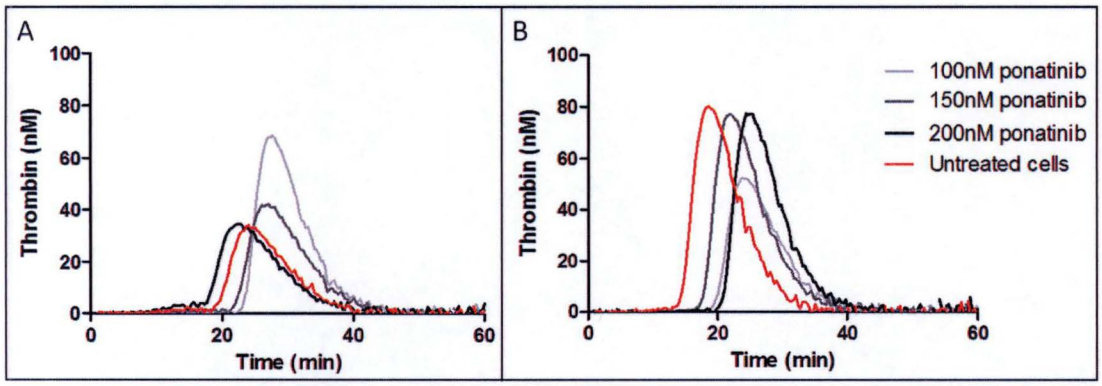

Figure IV.14: **Reproducibility of the thrombin generation assays performed on the HUVECs cells supernatant.** The thrombin generation curves obtained for tests performed on the cell supernatant treated with the three concentrations of ponatinib during a first test (A) and during another test (B) are represented on these graphs. Tests performed with untreated cells were used as reference (curves in red) and each thrombin generation curve represents a mean of n=3.

IV.2.2 Morphology of endothelial cells treated with ponatinib, imatinib or TNF-α

EAhy926 cells morphology after a 24-hour treatment with ponatinib, imatinib and TNF-α was not changed relative to the morphology of untreated cells (Figure IV.15).

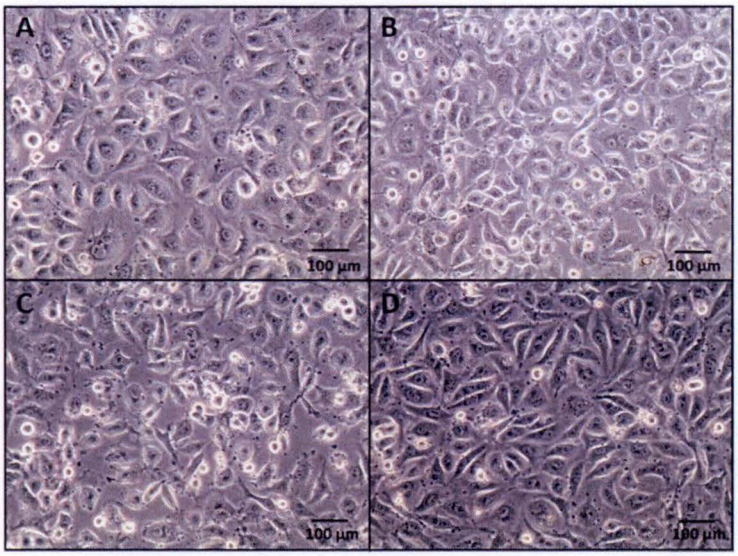

Figure IV.15: **EAhy926 cells morphology after 24-hour treatments with imatinib, ponatinib and TNF-α.** These pictures were taken by phase contrast microscopy with a magnification of 200x for the different test conditions: (A) untreated cells, (B) 1.5 µM imatinib, (C) 200 nM ponatinib and (D) 10 ng/ml TNF-α.

After 48 hours of treatment, the cells treated with the maximal tested dose of ponatinib (Figure IV.16 C) and TNF-α (Figure IV.16 D) had slightly changed their morphology. Indeed, these cells became elongated which revealed a large surface of the plate.

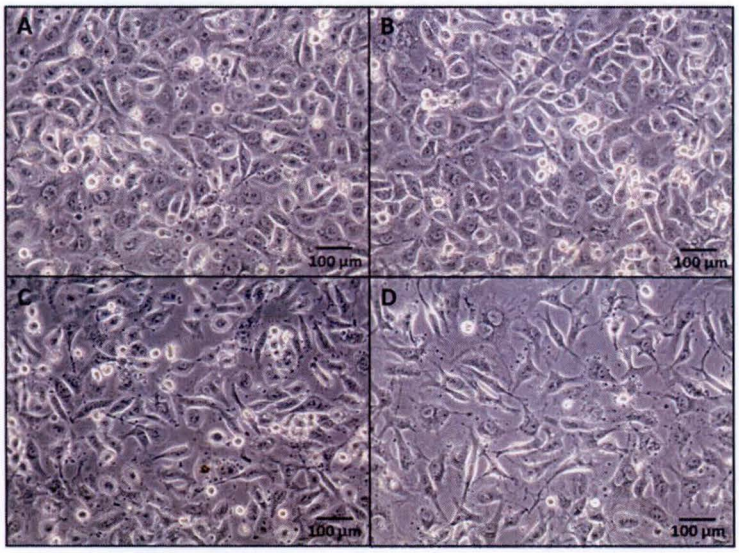

Figure IV.16: **EAhy926 cells morphology after 48-hour treatments with imatinib, ponatinib and TNF-α.** These pictures were taken by phase contrast microscopy with a magnification of 200x for the different test conditions: (A) untreated cells, (B) 1.5 µM imatinib, (C) 200 nM ponatinib and (D) 10 ng/ml TNF-α.

HUVECs cells morphology after a 24-hour treatment with ponatinib and imatinib was not changed relative to the morphology of untreated cells (Figure IV.17 B and C). Conversely, many cells treated with 10 ng/ml of TNF-α were dead and floating. The few cells which still adhered to the bottom of the plate had undergone a change in morphology and became more elongated so that the bottom of the plate was largely revealed in this condition (Figure IV.17 D).

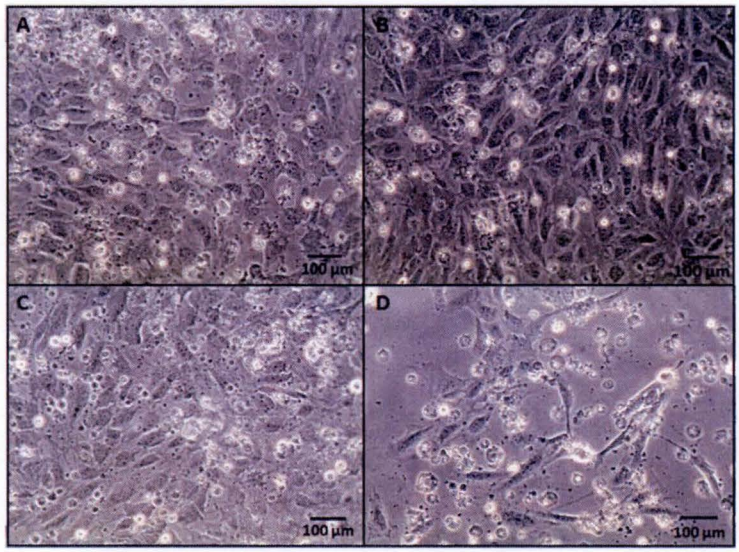

Figure IV.17: **HUVECs cells morphology after 24-hour treatments with imatinib, ponatinib and TNF-α.** These pictures were taken by phase contrast microscopy with a magnification of 200x for the different test conditions: (A) untreated cells, (B) 1.5 µM imatinib, (C) 200 nM ponatinib and (D) 10 ng/ml TNF-α.

IV.2.3 Metabolic changes of endothelial cells treated with ponatinib, imatinib or TNF-α

The impact of ponatinib, imatinib and TNF-α on the endothelial cells metabolism was assessed by MTS assay. After a 24-hour treatment of EAhy926 cells with 10 ng/ml of TNF-α, a significant decrease of the percentage of metabolically active cells could be observed relative to untreated cells. There was no significant difference of the metabolically active cells percentage for the EAhy926 cells treated with ponatinib and imatinib during 24 hours, whatever the concentration tested (Figure IV.18 A).

After 48-hour treatments, the percentage of metabolically active EAhy926 cells was significantly decreased for the cells treated with the two highest doses of ponatinib. Moreover, there was also a significant reduction of the metabolically active cells percentages obtained after a 48-hour treatment with 1 ng/ml and 10 ng/ml of TNF-α. Finally, no significant difference had been observed for the EAhy926 cells treated with imatinib during 48 hours, whatever the concentration tested (Figure IV.18 B).

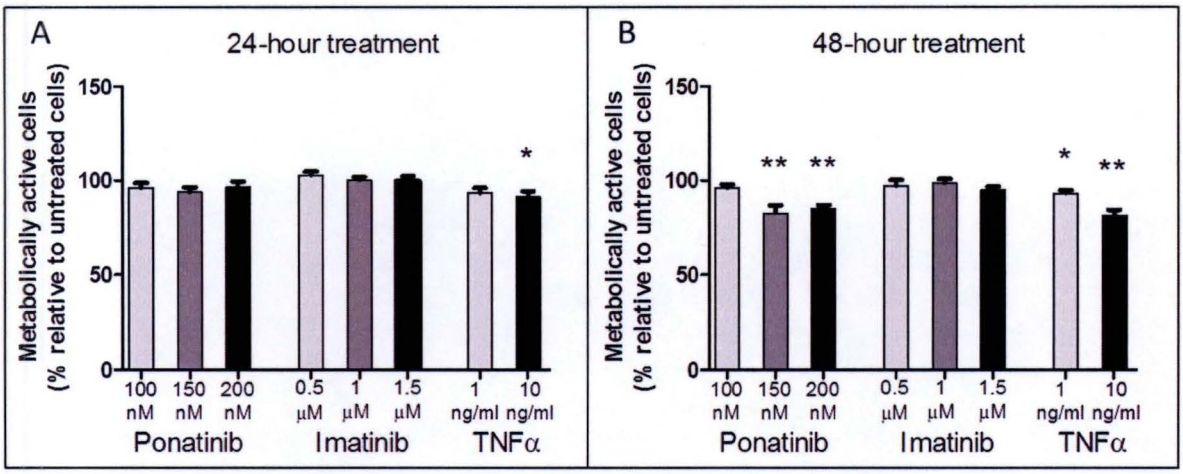

Figure IV.18: **Impact of ponatinib, imatinib and TNF-α on the metabolism of EAhy926 cells.** The mean percentages of metabolically active cells after a treatment with different concentrations of ponatinib, imatinib or TNF-α during 24 hours (A) or 48 hours (B) are represented on these graphs. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) and lower than 0.01 are represented by two asterisks (**) on these graphs.

Regarding the MTS assays performed on HUVECs after 24-hour treatments, a significant decrease of the metabolically active cells percentage relative to untreated cells was obtained for the two tested concentrations of TNF-α. This percentage was significantly increased for the HUVECs cells treated with 200 nM of ponatinib and 0.5 µM of imatinib during 24 hours (Figure IV.19).

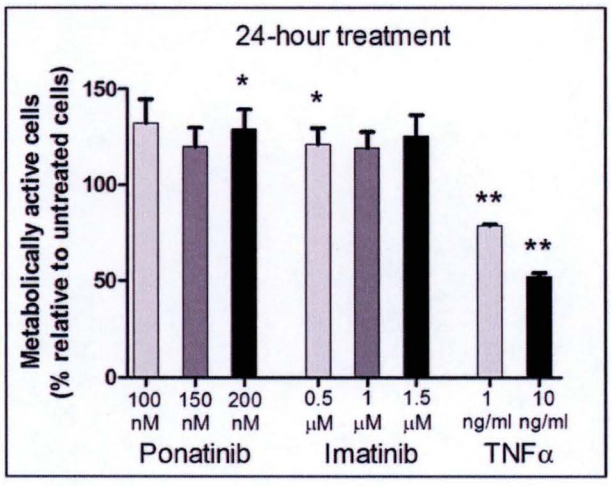

Figure IV.19: **Impact of ponatinib, imatinib and TNF-α on the metabolism of HUVECs cells.** The mean percentages of metabolically active cells after a treatment with different concentrations of ponatinib, imatinib or TNF-α during 24 hours are represented on this graph. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) and lower than 0.01 are represented by two asterisks (**) on this graph.

IV.2.4 Toxicity of ponatinib, imatinib and TNF-α on endothelial cells

The cytotoxicity of ponatinib, imatinib and TNF-α on endothelial cells was assessed by LDH assay. After 24-hour treatments of EAhy926 cells, there was no significant difference of LDH percentage released in the cell supernatant relative to untreated cells, whatever the treatment and the tested concentration (Figure IV.20 A).

After 48-hour treatments, the percentage of LDH released in the EAhy926 cells supernatant significantly increased for the cells treated with 150 nM of ponatinib and with 0.5 µM of imatinib. For the other concentrations of ponatinib and imatinib and for the two concentrations of TNF-α, no significant difference of LDH percentages released in the cell supernatant had been observed after 48-hour treatments (Figure IV.20 B).

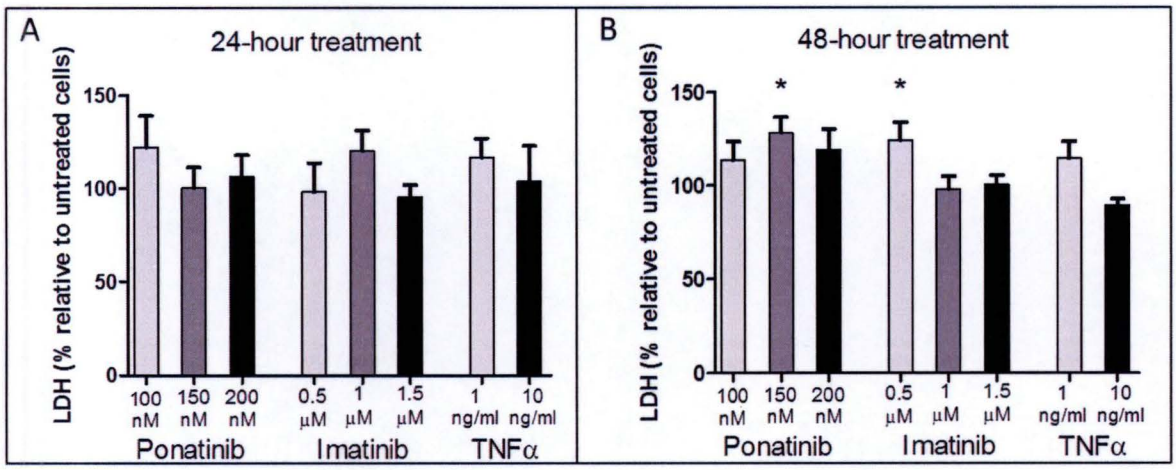

Figure IV.20: **Toxicity of ponatinib, imatinib and TNF-α on EAhy926 cells.** The mean percentages of LDH released in the cell supernatant after a treatment with different concentrations of ponatinib, imatinib or TNF-α during 24 hours (A) or 48 hours (B) are represented on these graphs. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) on these graphs.

Regarding the LDH assays performed on HUVECs after 24-hour treatments, a significant decrease of the LDH percentage released in the cell supernatant was obtained for the three concentrations of ponatinib. The higher decrease was observed for 150 nM ponatinib with a reduction of 27.56% relative to untreated cells. A significant decrease was also observed for the tests performed on the supernatant of cells treated with 1.5 µM of imatinib. Moreover, the LDH percentage in the cell supernatant was significantly increased for the HUVECs treated with the two concentrations of TNF-α. Indeed, these percentages were increased of 66% and 113.6% relative to untreated cells for 1ng/ml and 10 ng/ml of TNF-α, respectively. Finally, for the two lower concentrations of imatinib, there was no significant difference of LDH percentage released in the cell supernatant relative to untreated cells (Figure IV.21).

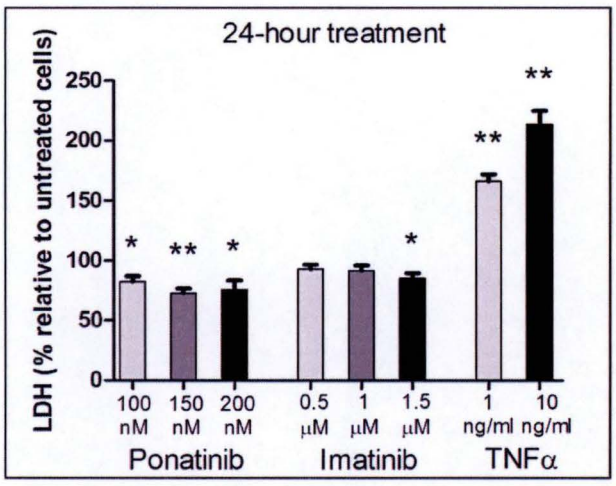

Figure IV.21: **Toxicity of ponatinib, imatinib and TNF-α on HUVECs cells.** The mean percentages of LDH released in the cell supernatant after a treatment with different concentrations of ponatinib, imatinib or TNF-α during 24 hours are represented on these graphs. Tests performed with untreated cells were used as reference. The results were normalized on this control and presented as means of n=9 ± SEM. P values lower than 0.05 are represented by one asterisk (*) and lower than 0.01 are represented by two asterisks (**) on this graph.

V. Discussion

V. Discussion

Ponatinib-treated patients have an increased risk of VOEs. Several hypotheses could be proposed to explain the induction of this prothrombotic phenotype. Two potential underlying mechanisms were studied during this master thesis. The potential impact of ponatinib on platelet aggregation and on endothelial cells was investigated.

Several TKIs are described in the literature to induce platelet dysfunction (Quintas-Cardama et al., 2009). Therefore, the thrombotic events of ponatinib-treated patients could be partly induced by an abnormal platelet function. To investigate the potential impact of ponatinib on platelet aggregation, optical aggregometry assays were carried out. During the first tests, the commonly used concentrations (recommended by the supplier) of different platelet agonists were employed to trigger platelet aggregation. Further to these assays, it appeared that platelet aggregation is not affected by ponatinib. A decreased concentration of these agonists was used to increase the sensibility of the tests and a prolonged incubation time was carried out to increase the exposure time of ponatinib with platelets. As expected, a decreased platelet aggregation with a reduced concentration of the ADP agonist and a prolonged incubation time was observed. A slight decrease was also observed with the highest concentration of imatinib for one donor sample. However, the same test conditions were repeated with the plasma of another donor but no more platelet aggregation decrease was observed. Another research group has found that ponatinib acted as a platelet antagonist especially by inhibiting platelet aggregation which was also assessed by light transmission aggregometry (Loren et al., 2015). Optical assay is not the most sensitive method to assess platelet aggregation. Consequently, we have performed the same test conditions (a reduced concentration of the ADP inducer and a prolonged incubation time) with the Multiplate® system which is based on the measurement of the electric current variation following platelet aggregation. The results of these tests showed different tendencies for the three ponatinib concentrations. The different studied parameters (area under the curve, velocity and maximal reached aggregation) of the platelet aggregation curves were decreased with the highest ponatinib concentration but not for the two other concentrations. It should be noticed that the results obtained with the technical duplicates by using the same ponatinib concentration were quite different. This difference could result from the use of a decreased concentration of platelet aggregation inducer relative to the recommended concentration. It is likely that the maximum accuracy of this measurement system was reached.

The observed tendency of ponatinib towards an antagonist effect on platelet aggregation is not consistent with the prothrombotic phenotype of patients. However, ponatinib was described many times and with different techniques as a platelet antagonist (Loren et al., 2015). Besides having shown that ponatinib decreased platelet aggregation, Loren et al. have also highlighted that this TKI inhibited platelet activation, spreading and granule secretion. This group especially described that ponatinib significantly decreases the platelet P-selectin exposure and the PS externalization on platelet surface, these two mechanisms being associated with platelet activation and aggregation (Loren et al., 2015). However, Loren et al. have used a concentration of 1 µM of ponatinib in most of their experiments. This ponatinib concentration

is quite higher than the physiological blood concentration (150 nM) in patients treated with the daily recommended dose. Moreover, even after a repeated administration, there is an accumulation by a factor 1.5 to 2.0 (Cortes et al., 2012a) which is still well lower than the concentration used by this research group. Therefore, the impact of ponatinib on the main platelet functions could be not found in treated patients. However, it was highlighted that ponatinib-treated patients have increased closure times during PFA-100 assays which also suggests an impairment of the primary hemostasis by ponatinib (Neelakantan et al., 2012). The targeting of key kinases of platelet aggregation and activation (such as PDGFR and SFK members) by ponatinib could partly explain this platelet dysfunction but the underlying mechanisms are still poorly understood. Recently, it was shown that ponatinib inhibited the phosphorylation of some Src family kinases members important for platelet function (Loren et al., 2015).

The second investigated assumption was the potential impact of ponatinib on endothelial cells. All the tests were also performed with imatinib to compare the results obtained with ponatinib to those observed with a TKI known to do not increase the VAEs development risk in treated patients. Regarding the choice of the ponatinib and imatinib concentrations used in all the tests, the intermediate concentrations (150 nM for ponatinib and 1 µM for imatinib) are the highest concentrations found in the blood of patients treated with the daily recommended dose of each TKI (Cortes et al., 2012a; Martins et al., 2011). For the two TKIs, a lower and a higher concentration were tested to assess if the obtained tendencies were more marked when the dose increased. Finally, TNF-α was used as a potential positive control of endothelial cells activation in the experiments which assess the impact of ponatinib on endothelial cells. Indeed, TNF-α is known to induce an inflammatory response of endothelial cells (Tellier et al., 2015). The highest tested concentration of this pro-inflammatory cytokine was already proven to increase the endothelial cells activation (Geenen et al., 2012).

Endothelial cells are one of the main players of the vascular homeostasis, preventing the coagulation steps in resting conditions. An endothelial dysfunction induced by ponatinib could partly explain the increased thrombotic risk of treated patients. To investigate the impact of ponatinib on the hemostatic balance of endothelial cells, thrombin generation assays on a monolayer of treated cells were used. Indeed, thrombin generation assays on endothelial cells were already performed by Ten Cate and his colleagues who have concluded that this method is valuable to assess the global coagulation profile of these cells (Geenen et al., 2012).

The first assays were performed on a monolayer of EAhy926 endothelial cells treated during 24 and 48 hours. The results obtained with this cell line were not reproducible. Indeed, despite having performed a great number of replicates of the same test condition (n=9), many lagtimes and peak heights differences relative to untreated cells were not significant. Overall, the tests performed on this cell line did not allow to highlight the impact of ponatinib on the endothelial cell coagulation profile. The variability of the results could partly result from the heterogeneity of the cell layer. Indeed, in accordance with the number of cells in each well, the well bottom is more or less covered. As we performed these assays on plates treated for cell culture, the uncovered surface of the well bottom influenced the obtained thrombin generation curves by promoting coagulation cascade. This impact of the plate surface on

coagulation was highlighted by our lab during the set-up of this experiment. Therefore, according to the number of cells and their distribution on the well bottom, there was a potential big interference of the plate in the global thrombin generation curves. A new microscopy system (Leica Phase Expert; Leica Microsystems, Leitz, Wetzlar, Germany) connected to a special picture processing software can provide the percentage of the well bottom covered by cells. The use of this microscope before each thrombin generation assay could allow to assess and take into account the percentage of covered surface for each replicate of a same test condition. Another limitation of this method is the fact that EAhy926 cells are immortalized and do not exactly express the same phenotype than primary endothelial cells. For example, EAhy926 cells have a low TF expression on their surface (Campbell et al., 2010) and do not express the vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Tellier et al., 2015). As they are immortalized, EAhy926 cells could be less sensitive to imatinib, ponatinib and TNF-α than primary cells. However, Ten Cate and co-workers have found that TNF-α-treated EAhy926 cells induced a lagtime decrease and a peak height increase relative to untreated cells in thrombin generation assay (Geenen et al., 2012). This research group did not demonstrate that this procoagulant profile resulted from a cell activation rather than from an uncovered well bottom as explained above. Indeed, TNF-α is known to induce endothelial cell death (Polunovsky et al., 1994) and, as dead cells become floating, the plate surface is largely uncovered which could partly explain the procoagulant profile they obtained. Pictures of the treated EAhy926 cells confirmed that these cells became elongated after their treatment with TNF-α which largely revealed the well bottom.

The HUVECs endothelial cell line was also used to perform thrombin generation assays. In this case, the different parameters (lagtime and peak height) of the thrombin generation curves obtained were more reproducible than those obtained with EAhy926 cells. We observed a significant increase of the main peak heights obtained for the HUVECs treated with two concentrations of ponatinib. This peak height increase could reflect an impact of ponatinib-treated cells on the produced thrombin amount but without impact on the initiation phase of coagulation. Indeed, the lagtime was not decreased during the tests. It is also important to note that we did not observe any change of cell morphology after the ponatinib treatment which can allow to exclude the well bottom contribution to the global thrombin generation profile obtained with ponatinib-treated HUVECs. Therefore, ponatinib could slightly activate HUVECs cells by inducing an overexpression of procoagulant molecules and/or a downregulation of anticoagulant molecules and/or an exposure of PS on the surface of HUVECs cells. Indeed, these three mechanisms could explain the increased peak height obtained with ponatinib-treated HUVECs cells. To determine if ponatinib-treated cells trigger the coagulation cascade by the intrinsic or extrinsic pathway, inhibitors of one of these two pathways could be added before the assays. For example, other research teams have used corn trypsin inhibitor (CTI) to inhibit contact activation and active site inhibited factor VIIa (ASIS) to block the extrinsic pathway (Geenen et al., 2012). The slight activation of endothelial cells by ponatinib could partly explain the prothrombotic state of treated patients.

Furthermore, a significant decrease of the lagtimes and a significant increase of the peak heights were obtained for the HUVECs treated with the two concentrations of TNF-α. We

have taken pictures of treated HUVECs cells by contrast phase microcopy and have observed that a lot of cells treated with TNF-α were dead and floating. The procoagulant profile obtained with these cells could partly be induced by the triggering of the intrinsic pathway by PS exposed on the surface of apoptotic cells during the thrombin generation assays (Spronk et al., 2014). Moreover, it was already highlighted that the treatment of endothelial cells with TNF-α decreased their expression of TM (Boffa and Karmochkine, 1998) and increased their expression of the intercellular adhesion molecule-1 (ICAM-1), VCAM-1 and E-selectin (Rafiee et al., 2003). TNF-α was also described to induce an increased expression of TF on the HUVECs cell surface (Camera et al., 1999). These mechanisms could explain the procoagulant profile obtained with TNF-α-treated HUVECs. However, as the well bottom was largely uncovered in this condition, it is likely that the obtained profiles may be partly influenced by this matrix interference effect.

The coagulation profiles and the morphology of imatinib-treated cells were not changed relative to untreated cells. Moreover, another research group has also found that imatinib does not induce the overexpression of endothelial cell activation markers like ICAM-1, VCAM-1, endothelin-1 and VEGF (Venalis et al., 2009). These observations confirm the vascular safety of imatinib.

It is also important to note that we have followed different protocols to culture the two cell lines in the 96-well plates before thrombin generation assays. Indeed, EAhy926 were placed in the wells with a concentration which allowed to rapidly reach cell confluence to perform the thrombin generation assays two days after having placed them in plates. As the cell dilutions were not exactly the same from a test to another and as the tests time line was predefined, the number of EAhy926 cells on which we have performed the tests was highly variable between replicates. Conversely, for HUVECs cells, we have placed cells with a low concentration in the wells and have waited a variable delay so that the cells were at confluence (visual check) before performing the tests. The cell concentration at test startup was less variable between replicates. This difference of followed protocols could also partly explain the higher inter and intra-assay variability obtained with EAhy926 cells.

In addition to having performed thrombin generation assays on treated endothelial cells, several tests were carried out on the supernatant of these cells. Activated endothelial cells may release EVs and molecules which can trigger primary and secondary hemostasis. Indeed, molecules such as t-PA, PAI-1, TF, vWF, soluble TM and soluble E-selectin can be found in the endothelial cell supernatant and influence thrombin generation profiles (Blann, 2012). Thrombin generation tests performed on the treated cells supernatant could therefore provide information on the coagulation profile of their secretion. The tests were performed on the supernatant of EAhy926 and HUVECs cells. Nine replicates were performed but few obtained results were significantly different relative to the tests performed on the untreated cells supernatant. Indeed, we did not obtain a significant increase of peak height as well as a significant decrease of lagtime with the ponatinib-treated cell supernatant, whatever the endothelial cell line and the treatment times used. This absence of significant difference could partly be explained by the lack of ponatinib impact on the hemostatic balance of the EVs and

molecules secreted by endothelial cells. However, the variability of the obtained results could also explain this absence of significant difference.

The VOEs development risk could also be increased as the ponatinib treatment is daily repeated in patients. In the present study, endothelial cells were once treated followed by a 24 and/or 48 hours period before performing tests. This could be too short periods to see the real impact of ponatinib. However, we have also performed 72-hour treatments of EAhy926 cells but a lot of cells were dead with this treatment time so that the obtained results were not treated.

A change in endothelial cells metabolism could also lead to the VOEs development in ponatinib-treated patients. In order to investigate the potential metabolic changes of endothelial cells after their treatment with ponatinib, MTS assays were performed. These tests allow to determine the number of metabolically active cells which can reflect a change in cell proliferation or cell death in a given condition. These assays were performed on EAhy926 and HUVECs cells treated with ponatinib, imatinib or TNF-α. A significant reduction of the metabolically active cells percentage relative to untreated cells was obtained for the cells treated with TNF-α, whatever the cell line and the treatment time used. Therefore, the TNF-α treatment could decrease the number of metabolically active endothelial cells either by decreasing their proliferation either by increasing cell death. Palmieri et al. have already highlighted that TNF-α induced the EAhy926 cells dysfunction through the modulation of different signaling pathways, including p38MAPK, JNK and Akt (Palmieri et al., 2012). Ponatinib and imatinib did not decrease the metabolism of HUVECs and EAhy926 cells after 24-hour treatments. Conversely, after a 48-hour treatment, the two highest tested concentrations of ponatinib significantly decreased the number of metabolically active EAhy926 cells. Therefore, ponatinib could affect the EAhy926 cells metabolism after 48 hours of treatment. This decrease of the percentage of metabolically active cells could reflect a decrease of cell proliferation as well as an increase of cell death.

Finally, LDH assays were performed to assess the cytotoxicity of the ponatinib, imatinib and TNF-α treatments. These tests assess the impact of compounds on cell membranes integrity. Indeed, during cell death, there could be an increase of cell membrane permeability which releases intracytoplasmic enzymes in the cell supernatant, including LDH. Therefore, LDH activity can be used as an indicator of cell membrane integrity and allows to assess cell viability by measuring plasma membrane permeability (Decker and Lohmann-Matthes, 1988). The results obtained after 24-hour treatments were different for the two tested cell lines. Indeed, ponatinib, imatinib and TNF-α did not change the percentage of LDH released in the EAhy926 cells supernatant whereas HUVECs treated with TNF-α increased their LDH release. So, TNF-α could impact the cell membrane integrity of HUVECs cells. TNF-α was already known to induce a release of LDH by cancer cells (Jurisic et al., 1999) but the impact of this cytokine on the integrity of endothelial cell membranes is ill-documented. It is well approved that TNF-α induce apoptosis by caspase-dependent pathways (Gaur and Aggarwal, 2003) but the LDH assay allows to show the loss of membrane integrity which is rather found during necrosis. However, it was also highlighted that TNF cytokines could induce necroptosis (Cho et al., 2011). This programmed caspase-independent cell death was

highlighted to induce the cell membrane disintegration (Vandenabeele et al., 2010) which could explain the increase of the LDH release in the supernatant of cells treated with TNF-α. Moreover, the pictures taken after a 24-hour treatment of HUVECs with TNF-α showed that a lot of cells were dead after this treatment. Overall, this cytokine seems to have a cytotoxic effect on HUVECs cells by decreasing their metabolism, inducing the loss of membrane integrity and cell death.

The three concentrations of ponatinib as well as the highest concentration of imatinib decreased the percentage of LDH in the supernatant of HUVECs cells treated during 24 hours. This decrease of the released LDH could be induced by a reduction of the cell membrane permeability. After 48-hour treatments, 150 nM of ponatinib and 0.5 µM of imatinib could affect the membrane integrity of EAhy926 cells. However, the other concentrations of ponatinib and imatinib did not seem to change the percentage of LDH released in the cell supernatant. Moreover, TNF-α did not seem to increase the permeability of the EAhy926 cell membranes after a 48-hour treatment.

The results obtained by MTS and LDH assays with imatinib-treated cells suggest that imatinib does not inhibit the proliferation and the metabolic activity of endothelial cells. Furthermore, imatinib does not seem to affect the endothelial cell membrane integrity which suggests that imatinib does not induce cell death. Another research group has also highlighted similar observations and has concluded that imatinib did not alter the main functions of endothelial cells (Venalis et al., 2009).

VI. Conclusion and perspectives

VI. Conclusion and perspectives

This master thesis aimed at studying the mechanisms by which ponatinib could induce VOEs in treated patients. This ADR often leads to the discontinuation of the ponatinib therapy. However, this TKI is a highly potent antileukemic drug and represents the last treatment option for patients suffering from Ph-positive leukemia (Cortes et al., 2013). It is important to understand how ponatinib induces thrombotic events to increase the survival of treated patients by a better management and prophylaxis of this ADR.

Firstly, the potential impact of ponatinib on platelet aggregation was studied. Indeed, the patients' prothrombotic profile could be induced by an impact on primary hemostasis. This assumption was investigated by light transmission aggregometry and Multiplate® assays. A slight decreased platelet aggregation was obtained by the two techniques with the highest tested concentration of ponatinib. However, this observation was not reproducible by light transmission assay and tested only in duplicate by Multiplate® assay. This decreased platelet aggregation is not consistent with the VOEs observed in treated patients but other research groups have described ponatinib as a platelet antagonist (Neelakantan et al., 2012; Loren et al., 2015). Moreover, our lab had previously assessed the impact of ponatinib on inactive platelets by studying platelet activation markers by flow cytometry. Further to these experiments, it was concluded that ponatinib did not seem to spontaneously activate platelets (unpublished data). Overall, it seems that ponatinib do not induce vascular thrombotic events by increasing platelet aggregation in patients.

The second investigated assumption was the potential impact of ponatinib on endothelial cells. Activated or damaged endothelial cells are able to exhibit a procoagulant phenotype, increasing their expression of procoagulant and proaggregant molecules and/or decreasing their expression of anticoagulant and antiaggregant molecules (Verhamme and Hoylaerts, 2006). Ponatinib could impair the hemostatic balance of endothelial cells, leading to an increased risk of thrombotic events development. An innovative technique of thrombin generation assay on a cell monolayer was used to investigate this assumption. The first tests were performed on EAhy926, an immortalized endothelial cell line. The results obtained with these assays were highly variable and only few obtained tendencies were significant. This lack of significant and reproducible results could be explained by two different parameters. Firstly, these assays were performed on 96-well plates treated for cell culture whose well bottom influences the thrombin generation curves by promoting coagulation cascade. So, the exact number of cells and their distribution on well bottom could influence the obtained thrombin generation curves. Secondly, EAhy926 cells are immortalized and have a less physiological phenotype than primary cells (Campbell et al., 2010). These cells could therefore be less sensitive to ponatinib. Indeed, the tests were also performed on cells treated with TNF-α, a pro-inflammatory cytokine already describe to induce endothelial cell activation and death (Pober, 1998). However, when EAhy926 cells were treated with TNF-α, no significant induction of procoagulant profile was observed which prove that these cells are very resistant.

The same assays were also performed on a primary endothelial cell line: the HUVECs. The thrombin generation assays performed on a HUVECs monolayer provided different significant tendencies. First of all, procoagulant thrombin generation curves were obtained with TNF-α-treated HUVECs cells. This increased peak height and decreased lagtime could be induced by an impact of this cytokine on the hemostatic balance of endothelial cells. Indeed, it was already highlighted that TNF-α induce an overexpression of molecules triggering coagulation cascade (Camera et al., 1999) and a downexpression of the anticoagulant pathways on the endothelial cells surface (Boffa and Karmochkine, 1998). However, the procoagulant curves observed with TNF-α-treated HUVECs were also probably partly induced by the well bottom which was largely uncovered in this condition. Indeed, pictures taken by contrast phase microscopy have shown that a lot of HUVECs cells were dead and floating after their 24-hour treatment with TNF-α.

HUVECs cells treated with ponatinib during 24 hours showed significantly increased peak height in thrombin generation assays with two of the three tested concentrations. When the morphology of ponatinib-treated cells was assessed, the covered surface of the well bottom did not seem to be changed relative to untreated cells. The increased peak height obtained with ponatinib-treated HUVECs cells could therefore be only induced by the impact of ponatinib on the hemostatic balance of endothelial cells. In thrombin generation assay, an increase of peak height without a decrease of lagtime could reflect an impact of ponatinib on the produced thrombin amount without any reduction of the initiation phase of coagulation. Overall, these results seem to show a slight activation of endothelial cells by ponatinib which could partly explain the increased thrombotic risk of treated patients.

Thereafter, thrombin generation assays could be performed on a monolayer of HUVECs cells pretreated with inhibitors of the activity of molecules which could induce the peak height increase observed with ponatinib-treated cells. Indeed, this method could provide information on the mechanism by which ponatinib could active endothelial cells. For example, the cells could be pretreated with Annexin V to block the PL-associated activity. The TF contribution to the procoagulant activity of ponatinib-treated cells could also be assessed by incubating cells with TF-activity neutralizing antibody before performing thrombin generation assays.

Regarding the coagulation profiles obtained with HUVECs cells treated with imatinib, it seems that this TKI did not impair the hemostatic balance of endothelial cells. This observation is consistence with the well-approved vascular safety of imatinib (Valent et al., 2015).

It could also be very interesting to perform thrombin generation assays on HUVECs cells treated with ponatinib during longer periods. Indeed, after a 24-hour treatment, the cell morphology and survival were not affected by ponatinib so that a longer treatment time could be considered. The cells could also be treated many times to assess the impact of the treatment repetition in treated patients. Before performing longer treatment periods, it could be necessary to check whether HUVECs can survive more than 24 hours without serum.

A new microscope system (Leica Phase Expert; Leica Microsystems, Leitz, Wetzlar, Germany) connected to a picture processing software can provide the percentage of the well

bottom covered by cells. This system could be systematically used before thrombin generation tests on a cell monolayer to perform the assays when a similar surface of the well bottom is covered, enhancing the reproducibility of these tests. Moreover, the exact percentage of covered surface could be taken into account during the processing of the results to correct the data obtained in a same test condition. It can also be noted that a visual check of the cell confluence before performing tests is highly important. Indeed, for the EAhy926 cells, the tests were performed with a predefined time line which strengthens the variability caused by the difference of cell number between replicates. The cell confluence could also be confirmed by the use of the microscope system cited earlier.

Thrombin generation assays were also performed on the supernatant of EAhy926 and HUVECs cells. The tendencies observed with these tests were not significant in most cases. This lack of significance could partly be explained by a lack of ponatinib impact on the hemostatic balance of the endothelial cells secretion but the high variability of results obtained could hide a potential impact. Thereafter, the hemostatic balance of EVs potentially secreted by endothelial cells treated with ponatinib could be assessed. In order to do this, the EVs fraction can be isolated by an ultracentrifugation of 90 minutes at 100 000g at 4°C. Then, the EVs can be sorted according to their size by filtration. Before performing such tests, transmission electron microscopy of ponatinib-treated cells should be used to check if these cells effectively produce EVs.

Other assumptions could explain the prothrombotic state of ponatinib-treated patients and be assessed by thrombin generation assay. Indeed, ponatinib could also impact the hemostatic balance of leukemic cells and induce a shedding of procoagulant EVs by these cells. As studied on endothelial cells, the coagulation profile of leukemic cells treated with ponatinib could be assessed by thrombin generation assay.

To further study the impact of ponatinib of endothelial cells, cytotoxic assays were performed. Indeed, ponatinib could deregulate the normal metabolism, proliferation or induce death of endothelial cells. To investigate these potential effects, LDH and MTS assays were carried out on EAhy926 and HUVECs cells. These tests have confirmed the toxic effect of TNF-α on endothelial cells (Madge and Pober, 2001). Indeed, this cytokine significantly decreased the metabolism of the two cell lines either by decreasing their proliferation either by increasing cell death. Moreover, TNF-α had a significant impact on the membrane integrity of HUVECs cells but not of EAhy926 cells. This could be due to the lower sensitivity of immortalized cells compared to primary cells. Together with the contrast phase microscopy pictures which clearly highlighted that a lot of TNF-α-treated HUVECs cells were dead, the increased LDH release observed in this condition seem to show that TNF-α induced a death of HUVECs cells. The increased membrane permeability could indicate a cell death by necroptosis which was already highlighted to be triggered by TNF cytokines in the literature (Cho et al., 2011).

After 24 hours of treatment, ponatinib did not seem to decrease proliferation or to increase death of EAhy926 and HUVECs cells. However, ponatinib significantly decreased the metabolism of EAhy926 cells after a 48-hour treatment. It could be very interesting to perform cytotoxic assays on HUVECs cells treated during 48 hours to assess if this decreased

metabolism is always observed with another cell type. The reduction of the number of metabolically active cells by ponatinib could reflect an increased cell death or a decreased cell proliferation. It is also important to note that a change in the EAhy926 cell morphology was observed when they were treated with ponatinib during 48 hours which strengthens the potential impact of this TKI after this treatment time.

Finally, imatinib did not seem to inhibit the endothelial cell proliferation and metabolic activity. The results obtained by LDH assay showed that imatinib did not significantly affect the membrane integrity which suggests that this TKI did not induce cell death. Together with the results obtained by thrombin generation assay, imatinib did not seem to activate or damage endothelial cells which confirm its well-admitted vascular safety (Valent et al., 2015).

VII. Bibliography

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