UNIVERSITÉ DU QUÉBEC À MONTRÉAL

# CIBLAGE PHARMACOLOGIQUE DU PHÉNOTYPE ANGIOGÉNIQUE ET INFLAMMATOIRE DES CELLULES ENDOTHÉLIALES CÉRÉBRALES

MÉMOIRE

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PAR

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## LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

| | |
|---|---|
| 2DG | 2-déoxy-D-glucose |
| ADN | Acide désoxyribonucléique |
| AKT | Enzyme appartenant à la famille des protéines kinases |
| AMPc | Adénosine monophosphate cyclique |
| ATP | Adenosine triphosphate |
| FGF | Facteur de croissance des fibroblastes |
| Bip | Protéine qui se lie à l'immunoglubuline |
| $Ca^{2+}$ | Ion calcium |
| CE | Cellules endothéliales |
| CHL | Acide chlorogénique |
| COX | Cyclooxygènase |
| DRO | Dérivé réactif de l'oxygène |
| G6P | Glucose-6-phosphate |
| G6PT | Transporteur du glucose-6-phosphate |
| GRP78 | Protéine controlée par le glucose |
| Glut1 | Transporteur de glucose 1 |
| HSP70 | Protéine de choc thermique |
| IkB | Inhibiteur kappa B |
| MEC | Matrice extracellulaire |
| MMP | Métalloprotéinase matricielle |
| MAPK | Protéine kinase activée par les agents mitogènes |
| mTOR | Cible de la rapamycine chez les mammifères |
| NF-kB | Facteur nucléaire-kappa B |
| NOS | Oxyde nitrique synthétase |
| OMS | Organisation Mondiale de la Santé |
| PGE2 | Prostaglandine 2 |
| PI3 kinase | Phosphatidylinositol 3-kinase |
| Ras | RAt Sarcoma |

| | |
|---|---|
| RE | Réticulum endoplasmique |
| TK | Tyrosine kinase |
| UPR | "Unfolded protein response" |
| VEGF | Facteur de croissance de l'endothélium vasculaire |
| $Zn^{2+}$ | Ion zinc |

# RÉSUMÉ

Les cellules endothéliales (CE) vasculaires cérébrales jouent un rôle important en tant que composantes structurales et fonctionnelles de la barrière hémato-encéphalique. L'angiogenèse tumorale, un processus important pour la croissance tumorale et pour le processus métastasique, régule le recrutement des CE qui, lorsqu'activées, prolifèrent et se différencient pour générer des structures capillaires afin de livrer les nutriments et l'oxygène au sein du compartiment tumoral. Les recherches rapportées dans ce mémoire nous ont permis de cibler, d'un point de vue pharmacologique, l'angiogenèse tumorale cérébrale. Dans le premier volet de cette étude, nous avons évalué l'impact anti-angiogénique *in vitro* de l'AD4-015, un inhibiteur du transporteur du glucose-6-phosphate (G6PT), sur les CE vasculaires cérébrales. Nous avons observé une inhibition de la migration cellulaire, de la tubulogenèse *in vitro* et de l'inhibition de la sécrétion et de l'expression génique de la métalloprotéinase matricielle-9 induite par le phorbol 12-myristate 13-acétate (PMA), un carcinogène promoteur de tumeur. L'expression de la cyclooxygénase-2 (cox-2) était aussi inhibée. L'inhibition pharmacologique de G6PT pourrait être une importante avenue thérapeutique ciblant la néo-vascularisation tumorale.

Dans le deuxième volet de notre étude, nous avons investigué les effets du 2-déoxy-D-glucose (2DG), un analogue du glucose ayant comme rôle d'inhiber la glycolyse par la déplétion d'ATP, sur les propriétés angiogéniques des CE vasculaires cérébrales. Nous avons observé une diminution de la tubulogenèse conséquente à une diminution du niveau d'ATP. L'induction de l'expression de cox-2 et de GRP78 a été potentialisée par la combinaison du 2DG avec le PMA. Le niveau de la sécrétion de protéine et de l'expression génique de MMP-9 a été diminué en présence du 2DG, un phénomène impliquant la signalisation par le facteur nucléaire kappa-B. Nous fournissons donc des preuves que l'ATP est nécessaire au processus de la tubulogenèse et nous établissons un lien entre le stress du réticulum endoplasmique et l'inflammation.

Dans le troisième volet de notre étude, nous avons investigué les propriétés inhibitrices de plusieurs flavonoïdes et la voie de signalisation du facteur nucléaire kappa-B, qui est une voie commune pour la régulation de l'expression de cox-2 et de MMP-9. Nous avons observé une diminution de l'expression génique et des niveaux de protéines de cox-2 et de MMP-9 par la fisetine, l'apigénine et la lutéoline. Il a également été démontré qu'il y a inhibition des niveaux de phosphorylation d'IkB prouvant leur impact sur l'activité de phosphorylation des IkB kinases. Nos résultats démontrent donc que la perturbation de la barrière hémato-encéphalique pourrait être minimisée par certains flavonoïdes qui agissent comme des inhibiteurs de la voie de signalisation de NF-κB.

Collectivement, nos résultats démontrent les effets de différents inhibiteurs sur la tubulogenèse médiée par les CE vasculaires cérébrales. Dans le futur, ceci permettra le ciblage pharmacologique de l'angiogenèse tumorale cérébrale avec l'utilisation de molécules inhibitrices comme l'analogue de la mumbaistatine, le 2DG et certains flavonoïdes.



Mots clés : cellules endothéliales vasculaires cérébrales, angiogenèse, cox-2, MMP-9.

# CHAPITRE I

# INTRODUCTION

## 1.1 Le Cancer

### 1.1.1. Quelques statistiques

En 2008, l'Organisation Mondiale de la Santé (OMS) a enregistré 12.7 millions de nouveaux cas de cancer et 7.6 millions de décès dans le monde. Plus de la moitié des nouveaux cas et des décès s'est répertoriée dans des pays en voie de développement (Globocan, 2008). La Société Canadienne du Cancer a estimé qu'en 2010 il y aurait eu 173,800 nouveaux cas de cancer et 76,200 décès causés par le cancer au Canada. Une augmentation de 1.6% pour les nouveaux cas et de 1.2% pour les décès comparé à l'année 2009. Parmi les cancers les plus répandus, le cancer de la prostate est le cancer le plus commun chez les hommes et le cancer du sein chez les femmes. Le cancer du poumon et le cancer colorectal sont les deuxièmes cancers les plus communs chez les êtres humains. Le cancer du poumon, du colon, du sein et de la prostate représenteraient plus de la moitié des cancers diagnostiqués au Canada en 2010. Le cancer du poumon était celui qui causait le plus de décès, le cancer du sein venait en second et le cancer de la prostate était classé au troisième rang (Société Canadienne du cancer, 2010). Selon l'OMS, la première cause de mortalité dans le monde est le cancer, estimant qu'il y aura 84 millions de morts entre les années 2005 et 2015 s'il n'y a pas d'importantes mesures prises pour combattre cette maladie. En 2010, le nombre de décès causé par le cancer du cerveau était estimé à 1,750 décès au Canada et 13,140 décès aux États-Unis. En effet selon l'Agence de la santé publique du Canada, le cancer du cerveau chez les enfants représentait 20% des nouveaux cas de cancer en 2004. La recherche sur le cancer, et particulièrement sur le cancer du cerveau, est donc très importante pour avoir une meilleure compréhension, un diagnostic accéléré et un développement de thérapies plus efficaces pour contrer cette maladie.

1.1.2. Processus de cancérogenèse

Les cellules retrouvées dans le corps humain se divisent et se développent d'une manière coordonnée pour la production d'autres cellules nécessaires au bon fonctionnement du corps. Ce processus subit des changements lorsqu'il y a des dommages au niveau de l'ADN des cellules, conduisant ainsi à des mutations. Ces mutations ont un impact sur le développement et la division de cellules normales, causant la formation non voulue de nouvelles cellules et la survie des cellules qui devraient aller en apoptose (Griffiths, 2002). Le cancer se définit, par ailleurs, comme étant la croissance anormale de cellules causée par des changements dans l'expression génique, menant à la perte de l'équilibre de la prolifération cellulaire et de la mort cellulaire, évoluant ainsi vers une population de cellules capables d'envahir les tissus (Ruddon, 1995).

Pour qu'il y ait transformation de ces cellules en tissu cancéreux, Hanahan et Weinberg ont suggéré six différentes altérations au niveau de la physiologie des cellules saines. Il doit y avoir invasion de tissus et métastase, angiogenèse soutenue, résistance à l'apoptose, une capacité proliférative illimitée, une insensibilité aux signaux inhibiteurs et une indépendance vis-à-vis des signaux stimulant la prolifération (figure 1).

La cancérogenèse est le processus par lequel une cellule normale se transforme en une cellule cancéreuse, par le biais de changements au niveau génétique et cellulaire. La production d'une tumeur cancéreuse nécessite plusieurs étapes dont l'initiation, la promotion et la progression (Hennings et *al.*, 1993). L'étape d'initiation se définit comme la conversion d'une cellule normale en cellule initiée ou précancéreuse. Il y a altération du génome induit par des agents cancérigènes causant des mutations géniques stables, des changements épigénétiques et l'immortalisation des cellules. Ensuite, plusieurs facteurs promoteurs, tels des cytokines et des facteurs de croissances, permettent aux cellules de subir une expansion clonale, ceci est l'étape de promotion. L'étape de la progression consiste à la transformation de ces cellules en cellules malignes, qui sont les cellules cancéreuses qui se propagent dans le corps par invasion (Trosko et Ruch, 1998) (figure 2). Le processus métastastasique est le phénomène par laquelle les cellules tumorales envahissent d'autres tissus et compartiments.

La diffusion des cellules cancéreuses pourrait diminuer les chances de survie des patients. Ces cellules se propagent par les vaisseaux sanguins ou par le système lymphatique pour atteindre d'autres organes (Nakajima et *al.*, 1989). Un des événements crucial du développement tumoral et du processus métastastasique est le phénomène appelé angiogenèse (Folkman, 2002).

### 1.1.3. Angiogenèse tumorale

L'angiogenèse est requise pour le développement tumoral et pour le processus métastasique (Folkman, 2002). L'angiogenèse se définit comme la néovascularisation ou la formation de nouveaux vaisseaux sanguins à partir de vaisseaux préexistants. L'angiogenèse est active dans de nombreux processus normaux mais particulièrement dans la pathologie de la progression tumorale (Folkman et Shing, 1992). Elle est régulée très précisément par une balance dynamique entre les facteurs positifs et négatifs de l'angiogenèse. Lorsque la tumeur devient hypoxique, elle synthétise des protéines pro-angiogéniques comme le facteur de croissance de l'endothélium vasculaire (VEGF) et le facteur de croissance des fibroblastes (FGF). Ces facteurs de croissance sont sécrétés par la tumeur dans le milieu environnant. Lorsqu'ils viennent en contact avec des cellules endothéliales (CE), ceux-ci s'associent à des récepteurs à activité tyrosine kinase (TK) retrouvés à la surface des cellules activant ainsi une cascade de signalisation (Ferrara et Davis-Smyth, 1997). Les CE actives produisent des métalloprotéases matricielles (MMPs), des enzymes qui dégradent les protéines de la matrice extracellulaire (MEC) permettant ainsi la migration des CE. Ceci permettrait aux stimuli angiogéniques de recruter les CE qui vont ensuite proliférer et se différencier pour former un réseau de structures capillaires (Hillen et Griffioen, 2007) (figure 3).

Par manque d'oxygène et de nutriments, la tumeur ne peut pas dépasser une taille plus élevée que 1-2 $mm^3$, l'angiogenèse joue donc un rôle très important pour la croissance de cette microtumeur. Comme il est bien connu que la néovascularisation est importante pour le processus tumoral, l'inhibition de ses voies de signalisation pourrait mener à de nouvelles approches thérapeutiques prometteuses qui permettraient une inhibition efficace et significative de l'angiogenèse tumorale et de la prolifération des cellules tumorales (Hillen et

Griffioen, 2007). En effet, plusieurs inhibiteurs de l'angiogenèse sont actuellement testés. Il existe des molécules qui interviennent dans la cascade de signalisation de l'angiogenèse, comme des molécules anti-VEGF et d'autres molécules inhibant l'activation des MMPs, bloquant ainsi la migration des CE dans d'autres tissus (Folkman J., 1971). Des études précliniques ont démontré que la combinaison d'agents anti-angiogéniques avec des agents cytotoxiques ou avec des radiothérapies résulte en des effets anti-tumoraux additifs et synergiques. Ainsi la co-administration d'agents anti-angiogéniques à certains agents chimiothérapeutiques naturels permettrait possiblement d'avoir une meilleure action anti-tumorale (Ferrara et Kerbel, 2005).

### 1.1.4. Les métalloprotéases matricielles dans l'angiogenèse et la métastasie

La MEC est composée d'un grand nombre de macromolécules telles les glycoprotéines, les protéoglycans et les glycosaminoglycans. Elle est impliquée dans plusieurs processus cellulaires, incluant la morphogenèse, la prolifération, l'attachement au substrat et la motilité des cellules (Miyakazi et *al.*, 1990). La dégradation de la MEC est également une étape clée dans les processus tels que la cicatrisation, l'invasion métastatique, etc. (Irwin et *al.*, 1996). Il existe plusieurs protéases capables de dégrader les protéines de la MEC, elles sont classées en quatre grandes familles : les cystéine-protéases, les aspartyl-protéases, les sérines protéases et les métalloprotéases (MMP). Ces dernières sont les plus diverses, et sont considérées comme les protagonistes physiologiques de la dégradation matricielle (Miyakazi et *al.*, 1990). Les MMP sont composées de trois domaines communs, le domaine pro-peptide, le domaine catalytique et le domaine C-terminal (figure 4). Les MMP sont synthétisées sous une forme zymogène inactif avec un domaine propeptide auto-inhibiteur. Le domaine propeptide protège le domaine catalytique contenant le zinc ($Zn^{2+}$). Pour que les MMP soient actives, il faut que le domaine pro-peptide soit clivé. Ce domaine contient un résidu cystéine interagissant avec le $Zn^{2+}$ dans le site actif, empêchant la liaison et le clivage du substrat, conservant ainsi l'enzyme sous sa forme inactive. Le domaine C-terminal confère la spécificité au substrat (Kandasamy et *al.*, 2009 ; Amalinei et *al.*, 2007) (Figure 4). La MMP-9, ou gélatinase B, est une protéase de 92 kDa fortement glycosylée, sécrétée dans le milieu extracellulaire par un grand nombre de types cellulaires. Cette enzyme a la capacité

d'hydrolyser plus d'une composante de la MEC, telle la gélatine (collagène dénaturé), le collagène (type I, IV, V, VII et X), l'élastine et des glycoprotéines (laminine et fibronectine). La MMP-2, quant à elle, est sécrétée d'une manière constitutive sous la forme de pro-enzyme par différents types cellulaires (Nagase et Woessner, 1999).

L'activité des MMP peut être modulée par une variété de stimuli incluant les hormones, les facteurs de croissances, les esters de phorbols et d'autres agents affectant la structure des cellules. Ces agents affectent l'activité des protéinases en augmentant ou diminuant le niveau de transcription des gènes des protéinases, la stabilité de l'ARNm, la sécrétion de protéines et la synthèse d'inhibiteurs endogènes. Une dégradation non controlée de la MEC est impliquée dans l'invasion tumorale et dans le processus métastasique (Apodaca et *al.*, 1990 ; Edvardsen et *al.*, 1993). La dégradation de la MEC permet notamment la migration des CE, qui se divisent et s'organisent en tubes, évoluant progressivement en un réseau de vaisseaux sanguins (Miyazaki et *al.*, 1990). Bien que plusieurs essais cliniques fussent déjà, la progression d'une tumeur est souvent associée à la surexpression des MMP, donc leur inhibition pourrait devenir une cible thérapeutique anticancéreuse intéressante.

## 1.2. Inflammation

### 1.2.1. L'inflammation associée à la progression tumorale

Un des événements essentiels du développement d'un cancer est l'apparition de lésions génétiques dans les cellules. Ces lésions jouent un role important dans l'initiation de la cancérogenèse mais peuvent également être impliquées dans les étapes de promotion et de progression du développement des tumeurs. Ces événements, incluant l'inactivation de gènes suppresseurs de tumeurs ou l'activation de proto-oncogènes, sont nécessaires pour la carcinogenèse mais ne sont pas suffisants. En effet, le développement tumoral et l'acquisition du phénotype malin résultent de l'implication combinée des cellules cancéreuses et non cancéreuses (Rakoff-Nahoum, 2006).

Il existe un lien entre l'inflammation et le développement d'un cancer : en effet, des études épidémiologiques ont démontré que 15% des cas de cancer dans le monde sont associés à des infections microbiennes. Lors d'une inflammation chronique, il y a présence de cytokines et chimiokines inflammatoires, une surproduction des dérivés réactifs de l'oxygène (DRO) et une stimulation de plusieurs voies de signalisations (Rakoff-Nahoum, 2006). Il y a plusieurs preuves de lien direct entre la cancérogenèse et la réparation de tissus endommagés. Les molécules telles la cyclooxygénase (cox)-1 et -2, qui sont impliquées dans la synthèse de prostaglandines médiant le processus de réparation de tissus (Houchen, Stenson et Cohn, 2000; Morteau et *al.*, 2000), jouent également un rôle important dans le développement de tumeurs (Oshima et al., 1996; Chulada et *al.*, 2000). L'activité des facteurs nucléaire-kappa B (NF-κB) est déclenchée en réponse à des agents infectieux et des cytokines pro-inflammatoires. Ils jouent un rôle important dans la promotion du cancer lors de l'inflammation et sont impliqués dans la carcinogenèse (Karin, 2006; Florian et *al.*, 2004). Glucose-regulated protein (GRP78) joue un rôle important dans la régulation de la signalisation du stress du réticulum endoplasmique (RE) et son expression est induite dans les tumeurs (Lee, 2007).

### 1.2.2. Implication de la voie de signalisation NF-κB dans l'inflammation

NF-κB est un facteur de transcription dimérique composé d'homodimères et d'hétérodimères appartenant à la famille de protéines Rel qui est composée de cinq sous-unités : RelA (p65), c-Rel, RelB, NF-κB1 (p50) et NF-κB2 (p52). Les dimères NF-κB se trouvent dans le cytoplasme sous une forme inactive lorsqu'associés à l'inhibiteur kappa B (IκB) (Elewaut et *al.*, 1999). Il y a sept différents IκB qui s'associent préférentiellement à une variété de protéines dimères de la famille des Rel, ces IκB sont : IκBα, IκBβ, IκBε, IκBγ, Bcl-3, p-100 et p-105. Les IκBs sont une famille de protéines contenant une répétition d'ankyrine qui interagit avec le domaine RDH des protéines NF-κB. IκBα, IκBβ, IκBγ et IκBε interagissent avec le dimère NF-κB et sont responsables de leur rétention dans le cytoplasme, tandis que Bcl-3 peut agir comme un co-activateur transcriptionnel lorsqu'il interagit avec les homodimères p50 et p52 (Dolcet et *al.*, 2005).

La voie de NF-κB est activée par différents stimuli comme des cytokines, des facteurs de croissances, des esters de phorbols et des signaux de stress (Dolcet et al., 2005). L'expression à la hausse des membres de familles du récepteur des facteurs de croissances de l'épiderme, du récepteur des facteurs de croissance de l'insuline et du récepteur du facteur de nécrose tumorale peuvent être responsables de l'activation de NF-κB. L'activation des voies de signalisation des protéines kinases activées par les agents mitogènes (Ras/MAPK) et des protéines kinases B (Akt) sont aussi impliquées dans l'activation de NF-κB.

Pour que NF-κB soit activé, il doit être relâché de son inhibiteur IκB et ensuite migrer du cytoplasme au noyau. Les hétérodimères p50 et p65 sont les sous-unités prédominantes qui migrent dans le noyau et qui s'associent avec les régions promotrices de plusieurs gènes. La régulation de l'expression de ces gènes est contrôlée par les protéines inhibitrices d'IκB, dont l'IκBα, qui est rapidement phosphorylé et dégradé lorsque stimulé, via la voie de l'ubiquitine protéasome (Huang et *al.*, 2001) (figure 5).

Les facteurs de transcription NF-κB sont des régulateurs clés de la réponse immunitaire et inflammatoire et sont aussi impliqués dans le contrôle de la prolifération cellulaire et de l'apoptose. L'amplification chromosomale, la surexpression et le réarrangement des gènes codant pour NF-κB ont été observés dans les tumeurs cancéreuses. L'activation constitutive des voies de signalisation impliquant des kinases ou des mutations causant l'inactivation de l'inhibiteur IκB permettent à NF-κB d'avoir une activité persistante. Le NF-κB est aussi impliqué dans le contrôle de la réponse à l'apoptose supportant ainsi son rôle dans l'oncogenèse et la résistance des cellules tumorales à la chimiothérapie. Il a aussi été démontré que NF-κB peut activer l'expression de gènes importants dans l'invasion et dans le processus métastasique, comme par exemple les facteurs de croissance de l'endothélium vasculaire et les enzymes protéolytiques comme les MMPs (Rayet et Gélinas, 1999).

### 1.2.3. Induction de la cyclooxygénase-2 dans l'angiogenèse tumorale

Les prostaglandines, telle PGE2, sont produits particulièrement à partir de l'acide arachidonique (Herschman et *al.*, 1994). Elles sont impliquées dans la différenciation, la

croissance et l'adhésion cellulaire. PGE2 joue un rôle important dans la réparation de blessures et dans le renouvellement des cellules normales et néoplasiques qui survient au niveau de l'épithélium gastro-intestinal. La synthèse de PGE2 est catalysée par deux isoformes, cox-1 et cox-2, possédant des rôles biologiques différents (Houchen et *al.*, 2000).

Cox-1, une enzyme constitutive, se retrouve dans plusieurs tissus et cellules, alors que cox-2, une enzyme inductible, est mesurable dans des cellules en réponse à des facteurs de croissances, des mitogènes et des cytokines pro-inflammatoires. Cox-1 a un rôle permettant le maintien des fonctions physiologiques comme la cytoprotection (Shattuck-Brandt et *al.*, 1998). Cox-2 n'est pas présent dans des conditions physiologiques normales mais est régulée à la hausse suite à une stimulation pro-inflammatoire (Seibert et *al.*, 1994). Plusieurs études ont démontré que cox-2 était surexprimé dans le cancer du colon, de la prostate, du sein et du pancréas. L'expression de cox-2 est généralement à la hausse dans les tumeurs différenciées et lors du processus métastasique. La transcription de cox-2 est stimulée par des facteurs de croissances, des cytokines, des oncogènes et des promoteurs de tumeurs via la protéine kinase-C et la signalisation médiée par RAS (Singer et *al.*, 1998).

Des études *in vitro* ont démontré que cox-2 était possiblement impliquée dans l'invasion tumorale, en induisant la dégradation de la MEC par des MMP. Cox-2 est induite par plusieurs facteurs pro-angiogéniques comme le VEGF, le nitric oxyde synthase (NOS), l'intérleukine 6 et 8, et elle produit la PGE2 qui a des effets autocrines et paracrines au niveau de la prolifération et de la migration *in vitro* des cellules endothéliales (Wu et *al.*, 2006). Il a été démontré que cox-2 est surexprimée dans les tumeurs. Cox-2 a aussi été associé à la vascularisation tumorale et au VEGF dans le cancer du cou et de la tête. Plusieurs modèles *in vivo* ont démontré que PGE2 produit par cox-2 stimule l'angiogenèse, et que l'inhibition de cox-2 ralentit la néovascularisation (Gately et Li, 2004).

### 1.2.4. Contribution de GRP78 au niveau du stress du réticulum endoplasmique

Le réticulum endoplasmique (RE) est une organelle cellulaire où les protéines membranaires sont synthétisées et modifiées. Il est aussi un important compartiment pour le stockage du

calcium intracellulaire (Lee, 2001). Des études soulignent que le RE est aussi important pour la régulation de l'apoptose. Les mécanismes "Unfolded protein response" (UPR) déclenchent plusieurs voies de signalisations permettant aux cellules de répondre au stress du RE (Ma et Hendershot, 2004). Les cellules cancéreuses démontrent un métabolisme de glucose élevé en conséquence à leur exposition à l'hypoxie, causant le stress du RE. Le GRP78/BiP, une protéine chaperonne du RE, possède des propriétés anti-apoptotiques et est un régulateur central de l'homéostasie du RE (Lee, 2007).

BiP (immunoglobin heavy chain binding protein) est aussi connu sous l'appellation glucose-regulated protein (GRP78) (Wang et *al.*, 2009). GRP78, qui se trouve principalement dans le RE, appartient à la famille des protéines de choc thermique (HSP), qui joue un rôle important au niveau du stress de l'oncogenèse. GRP78 a plusieurs rôles, il prévient l'agrégation des intermédiaires protéiques, facilite le repliement des protéines, cible les protéines immatures pour leur dégradation par le protéasome, il s'associe au $Ca^{2+}$ et sert de régulateur pour la signalisation du stress du RE (Lee, 2001 ; Hendershot, 2004). Le stress du RE induit GRP78 reflétant la perturbation de la fonction du RE et de l'homéostasie et protège contre les lésions au niveau des tissus et organes sous des conditions comme le stress neurotoxique, l'infarctus du myocarde et l'artériosclérose (Lee, 2001). L'expression de GRP78 est maintenue à un niveau basal dans plusieurs organes mais il est induit fortement dans les tumeurs (Li et *al.*, 2006 ; Dong et *al.*, 2004). Il a aussi été démontré que le niveau de GRP78 est élevé dans les cellules cancéreuses métastasiques et que sa présence est détectée dans le cancer métastasique de la prostate et pourrait médier des voies de signalisation induisant l'invasion et la prolifération (Fu et Lee, 2006) L'induction de GRP78 se produit durant le développement embryonnaire et est aussi corrélée au développement tumoral. Dans les cellules cancéreuses, GRP78 promouvoit la survie et la chimiorésistance dans les cellules tumorales dormantes et prolifératives (Lee, 2007).

## 1.3. Ciblage pharmacologique de l'angiogenèse tumorale cérébrale

### 1.3.1. Ciblage angiogénique de la mumbaistatine et son implication sur le système glucose-6-phosphatase

La glucose-6-phosphate translocase (G6PT) a comme rôle principal de transporter le glucose-6-phosphate (G6P) du cytosol vers le RE, permettant l'hydrolyse du G6P en glucose et en phosphate inorganique (Van Schaftingen et Gerin, 2002) (Figure 6a). G6PT fait partie du système G6Pase composé d'un transporteur de G6P, le G6PT ou T1, d'une unité catalytique, la G6Pase, et de deux translocases, T2 et T3, responsables respectivement de l'exportation du phosphate inorganique et du glucose vers la circulation sanguine (Chen et *al.*, 2008). G6PT régule aussi plusieurs fonctions métaboliques telles la glycémie, la lipidémie, l'uricémie et l'acidémie lactique. La déficience en G6PT cause la glycogénose de type 1b, qui est caractérisée non seulement par un débalancement de l'homéostasie glucidique mais aussi par une dysfonction sévère des cellules myéloïdes (Chou et *al.*, 2002). G6PT a aussi un rôle dans la promotion de la survie cellulaire et dans l'adaptation métabolique à l'hypoxie. Il joue aussi un rôle dans le contrôle du flux calcique, dans la chimiotaxie des neutrophiles et des cellules de la moëlle osseuse (Kim et *al.*, 2006 ; Yiu et *al.*, 2007 ; Fortier et *al.*, 2008). L'expression génique de G6PT est régulée en réponse à des changements métaboliques impliquant l'adénosine monophosphate cyclique (AMPc), le glucose et l'insuline (Van de Werve et *al.*, 2000). Cette adaptation métabolique médiée par le G6PT, qui permettrait la survie des cellules dans un environnement hypoxique, pourrait devenir une cible thérapeutique permettant d'altérer les nouvelles fonctions attribuées à G6PT comme la survie cellulaire, la séquestration du calcium, la migration cellulaire et la prolifération cellulaire (figure 6b).

La mumbaistatine est une anthraquinone aromatique polycétide isolée de streptomyces DSM 11641(Kaiser et *al.*, 2003). Elle est un des plus puissants inhibiteurs de G6PT (Vertesy et *al.*, 2001). La disponibilité de cette molécule est cependant limitée du au faible rendement d'extraction et de la rareté de la source mère. Une méthode semi-synthétique en 5 étapes a donc été développée pour générer une variété d'analogues de la mumbaistatine avec des hauts

rendements, pour une plus grande disponibilité (Kaiser et *al.*, 2003). Il a été démontré par notre équipe que l'AD4-015, qui est un des analogues de la mumbaistatine, réduit la survie des cellules adaptées à l'hypoxie (Lord-Dufour et *al.*, 2009). L'effet de l'acide chlorogénique (CHL), qui est aussi un inhibiteur du G6PT, était moindre en comparaison à celui de la mumbaistatine (Hemmerle et *al.*, 1997). Il est important de noter que G6PT peut être ciblé spécifiquement au sein de tumeurs. Il n'y a pas beaucoup d'études consacrées sur le lien entre l'angiogenèse et G6PT, ceci pourrait donc être un aspect intéressant pour la recherche.

### 1.3.2. Ciblage métabolique par le 2-déoxy-D-glucose

Afin de combler leur besoin métabolique, les cellules tumorales dépendent en partie du processus de la glycolyse. Les cellules malignes cultivées ont démontré une augmentation du transport du glucose dans la cellule. Ceci est médié par l'activation d'un des transporteurs du glucose, le Glut1 (Yamamoto et *al.*, 1990). Donc, la consommation élevée de glucose peut représenter un point important de régulation dans le maintien de la croissance et de l'activité des cellules malignes et de la suppression de l'apoptose. Des études *in vitro* et *in vivo* ont démontré que les analogues de glucose inhibent le métabolisme glucidique dans les cellules cancéreuses (Ball et *al.*, 1957 ; Laszlo et *al.*, 1958 ; Kaplan et *al.*, 1990). Des études ont démontré que le 2-déoxy-D-glucose (2DG) inhibe la production d'adénosine triphosphate (ATP) (Laszlo et *al.*, 1958 ; Jain et *al.*, 1985). Le 2DG est un analogue du glucose qui diffère au niveau du deuxième carbone par la substitution de l'hydrogène avec un groupement hydroxyle (Aft et *al.*, 2002). Les cellules tumorales qui croissent en absence d'oxygène sont dépendantes en premier lieu de la glycolyse pour la production d'ATP. Lorsqu'il y a utilisation d'inhibiteur glycolytique, comme le 2DG, il n'y a plus de synthèse d'ATP. Cependant, dans le cas où il y a présence d'oxygène, la cellule peut utiliser des sources alternatives d'énergie pour la production d'ATP (Maschek et *al.*, 2004). L'utilisation d'inhibiteur de glucose permet d'inhiber la prolifération tumorale, donc la combinaison de ces inhibiteurs avec des médicaments chimiothérapiques, qui ciblent les cellules en état de division, pourrait augmenter l'efficacité des traitements anticancéreux.

En effet, dans des études cliniques de phase I, l'administration orale de 2-DG en combinaison au taxotère n'a causé aucun effet secondaire sérieux (Merchan et *al.*, 2010). Stein et al. ont rapporté que l'administration du 2-DG chez les patients atteints du cancer de la prostate avancé donnait des résultats concluant (Merchan et *al.*, 2010). Donc, l'effet du 2DG qui, lorsque phosphorylé par l'hexokinase en 2DG-6-phosphate conduit au blocage de la glycolyse et à la déplétion de l'ATP intracellulaire, pourrait être une molécule importante à analyser pour ses propriétés anti-angiogéniques.

### 1.3.3. Effet anti-inflammatoire par les flavonoïdes

Les flavonoïdes sont des substances naturelles retrouvées dans les fruits, les végétaux, les racines, les fleurs, le thé et le vin. Plus de 4000 variétés de flavonoïdes ont été identifiées, dont plusieurs sont responsables de la coloration retrouvée sur les fleurs, les fruits et les feuilles. (Nijveldt, Van Nood et *al.*, 2001) (figure 7). Les flavonoïdes ont des propriétés antibactériennes, anti-oxydantes, antivirales, antiallergènes, hépatoprotectives, immunomodulatrices et anti-inflammatoires (Medic-Saric et *al.*, 2009). Chez la population méditerranéenne, le faible taux de mortalité par maladie cardiovasculaire est associé à la consommation de vin rouge et à la consommation d'acides gras insaturés. Les flavonoïdes retrouvés dans le vin rouge sont en partie responsables de cet effet. D'autres études épidémiologiques suggèrent un rôle protecteur contre l'insuffisance coronarienne (Nijveldt, Van Nood et al., 2001).

Les flavonoïdes peuvent être divisés en différentes classes selon leur structure moléculaire. Les flavones sont caractérisées par leur structure planaire et par la présence d'une liaison double dans le cycle aromatique, comme c'est le cas pour l'apigenine et la lutéoline. La fisetine est présente chez plusieurs plantes et fruits, tels les pommes, les fraises et les oignons (Kawai et *al.*, 2007). L'arbutine est un des agents d'hypo-pigmentation le plus utilisé dans l'industrie de la cosmétique (Sapkota et *al.*, 2010). La salicine, un autre flavonoïde, a des effets anti-inflammatoires et des études ont démontré qu'elle a des propriétés anti-leucémiques (El-Shemy et *al.*, 2003). La phlorizine appartient au groupe dihydrochalcone, un type de flavonoïde retrouvé dans plusieurs fruits, elle a été utilisée comme agent

pharmaceutique (Ehrenkranz JR. et *al.*, 2005). La coniferine et la quercitrine appartiennent aussi à la famille des flavonoïdes. Certains flavonoïdes exercent des activités anticancéreuses, d'autres possèdent des activités inhibitrices sur une variété de systèmes enzymatiques incluant la protéine kinase C, les protéines à activité tyrosine kinase et d'autres (Middleton 1998).

## 1.4. La mort cellulaire

### 1.4.1. L'apoptose

Il existe plusieurs processus pour l'élimination des cellules endommagées. Ces processus régulent la mort cellulaire par l'apoptose, la nécrose et l'autophagie (figure 8). L'apoptose, aussi connue sous le nom de "suicide cellulaire", est une cascade d'événements résultant en la mort cellulaire. Durant l'apoptose toute la cellule, incluant le noyau, se sépare en différents fragments, les corps apoptotiques. Ceux-ci contiennent toutefois des organites fonctionnels puisque la cellule continue de produire des protéines et de l'ATP (Nanji et Hiller-Sturnmhofel, 1997) (figure 9). L'apoptose joue un rôle important dans le maintien et le développement tissulaire en éliminant les cellules non nécessaires et malsaines. Les caractéristiques cytologiques de l'apoptose sont la condensation et la fragmentation de la chromatine, la préservation de la membrane, le rétrécissement de la cellule et la formation de corps apoptotiques contenant des matériaux nucléaires ou cytoplasmiques. Trop ou pas assez d'apoptose pourrait avoir des conséquences pathologiques, menant à des maladies auto-immunes, neuro-dégénératives, à des défauts de développement, et même le cancer (Lowe et Lin, 2000). Plusieurs études ont démontré que l'apoptose des cellules endothéliales pourrait jouer un rôle important dans la néovascularisation. Des facteurs de croissances, qui sont essentiels pour l'angiogenèse, stimulent la prolifération et la migration des cellules endothéliales mais inhibent l'apoptose des ces cellules. En effet, le VEGF et le bFGF peuvent potentiellement bloquer l'exécution de l'apoptose des cellules endothéliales (Dimmeler and Zeiher 2000). L'apoptose est importante dans l'élimination des cellules anormales au stade précoce de la cancérogenèse, elle est aussi une réponse cellulaire commune au stress, dont la plupart des événements promouvant la cancérogénèse créée ou amplifie le stress cellulaire.

Des changements dans l'apoptose se produisent durant la cancérogénèse promouvant ainsi la croissance tumorale et la résistance aux traitements (Jin et White, 2007). L'inhibition de l'angiogenèse limite la croissance tumorale en augmentant les événements apoptotiques. Plusieurs études cliniques ont démontré que l'angiogenèse et des facteurs impliqués dans l'apoptose sont des indicateurs du cancer et peuvent servir de cible pour des traitements anticancéreux. Dans l'ensemble, les études suggèrent que l'inhibition progressive de l'apoptose et l'induction de l'angiogenèse contribue à l'initiation, la croissance et à la formation de métastases. Ces marqueurs tumoraux ont le potentiel d'être ciblés à l'intérieur de stratégies thérapeutiques visant la survie cellulaire et la néovascularisation tumorale par l'induction de l'apoptose ou l'inhibition de l'angiogenèse (WU, 1996).

### 1.4.2. L'autophagie

L'autophagie est un processus catabolique permettant aux cellules de dégrader leurs propres organelles intracellulaires. L'autophagie est importante dans le développement des maladies dégénératives non controlées, incluant les infections virales et bactériennes, les désordres neurodégénératifs et cardiovasculaire. (Shintani et Klionsky, 2004). C'est un processus contrôlé génétiquement qui résulte en la formation d'une vésicule composée d'une double membrane, qui encapsule du cytoplasme et des organelles et qui fusionne avec des lysosomes, résultant ainsi en la dégradation des composants retrouvés à l'intérieur de la vésicule (Hippert M. M., et al, 2006) (Figure 10). Ceci pourrait permettre la régulation du renouvellement normal des organelles et l'élimination de celles ayant des fonctions compromises.

Lorsqu'une cellule est privée de nutriments, le mécanisme d'autophagie est augmenté permettant la dégradation de protéines et d'organelles dans le but d'obtenir une source d'énergie alternative. Donc le processus d'autophagie aurait un rôle protecteur permettant aux cellules de survivre lorsque privées de nutriments (Yorimitsu et Klionsky, 2005). Le contrôle de l'autophagie se fait par mTOR (cible de rapamycine chez les mammifères) qui se trouve sous la voie PI-3 kinase/AKT et qui régule la croissance cellulaire et la synthèse de protéines en réponse à la disponibilité des nutriments et des facteurs de croissances. L'autophagie est

donc inactive lorsque la réserve en nutriments est suffisante pour supporter le métabolisme cellulaire (Dengenhradt et *al.*, 2006). Des études ont démontré que lorsque les cellules tumorales étaient privées de facteurs de croissance et de facteurs de survie, cela menait à une augmentation de l'autophagie empêchant la mort des cellules. Dans une tumeur, l'autophagie garde les cellules tumorales en vie lorsque privées de nutriments et lorsqu'il y a hypoxie causée par la manque d'angiogenèse, promouvant ainsi la croissance des tumeurs lors d'une augmentation en autophagie. À l'inverse, une diminution de l'autophagie permettrait de limiter la croissance tumorale (Hippert et *al.*, 2006). L'inhibition de l'autophagie pourrait limiter la progression d'une tumeur et augmenter l'efficacité des traitements.

### 1.4.3. La nécrose

La mort des cellules par nécrose est caractérisée par la perte de fonctions métaboliques et de l'intégrité de la membrane cellulaire. Les cellules nécrotiques arrêtent leur production de protéines et d'ATP. En effet, la mort cellulaire par nécrose est associée à la perte de la régulation osmotique, à la déplétion en ATP, à la lyse cellulaire et à une réponse inflammatoire qui faciliterait la guérison d'une blessure (Dengenhradt et *al.*, 2006). D'un point de vue structural, les organelles des cellules deviennent enflées et deviennent non fonctionnelles durant les premiers stades de la nécrose. De plus, la membrane des cellules forme des projections en forme de bulles. Ces bulles, ne contenant pas d'organelles, fusionnent et augmentent en taille. Finalement, la membrane cellulaire se déchire résultant en le relâchement des composantes de la cellule dans le milieu environnant. Ce processus de dissolution est appelé cytolyse. Les composés relâchés induisent alors une réponse inflammatoire dans les tissus affectés (Nanji et Hiller-Sturmhofel, 1997).

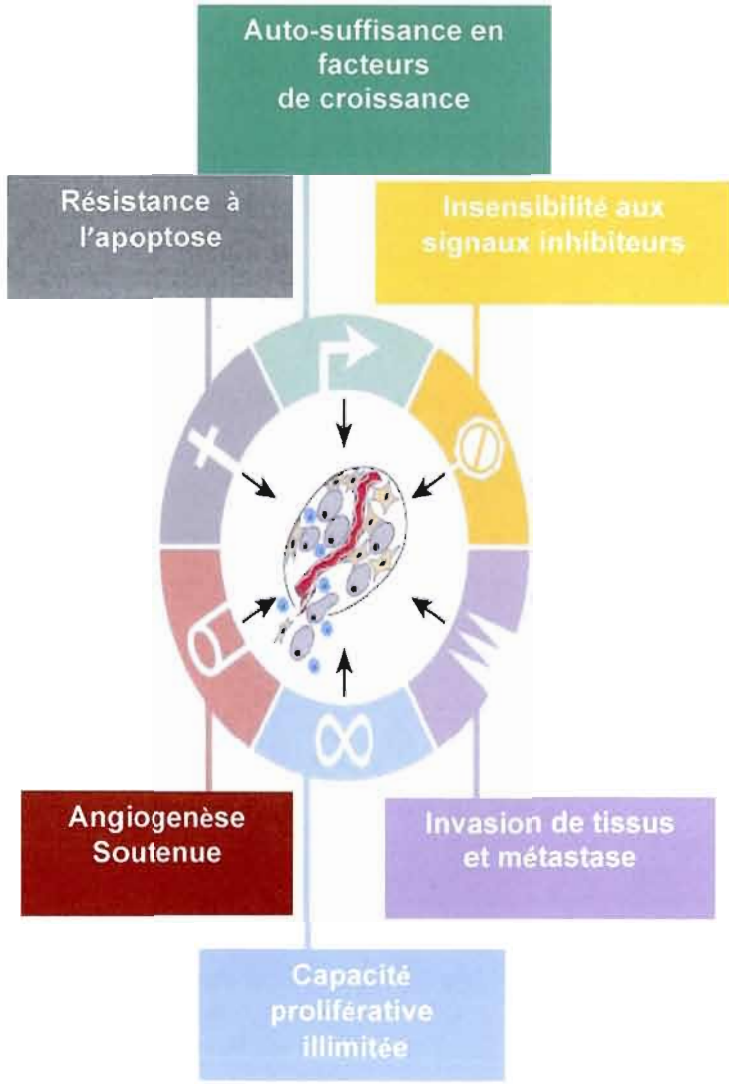



**Figure 1.1 : Les six propriétés acquises par une cellule pour sa transformation tumorale.** Selon Hanahan et Weinberg 2000, il y a six différentes altérations au niveau de la physiologie des cellules saines pour leur transformation en cellules cancéreuses: invasion de tissus et métastase, angiogenèse soutenue, inhibition de l'apoptose, capacité proliférative illimitée, insensibilité aux signaux inhibiteurs et indépendance vis-à-vis des signaux stimulant la prolifération.

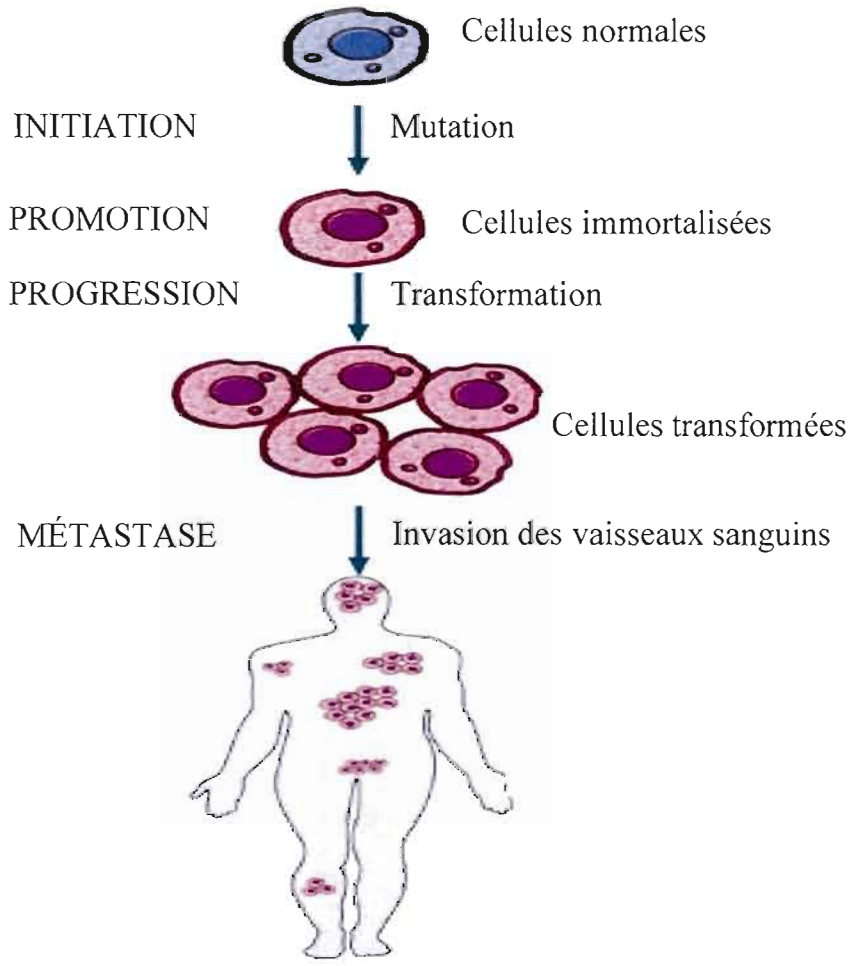



**Figure 1.2** : **Les étapes menant à la cancérogenèse et à la formation de métastases.** La cancérogenèse est la transformation d'une cellule normale en cellule cancéreuse. Cela nécessite plusieurs étapes dont l'initiation, la promotion et la progression. L'étape d'initiation est l'altération du génome qui est induite par des agents cancérigènes causant des mutations et causant ainsi la conversion d'une cellule normale en cellule initiée. Dans l'étape de promotion, les cellules initiées subissent une expansion clonale par l'induction de facteurs promoteurs comme des cytokines et des facteurs de croissances. L'étape de progression est la transformation des cellules initiées en cellules malignes. Le processus métastasique est le phénomène par lequel les cellules tumorales envahissent et se développent dans de nouveaux sites.

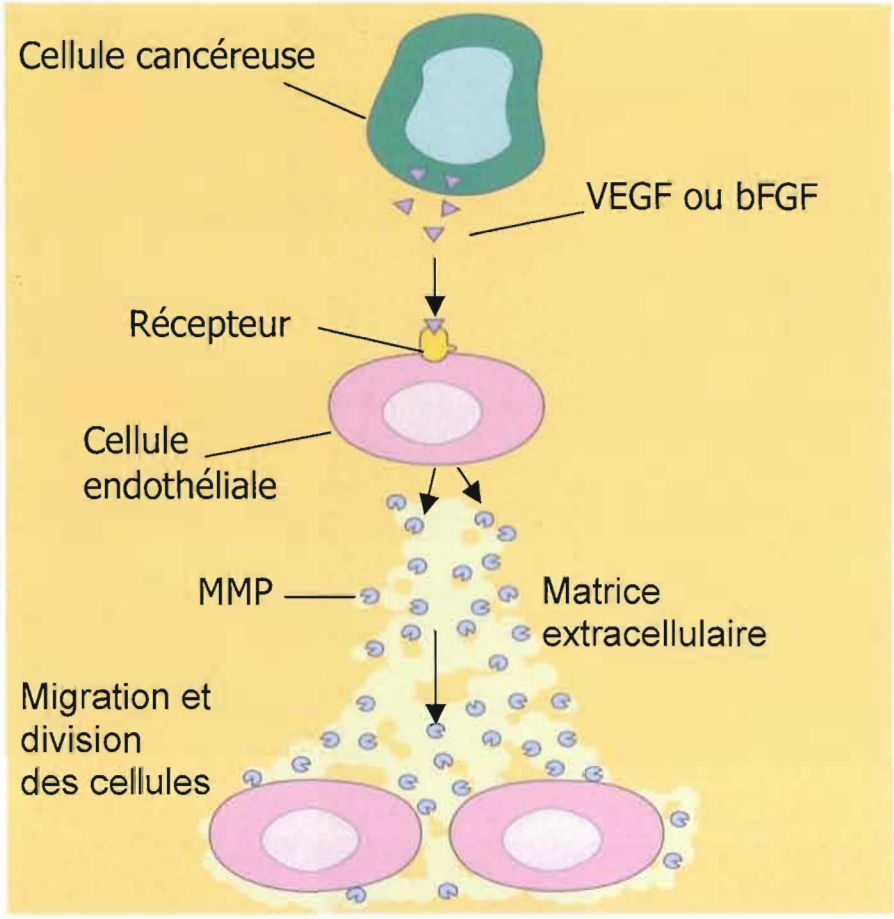



**Figure 1.2: Les différentes étapes par lesquelles les cellules endothéliales forment des vaisseaux sanguins.** L'angiogenèse est la formation de nouveaux vaisseaux sanguins à partir de vaisseaux préexistants. Lorsque la tumeur devient hypoxique elle sécrète des facteurs de croissance, comme le facteur de croissance de l'endothélium vasculaire (VEGF) et le facteur de croissance des fibroblastes (FGF). Ces facteurs de croissance, lorsqu'ils viennent en contact avec des cellules endothéliales, s'associent sur les récepteurs retrouvés à leur surface. Les cellules endothéliales produisent ainsi des métalloprotéases matricielles (MMP), qui sont des enzymes capables de dégrader la matrice extracellulaire et ceci permet aux cellules endothéliales de migrer et de se diviser. Permettant ainsi la formation de structures en forme de capillaires alimentant la tumeur.

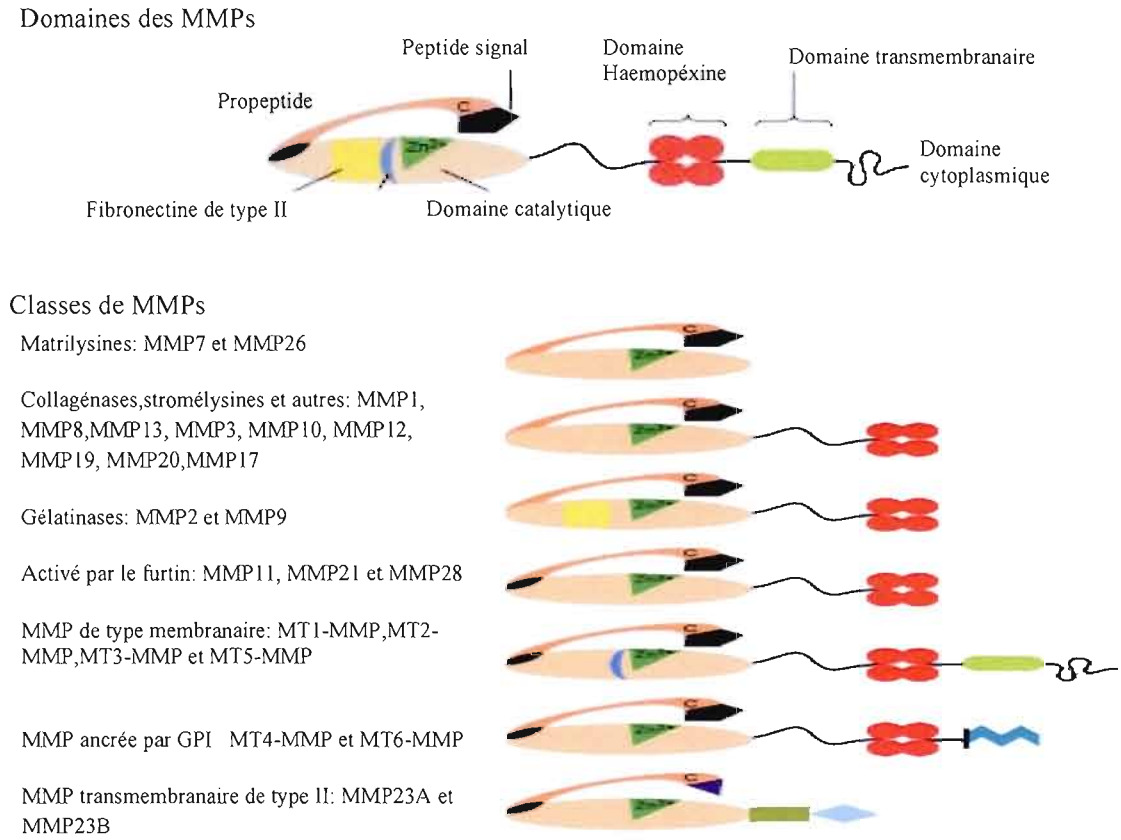



**Figure 1.3: Les différentes classes de MMPs.** Les MMPs permettent la dégradation de la matrice extracellulaire, elles sont classées en différents groupes selon leur structure. Elles sont composées de trois domaines communs, le domaine pro-peptide, le domaine catalytique et le domaine C-terminal. Elles sont synthétisées sous une forme inactive avec le domaine propeptide comme domaine autoinhibiteur. Le domaine catalytique a la capacité de lier le zinc. Le domaine propeptide contient un résidu cystéine qui interagit avec le zinc, et empêche la liaison et le clivage du substrat.

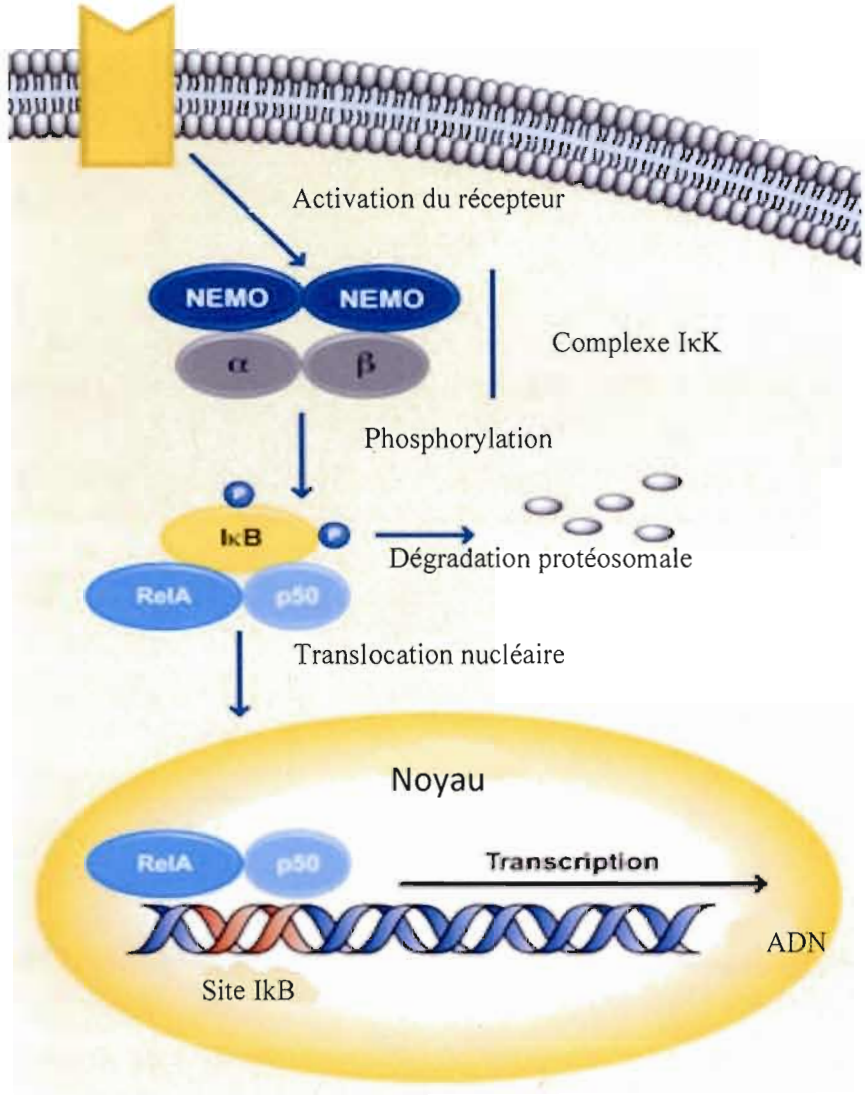
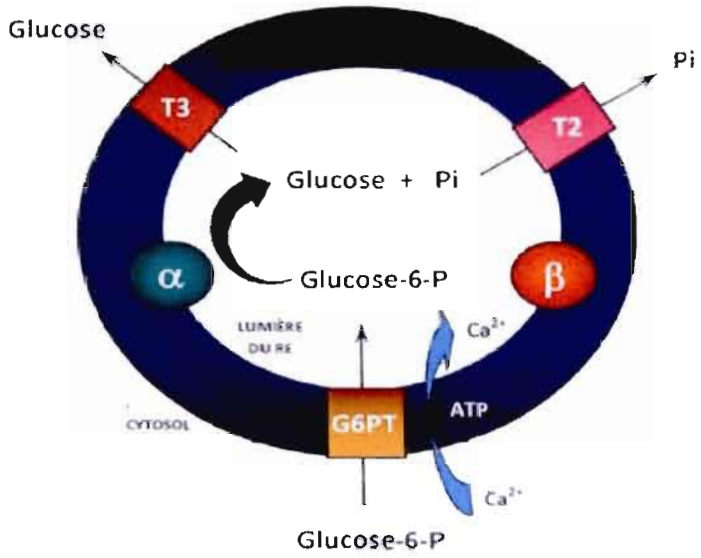



**Figure 1.4: Voies de signalisation pour l'activation de NF-κB.** NF-B est un facteur de transcription appartenant à la famille des protéines Rel. Il est activé par différents stimuli comme des cytokines, des esters de phorbols et des signaux de stress. L'activation d'un récepteur, permet le recrutement du complexe IκK, composé de sous-unités catalytiques (alpha et beta) et d'une protéine régulatrice non-enzymatique (NEMO). Le IκK permet la phosphorylation et la dégradation par le protéasome de l'inhibiteur IkB, associé à p50 et à p65 (RelA). NFkB est ensuite transloqué au noyau pour l'activation des gènes.

a)

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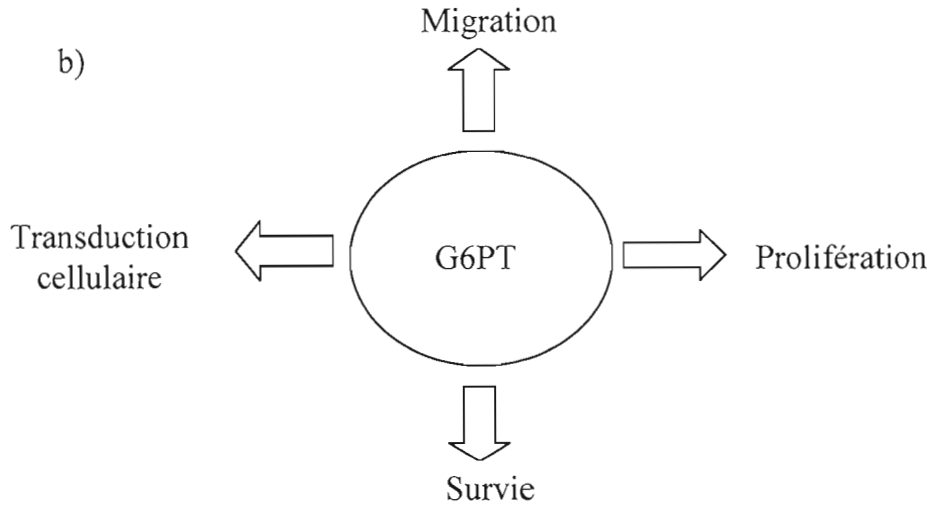



**Figure 1.5: Le système glucose-6-phosphatase.** a) Le système glucose-6-phosphatase est composé de trois translocases et de deux phosphatases, α et β. Le Glucose-6-Phosphate Transférase (G6PT) a comme rôle principal de transporter le glucose-6-phosphate du cytosol vers le réticulum endoplasmique, où il sera hydrolysé en glucose et en phosphate inorganique (Pi). Les translocases T2 et T3 vont par la suite permettre le transport du glucose et du Pi dans le cytosol. b) Le G6PT est impliqué dans la survie cellulaire, dans la prolifération cellulaire, dans la migration cellulaire et dans la transduction cellulaire qui régule la mobilisation du calcium.

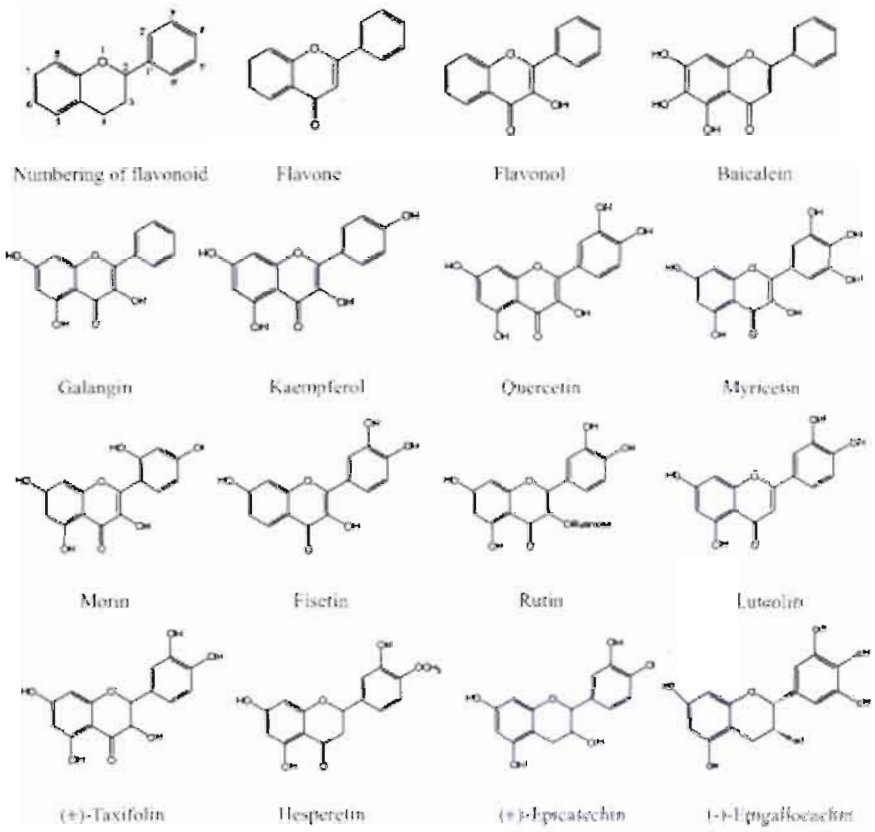
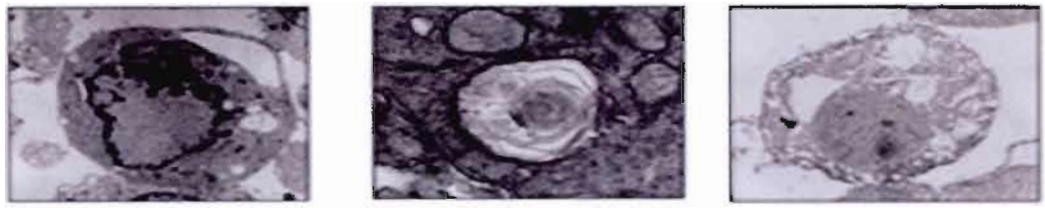



**Figure 1.7: Structure de certains flavonoïdes.** Les flavonoïdes avec différentes structures chimiques sont largement distribués chez les plantes, les végétaux, les fruits, le thé et le vin, et sont donc ingérés régulièrement via la diète humaine. Les flavonoïdes sont connus pour avoir des actions biologiques et sont donc importants pour la santé.



| Types: | apoptose | autophagie | nécrose |
|---|---|---|---|
| Morphologie: | rétrécissement cellulaire<br>corps apoptotiques<br>condensation de la chromatine<br>dégradation de l'ADN<br>fragmentation du noyau | diminution de la taille<br>vésicules à double membrane<br>dégradation des organelles | perte de l'intégrité membranaire<br>enflure de la cellule<br>enflure des organelles |
| Régulation | famille de BCL-2<br>caspases<br>protéines adaptrices<br>kinases, phosphatases<br>calcium | mTOR, PI3-kinase<br>famille ATG<br>kinases (ex:JNK)<br>famille BCL-2<br>récepteurs IP3 | calcium<br>PARP<br>protéines régulées par le calcium<br>kinase RIP<br>récepteurs de mort |
| Stimuli | stress oxydatif<br>dommage à lADN<br>ligands des récepteurs de mort<br>médicaments anti-cancéreux<br>stress des organelles<br>infections aux pathogènes | déplétion en nutriments<br>stress du RE<br>surplus de calcium<br>hypoxie, ischemie<br>dommages aux organelles | toxines bactériennes<br>poisons métaboliques<br>surplus de calcium<br>ischémie |

**Figure 1.8: Comparaison entre l'apoptose, la nécrose et l'autophagie.** Il existe plusieurs systèmes pour l'élimination des cellules endommagées. Ces systèmes régulent la mort cellulaire par l'apoptose, la nécrose et l'autophagie.

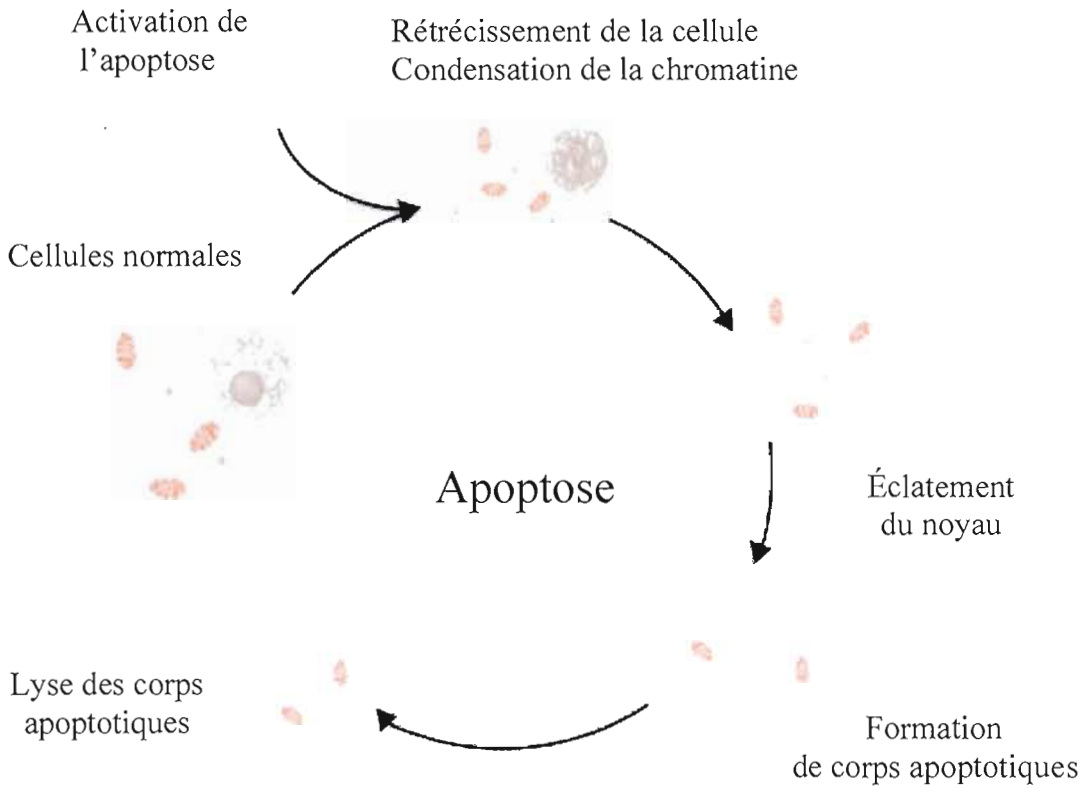



**Figure 1.9: Les mécanismes de l'apoptose.** La mort cellulaire programmée ou apoptose est le processus par lequel les cellules se suicident pour l'élimination de cellules non fonctionnelles. Durant l'apoptose, le génome de la cellule va rétrécir et une partie de la cellule va se désintégrer en des petits corps apoptotiques. L'apoptose est un processus contrôlé où les composantes des cellules sont dégradées dans la membrane cellulaire. Ces cellules vont être lysées à la fin du cycle apoptotique.

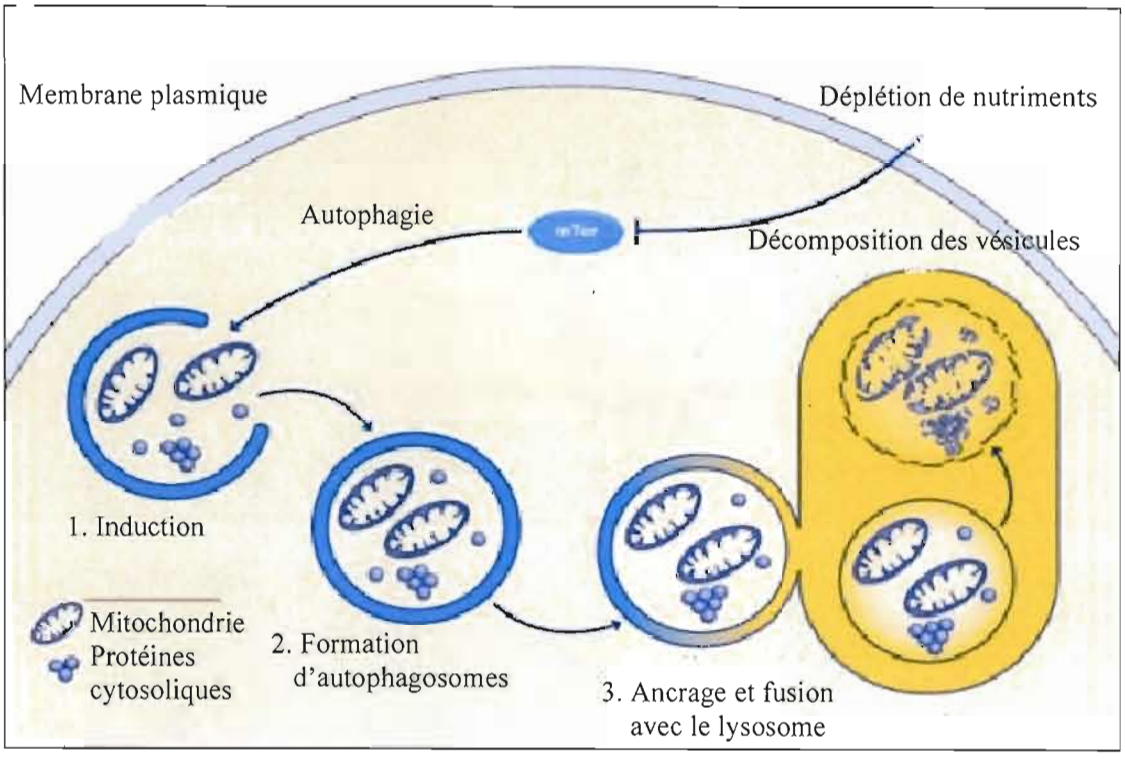



**Figure 1.10: Le processus d'autophagie.** Lorsqu'il y a une déplétion en nutriments (ou activation d'autres stimuli), il y a inhibition de mTOR et le processus d'autophagie est alors activé. Les protéines cytosoliques et les organelles sont séquestrées par une vésicule à double membrane. Il y a ensuite fusion avec le lysosome et décomposition des vésicules autophagiques.

# CHAPITRE II

## 2.1. Mise en contexte

Dans le cerveau, entre la circulation sanguine et le système nerveux central (SNC), se trouve la barrière hémato-encéphalique. La barrière hémato-encéphalique contrôle l'entrée des nutriments et limite l'entrée des substances toxiques au sein du SNC contribuant ainsi à l'homéostasie des fonctions cérébrales. Les CE cérébrales sont les composantes structurales et fonctionnelles de la barrière hémato-encéphalique (Annabi et *al.*, 2010). Ces cellules sont reliées entre elles par des jonctions serées. Ces jonctions entourent complètement les cellules endothéliales et joignent les cellules adjacentes. En général, les particules ne peuvent pas passer au travers de ces jonctions (Pardridge, 1999). Comme la plupart des médicaments ne peuvent pas traverser la barrière hémato-encéphalique, les CE sont considérées comme des cibles thérapeutiques intéressantes, particulièrement pour traiter les tumeurs cérébrales hautement vascularisées. Les CE microvasculaires cérébrales (HBMEC), représentent un modèle qui se rapproche le plus du phénotype et des fonctions des CE dérivées de tumeurs cérébrales. Ceci nous permet d'évaluer l'impact de différentes molécules inhibitrices dans un environnement promouvant l'angiogenèse tumorale (McLaughlin et *al.*, 2006).

## 2.2. Objectif de recherche

Étant donné que les CE vasculaires cérébrales jouent un rôle détérminant dans l'angiogenèse tumorale cérébrale, mon premier objectif était de déterminer si l'analogue semi-synthétique de la mumbaistatine, pouvait cibler les propriétés angiogéniques des HBMEC incluant la sécrétion de MMP-9, la migration cellulaire et la tubulogenèse *in vitro*. Mon deuxième objectif était d'analyser les effets du 2DG, un inhibiteur du métabolisme du glucose et de la production d'ATP, sur le stress du réticulum endoplasmique et sur les marqueurs de l'inflammation (cox-2) et de l'angiogenèse tumorale (MMP-9, tubulogenèse). Mon troisième objectif était d'évaluer les propriétés inhibitrices des flavonoïdes, sur la voie de signalisation de NF-κB et sur la régulation de MMP-9 et de cox-2.

## 2.3. Hypothèses de travail

Volet 1 : Les CE vasculaires cérébrales, jouent un rôle important en tant que composantes structurales et fonctionnelles dans l'angiogenèse tumorale. G6PT étant impliqué dans la survie cellulaire et dans l'adaptation métabolique à l'hypoxie des cellules cancéreuses et des cellules hypoxiques, en général nous avons émis l'hypothèse que l'AD4-015, un inhibiteur de G6PT, pourrait contrer la tubulogenèse *in vitro* des CE.

Volet 2 : Les cellules tumorales en hypoxie sont dépendantes de l'ATP produit par une glycolyse accrue. Nous avons donc émis l'hypothèse que le 2DG, un analogue du glucose qui a comme rôle d'inhiber la glycolyse par la déplétion d'ATP, pourrait inhiber les propriétés angiogéniques des cellules endothéliales vasculaires cérébrales.

Volet 3 : La voie de signalisation NF-κB est impliquée dans le processus inflammatoire et est reliée à la régulation de l'expression de cox-2 et de MMP-9. Nous avons émis l'hypothèse que les flavonoïdes, des produits naturels retrouvés dans plusieurs plantes et fruits qui ont des propriétés anti-inflammatoires, peuvent cibler la phosphorylation de IkB induite par un agent carcinogène dans les CE vasculaires cérébrales.

## CHAPITRE III

### Article 1

**Inhibition of tubulogenesis and of carcinogen-mediated signalling in brain endothelial cells highlight the antiangiogenic properties of a mumbaistatin analog**

Elizabeth Tahanian, Simon Lord-Dufour, Abhirup Das, Chaitan Khosla, René Roy et Borhane Annabi

Description de la contribution des auteurs de cet article :

Tahanian : Expérimentation essais biologiques, analyse des résultats, montage des figures, rédaction

Lord-Dufour : Expérimentation essais biologiques, analyse des résultats

Das : Expérimentations synthèse chimique, montage des figures, rédaction

Khosla : Analyse des résultats, rédaction, financement

Annabi : Analyse des résultats, montage des figures, rédaction, financement

## ARTICLE 1

# Inhibition of tubulogenesis and of carcinogen-mediated signaling in brain endothelial cells highlight the antiangiogenic properties of a mumbaistatin analog

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*Running title :* Antiangiogenic properties of G6PT inhibitors

*Key words :* Brain endothelial cells, angiogenesis, carcinogenesis, MMP-9, COX-2, G6PT inhibitors

*The abbreviations used are :* BBB, blood-brain barrier; COX-2, cyclooxygenase-2; EC, endothelial cells; G6PT, glucose-6-phosphate translocase; HBMEC, human brain microvascular EC; MMP-9, matrix metalloproteinase-9; PMA, phorbol 12-myristate 13-acetate

## Abstract

A better understanding of the metabolic adaptations of the vascular endothelial cells that mediate tumour vascularisation would help the development of new drugs and therapies. Novel roles in cell survival and metabolic adaptation to hypoxia have been ascribed to the microsomal glucose-6-phosphate translocase (G6PT). While anti-tumorigenic properties of G6PT inhibitors such as chlorogenic acid (CHL) have been documented, those of the G6PT inhibitor and semi-synthetic analog AD4-015 of the polyketide mumbaistatin are not understood. In the present study, we evaluated the *in vitro* antiangiogenic impact of AD4-015 on human brain microvascular endothelial cells (HBMEC), which play an essential role as structural and functional components in tumour angiogenesis. We found that *in vitro* HBMEC migration and tubulogenesis were reduced by AD4-015, but not by CHL. The mumbaistatin analog significantly inhibited the phorbol 12-myristate 13-acetate (PMA)-induced matrix-metalloproteinase (MMP)-9 secretion and gene expression as assessed by zymography and RT-PCR. PMA-mediated cell signalling leading to cyclooxygenase (COX)-2 expression and I□B downregulation was also inhibited, further confirming AD4-015 as a cell signalling inhibitor in tumor promoting conditions. G6PT functions may therefore account for the metabolic flexibility that enables endothelial cell-mediated neovascularisation. This process could be specifically targeted within the vasculature of developing brain tumors by G6PT inhibitors.

## Introduction

Tumor-associated angiogenesis, a fundamental process in cancer tissue growth, consists of recruiting endothelial cells (EC) toward an angiogenic stimulus (1). The cells subsequently proliferate and differentiate to form endothelial tubes and capillary-like structures. Although little is known about the metabolic adaptation of EC through such a transformation, it has been suggested that the inhibition of metabolic pathways may offer a novel and powerful therapeutic approach which would simultaneously inhibit tumor cell proliferation and tumor-induced angiogenesis (2, 3). In fact, brain tumor-associated microvasculature is thought to become aberrant and to undergo a sequence of adaptive changes, such as during the development of gliomas. These changes are characterized by important metabolic adaptations, primarily involving hypoxia-regulated genes (4). Among those, transcription of genes responsible for glucose and energy metabolism, such as glucose transporters and glycolytic enzymes, has been documented. These proteins take up glucose and convert it to lactate, and include pyruvate dehydrogenase kinase 1, which shunts pyruvate away from the mitochondria, and BNIP3, which triggers selective mitochondrial autophagy (5). The adaptative shift from oxidative to glycolytic metabolism also allows maintenance of redox homeostasis and cell survival under conditions of prolonged hypoxia.

Recently, our group ascribed a dual role to the microsomal glucose-6-phosphate translocase (G6PT) in cell survival and metabolic adaptation to hypoxia in cancer cells and in hypoxic cells (6-8). In fact, hypoxia inducible factor (HIF)-1$\alpha$ was found to regulate G6PT gene expression (8). G6PT has originally been shown to transport G6P from the cytosol to the lumen of the endoplasmic reticulum, thereby performing the rate-limiting step for G6P hydrolysis into glucose and inorganic phosphate by the glucose-6-phosphatase system (9). G6PT is also known to integrate and regulate many metabolic functions such as glycemia, lipidemia, uricemia, and lactic acidemia (10). More importantly, its activity cannot be substituted as G6PT deficiencies lead to glycogen storage disease type Ib characterized not only by disturbed glucose homeostasis but also by severe myeloid dysfunctions (10). G6PT was further shown to play a role in bone marrow cell and neutrophil chemotaxis (11, 12), in calcium flux control (13-16), and in U87 glioma cell survival (6, 7). In turn, G6PT gene

expression is regulated in response to adaptive metabolic changes involving glucose, insulin and cyclic AMP (17). G6PT-mediated metabolic adaptation, which could enable cells to survive under conditions characterized by hypoxia (8) may therefore become an appealing therapeutic prospect through the design of efficient anti-G6PT molecules targeting those cells proliferating within the tumor microenvironment.

Among anti-G6PT targeting strategies, the anthraquinone natural product mumbaistatin is one of the most potent known functional inhibitors of G6PT. Its availability, however, has been limited due to its extremely low yields from the natural source cultures of *Streptomyces* sp (18). A facile semisynthetic method that afforded high yields and generation of a variety of mumbaistatin analogs within five steps was therefore developed starting from DMAC (5, 3,8-dihydroxyanthraquinone-2-carboxylic acid), a structurally related polyketide product of engineered biosynthesis (19). Among those semisynthetic analog of the polyketide mumbaistatin, AD4-015 was found by us to potently and specifically reduce cell survival of those cells that adapted to hypoxia (8). Such anti-G6PT effect was better than that of chlorogenic acid (CHL) which pharmacological functions also target G6P transport functions (20) and, in recent years, shown to affect hepatoma and glioblastoma cell proliferation and survival (21, 22). Collectively, our data therefore suggest that G6PT may account for important molecular adaptation that enables cells to survive under conditions characterized by hypoxia and could be specifically targeted within developing tumors. To date, no roles for G6PT have yet been proposed in angiogenesis, and little is known about metabolic targeting of tumor-associated EC as part of cancer treatments.

Human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components of the blood-brain barrier (BBB). Most importantly, recent studies delineated a unique adaptative phenotype of brain EC in which matrix metalloproteinase (MMP)-9 secretion in HBMEC was increased by the tumor-promoting agent phorbol 12-myristate 13-acetate (23, 24), a condition that favors disruption of the BBB by MMP-9 and tumor invasion (25). Given that HBMEC play a role in angiogenesis within hypoxic tumors, we questioned in this study whether the semi-synthetic analog of the polyketide mumbaistatin, a potent G6PT inhibitor, could target HBMEC's angiogenic

properties including carcinogen-induced MMP-9 secretion, cell migration and *in vitro* tubulogenesis.

## Materials and methods

*Materials :* Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma-Aldrich Canada.

*Cell culture :* Human brain microvascular endothelial cells (HBMEC) were characterized and generously provided by Dr Kwang Sik Kim of Johns Hopkins University School of Medicine (Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV and Ulex Europeus Agglutinin I; they took up fluorescently labelled, acetylated low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain EC-specific phenotype (26). HBMEC were immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages (27). HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%) (Gibco), sodium pyruvate (1 mM) and EC growth supplement (30 μg/ml). Cells were cultured at 37°C under a humidified atmosphere containing 5% $CO_2$. All experiments were performed using passages 3 to 28.

*cDNA synthesis and semi-quantitative real-time RT-PCR :* Total RNA was extracted from cultured HBMEC using TRIzol reagent (Invitrogen, Burlington, ON). Semi-quantitative RT-PCR analysis was performed starting with 2 micrograms of total RNA for first strand cDNA synthesis, followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON). Primers for MMP-9 (forward : 5'-AAGATGCTGCTGTTCAGCGGG-3', reverse : 5'- GTCCTCAGGGCACTGCAGGAT-3') were derived from human sequences. PCR conditions were optimized so that the gene

products were examined at the exponential phase of their amplification and the products were resolved on 1.8% agarose gels containing 1 □g/ml ethidium bromide.

*Analysis of HBMEC migration :* HBMEC migration was assessed using modified Boyden chambers. The lower surfaces of Transwells (8-μm pore size; Costar, Acton, MA) were pre-coated with 0.2% type-I collagen for 2 hours at 37°C. The Transwells were then assembled in a 24-well plate (Fisher Scientific Ltd, Nepean, ON). The lower chamber was filled with serum-free HBMEC medium in the presence or absence of the G6PT inhibitors. HBMEC were collected by trypsinization, washed and resuspended in serum-free medium at a concentration of $10^6$ cells/ml; $10^5$ cells were then inoculated onto the upper side of each modified Boyden chamber. The plates were placed at 37°C in 5% $CO_2$/95% air for 30 minutes after which various concentrations of G6PT inhibitors were added to the lower chambers of the Transwells. Migration then proceeded for 6 hours at 37°C in 5% $CO_2$/95% air. Cells that had migrated to the lower surfaces of the filters were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet-20% methanol (v/v). Images of at least five random fields per filter were digitized (10X magnification). The average number of migrating cells per field was quantified using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON). Migration data are expressed as a mean value derived from at least three independent experiments.

*Endothelial cell morphogenesis assay :* Tubulogenesis was assessed using Matrigel aliquots of 50 μL, plated into individual wells of 96-well tissue culture plates (Costar, Amherst, MA) and allowed to polymerize at 37°C for 30 minutes. After brief trypsination, HBMEC were washed and resuspended at a concentration of $2.5x10^5$ cells/ml in serum-free medium. One hundred μL of cell suspension (25,000 cells/well) and 75 μL of medium with serum were added into each culture well. Cells were allowed to form capillary-like tubes at 37°C in 5% $CO_2$/95% air for 18 hours in the presence or absence of different AD4-015 concentrations. The formation of capillary-like structures was examined microscopically and pictures (10x) were taken using a Retiga 1300 camera (QImaging) and a Nikon Eclipse TE2000-U microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like

network, using a commercially available image analysis program (Northern Eclipse) as described and validated previously (28, 29). For each experiment, four randomly chosen areas were quantified by counting the number of tubes formed. Tubulogenesis data are expressed as a mean value derived from at least three independent experiments.

*Gelatin zymography :* Gelatin zymography was used to assess the extent of proMMP-2 and proMMP-9 activity as previously described (30). Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled $H_2O$. Gels were further incubated at 37°C for 20 hrs in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in $H_2O$. Gelatinolytic activity was detected as unstained bands on a blue background.

*Immunoblotting procedures :* Cytosolic proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes which were then blocked for 1 hr at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.1% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary polyclonal anti-HuR antibody (1/1,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/2,500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by ECL.

*Cell survival assay :* Cell survival was assessed by measuring fluorometric caspase-3 activity. HBMEC were grown to 60% confluence and treated with different concentrations of the mumbaistatin analog. Cells were collected and washed in ice-cold PBS pH 7.0. Cells were subsequently lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for one hour at 4°C and the lysates were clarified by centrifugation at 16,000g for 20 minutes. Caspase-3 activity was determined by incubation with 50 μM of the caspase-3-specific fluorogenic

peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0,1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates (31). The release of AFC was monitored for at least 30 minutes at 37°C on a fluorescence plate reader (Molecular Dynamics) ($\lambda_{ex}$= 400nm, $\lambda_{em}$=505nm).

*Statistical data analysis :* Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test and probability values of less than 0.05 were considered significant; an asterisk (*) identifies such significance in each figure.

## Results

*Polyketide derivative AD4-015 inhibits in vitro HBMEC cell migration.* The effects of chlorogenic acid (CHL; Fig.1a upper panel), a natural product with weak G6PT inhibitory activity (20) and of a more potent semi-synthetic polyketide analog (AD4-015, Fig.1a middle panel) of a different natural product, mumbaistatin (19) (Fig.1a lower panel), were tested on HBMEC migration (Fig.1b). While CHL had no significant effect, HBMEC migration was inhibited dose-dependently by AD4-015 with a calculated $IC_{50}$ of 11.2 ± 3.1 μM (Fig.1c). Because AD4-015 must cross the plasma membrane, this level of inhibition by AD4-015 is slightly higher than those $IC_{50}$ parameters ascertained from dose-response curves generated for G6PT inhibition in acellular rat liver microsomal assays ($IC_{50}$ of 2.5 μM) (19). The latter assay allowing direct inhibition of [$^{14}$C] G6P transport/hydrolysis functions performed by the microsomal glucose-6-phosphatase system (9).

*Mumbaistatin analog AD4-015 inhibits HBMEC in vitro capillary-like structure formation.* In order to next assess the antiangiogenic potential of G6PT inhibitors, we used the classical Matrigel angiogenesis assay (32). Cells were seeded on top of Matrigel and left to adhere as described in the Methods section. Various concentrations of AD4-015 were then added, and capillary formation was left to proceed for 18 hours. While a well defined capillary-like network formed in vehicle-treated cells (Fig.2a), tubulogenesis was inhibited by AD4-015 with an $IC_{50}$ of 3.5 ± 0.8 μM (Fig.2b, closed circles). Cell viability, based on caspase-3 activity, was not significantly affected by AD4-015 (Fig.2b, open circles). Lack of cytotoxic effect, combined with the inhibition of structure formation within Matrigel, prompted us to investigate whether any extracellular matrix (ECM) degrading events were involved in AD4-015 inhibition.

*AD4-015 inhibits PMA-induced MMP-9 gene expression and secretion in HBMEC.* Among the secreted enzymes involved in ECM degradation, matrix metalloproteinases (MMP) are well-documented as being involved in cell migration and tubulogenesis (23, 33). More specifically, MMP-2 and MMP-9 are secreted by numerous cell types and their presence is often representative of angiogenesis (34, 35). HBMEC were serum-starved,

treated for 18 hours with AD4-015, and the conditioned medium was harvested. The levels of MMP-2 and of MMP-9 were measured by gelatin zymography in media samples derived from control and phorbol 12-myristate 13-acetate (PMA)-treated cells. While extracellular MMP-2 levels remained unaffected by PMA or AD4-015, MMP-9 activity was significantly increased upon PMA treatment (Fig.3a). Addition of AD4-015 to PMA-treated cells resulted in inhibition of extracellular MMP-9 activity (Fig.3a). Consistent with this observation, PMA increased MMP-9 gene expression in HBMEC, whereas AD4-015 blocked this increase in a dose-dependent fashion (Fig.3b).

*AD4-015 antagonizes carcinogen-mediated cell signalling leading to COX-2 expression in HBMEC.* Cyclooxygenase (COX)-2 expression is increased in tumor-associated inflammation and angiogenesis (36). Recently, the antiangiogenic effect of statins was explained by their ability to inhibit COX-2 and MMP-9 in human EC (37). In order to better understand the effect of AD4-015 on PMA-induced cell signaling in HBMEC, cells were treated with PMA in the presence of either CHL or AD4-015, and COX-2 expression was evaluated in cell lysates. PMA induced COX-2 expression in HBMEC, and AD4-015 efficiently inhibited this increase (Fig.4a) in a dose-dependent manner (Fig.4c). In contrast, total extracellular signal-regulated kinase (ERK) expression was unaffected by CHL, which was also unable to block PMA-induced COX-2 expression (Fig.4a). The effect of AD4-015 was possibly mediated through preventing dissociation of the inhibitor of nuclear factor kappaB (IκB) from the NF-κB complex, which is rapidly degraded through an ubiquitin-mediated process (Fig.4b)

## Discussion

G6P is not only an intermediate in all major metabolic pathways for glucose utilization and biosynthesis, but is also an allosteric regulator of enzyme activity and gene expression. Such crucial roles for G6P has given rise to a possible subcellular compartmentation of G6P pools that may channel flux through multiple pathways (38). As such, cytoplasmic G6P levels are also strategically positioned to link the cell's energy state to the regulation of multiple functions, including for example its pro-inflammatory response to certain stresses (39). Among newly discovered intracellular glucose generating systems, a microsomal glucose-6-phosphatase complex composed of a G6PT and a G6Pase-β catalytic subunit isoform (G6PC-3) is thought to be capable of endogenous glucose production in brain (40). Whether this complex is regulated in brain EC is unknown. Interestingly, transcriptional regulation of G6PT, but not of G6PC-3, by hypoxia through HIF-1α is suggestive of metabolic adaptation to low oxygen such as that present in developing tumors (8). While the physiological consequences of this specific adaptive phenotype remain to be elucidated, alternate roles for G6PT in chemotaxis, intracellular calcium flux control, and cell survival have already been proposed. Altogether, those documented G6PT-regulated cellular functions are a common feature also found in tumor growth factors' paracrine regulation of angiogenesis (1). The novelty of our study therefore demonstrates that the G6PT inhibitor AD4-015 may act as a potent antiangiogenic agent in that it inhibits *in vitro* tubulogenesis and HBMEC migration, both of which may be modulated through G6PT metabolic control.

It is well known that angiogenesis and hypoxia determine the vessel density of a tumor, partially through the modulation of glucose transporter expression by several other genes (41). Among these, a significant correlation of HIF-1α to GLUT-1 and GLUT-3 expression was found in renal cell carcinomas (42), MCF7 breast cancer cells (43) and in hepatoma models (44). HIF-1α-induced glycolytic enzymes, including HKI, HKII, PFK-L, ALD-A, ALD-C, PGK1, ENO-alpha, PYK-M2, LDH-A and PFKFB-3, were also examined in cancer cells. It was concluded that targeting the HIF-1α-induced glucose transporter and hexokinase might provide better therapeutic targets for inhibiting tumor growth and

progression than would targeting HIF-1α itself (45). Accordingly, this strategy was recently put forward in the targeting of hypoxic mesenchymal stromal cells (8), which are believed to be recruited and to contribute to brain tumor growth (46, 47). It remains unknown whether metabolic targeting could be used as a therapeutic strategy.

Only recently have brain EC been considered as targets for antiangiogenic agents but also for ionizing radiation modalities in highly vascularized tumours such as glioblastoma (28, 48). Here we have shown that the G6PT inhibitor AD4-015 inhibits MMP-9 and COX-2 expression. Recent studies have delineated a unique brain endothelial phenotype in which MMP-9 secretion in HBMEC was increased by the tumor-promoting agent PMA (23, 24). MMP-9 is required for EC migration and tube formation, and inhibition of MMP-9 secretion was demonstrated to reduce both *in vitro* invasion and angiogenesis in human microvascular EC (49). Therefore, one major implication of our study is that any therapeutic strategies resulting from discovery of lead products, such as AD4-015, and which inhibit endothelial MMP-9 expression, are likely to be of utility in treating brain tumor-associated angiogenesis. Interestingly, small molecule inhibitors of MMP-9 secretion, such as the green tea polyphenol epigallocatechin gallate and the isothiocyanate sulforaphane, do so by decreasing the expression of the MMP-9 mRNA stabilizing factor HuR (23, 50). Given that HuR levels are elevated in cancer (51), and have a pivotal role in promoting angiogenesis (52, 53), it remains to be determined whether HuR expression is also affected upon AD4-015 treatment. The inhibition of PMA-induced MMP-9 secretion by the G6PT inhibitor AD4-015 is therefore of importance given that MMP-9 is significantly increased during tumor progression and is associated with the opening of the BBB.

Until recently, most of the anti-angiogenic or anti-cancerous studies performed on EC *in vitro* involved macrovascular endothelial models such as human umbilical vein EC or bovine aortic EC. However, microvascular EC, such as brain EC, display a selective phenotype which differs from macrovascular EC. The availability of a stable human model of brain EC has only very recently allowed the emergence of studies which can now more closely represent cerebral endothelial microvasculature and mimic the impact of anti-cancerous treatments on the brain vasculature (54). Our study is thus among the pioneering

ones that are being reported. Currently, the HBMEC model is the best surrogate model that approximates tumour-derived EC available for long-term *in vitro* studies (26, 28, 55). Although human EC isolated from glioblastoma specimens have also been studied, these cells are not ideal targets for long-term *in vitro* studies as they de-differentiate in culture and have inherently limited proliferative potential before senescence (56).

In summary, we have provided pharmacological evidence for the role of G6PT in EC-mediated neovascularisation. The activity of the semisynthetic polyketide AD4-015 in our assays represents a first step towards targeting angiogenic processes within the vasculature of developing brain tumors using small molecules.

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Fig.1

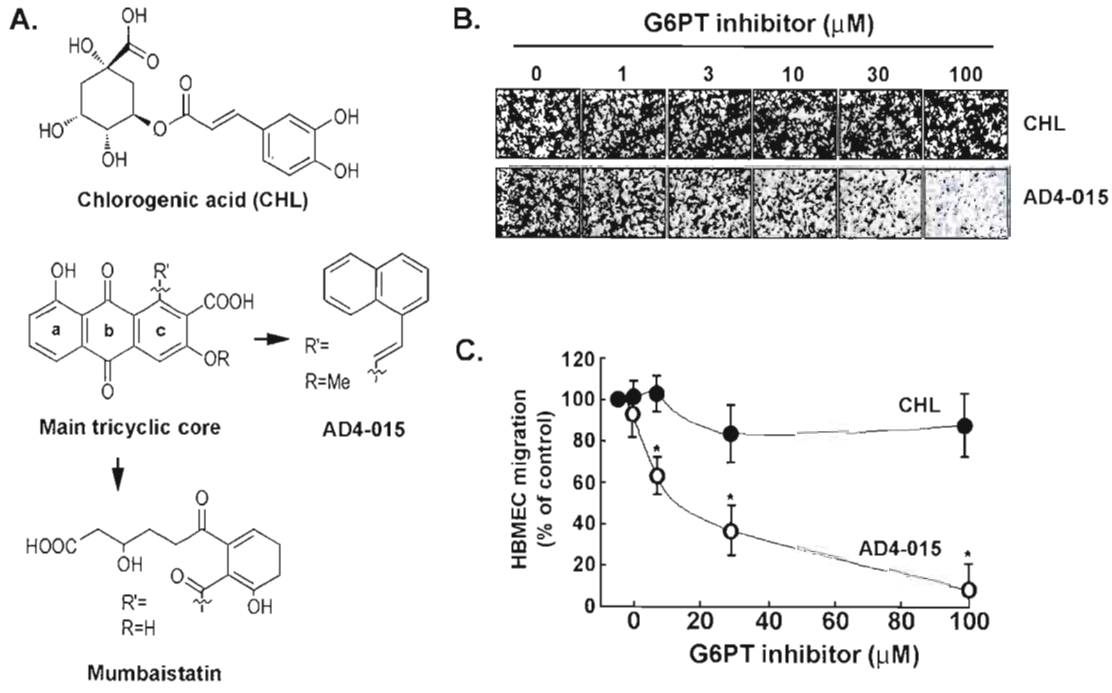


**Fig.1 : Polyketide derivative AD4-015 inhibits *in vitro* HBMEC cell migration.** (A) Structures of chlorogenic acid (CHL), parental mumbaistatin and AD4-015. Mumbastatin and AD4-015 have the same main tricyclic core; the hydroxyl group of ring c present in mumbaistatin has been exchanged by a methoxy group, and the ketone moiety by a trans-alkene naphthalenyl group to generate AD4-015. (B) HBMEC were treated with varying CHL or AD4-015 concentrations. At the end of the treatment, cells were harvested and seeded on top of gelatin-coated filters using modified Boyden chambers. Migration proceeded for 6 h, and pictures were taken of the stained filters. (C) Densitometric quantification of cell migration was performed from stained filters of AD4-015- (open circles) and CHL- (closed circles)-treated HBMEC.

Fig.2

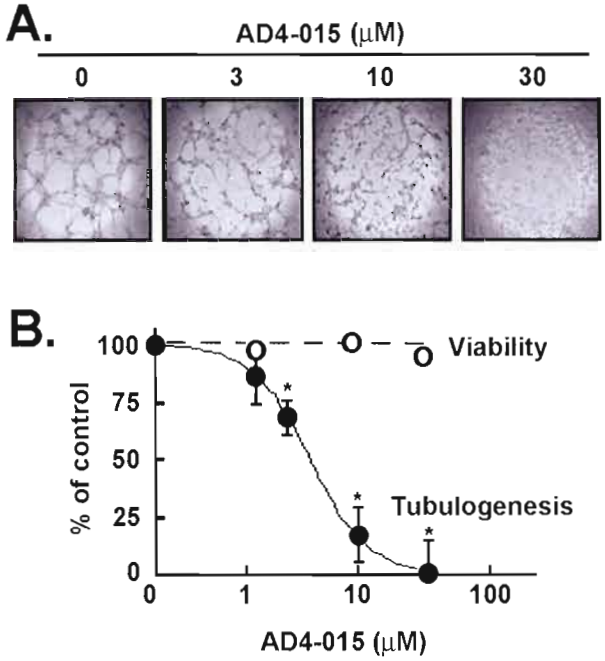


**Fig.2: Polyketide derivative AD4-015 inhibits HBMEC *in vitro* capillary-like structure formation.** (A) In order to assess *in vitro* tubulogenesis, HBMEC were seeded on top of Matrigel as described in the Methods section, and then treated with varying concentrations of AD4-015 for 18 hrs. Representative phase contrast pictures are shown. (B) The extent of cell survival (open circles) and of three-dimensional capillary-like structure formation (closed circles) was quantified as described in the Methods section. Data are presented as percent of vehicle-treated cells (control).

# Fig.3

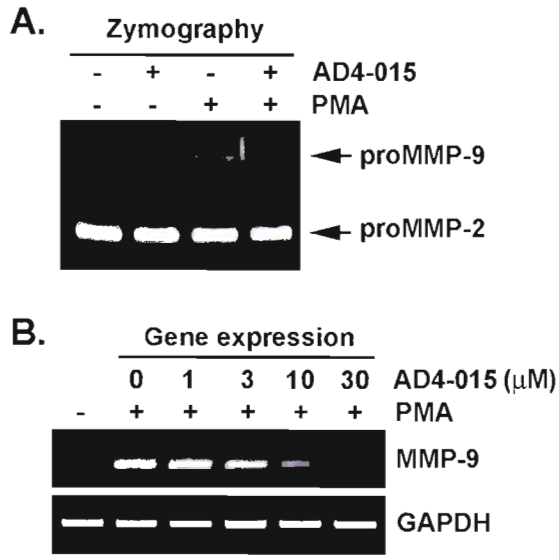


**Fig.3: AD4-015 inhibits PMA-induced MMP-9 gene expression and secretion in HBMEC.** HBMEC were serum-starved in the presence of 30 mM AD4-015, 1 mM PMA, or a combination of both for 18 hrs. (A) Conditioned media were then harvested and gelatin zymography was performed in order to detect proMMP-9 and proMMP-2 hydrolytic activity as described in the Methods section. (B) Total RNA was extracted from HBMEC treated with various concentrations of AD4-015 as described in the Methods section. Semi-quantitative RT-PCR was performed to confirm the presence of a single amplicon for MMP-9 and for GAPDH. Data are representative of three independent experiments.

Fig.4

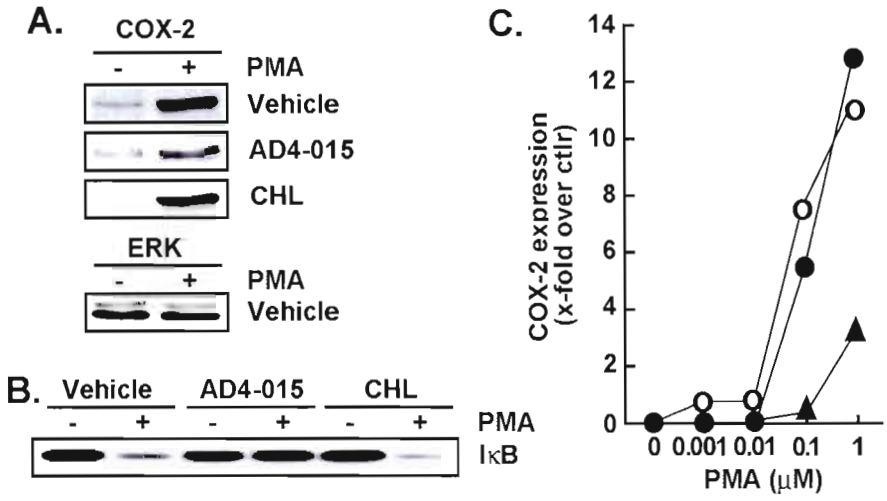


**Fig.4: AD4-015 antagonizes carcinogen-mediated cell signalling leading to COX-2 expression in HBMEC.** Lysates of serum-starved PMA-treated HBMEC were isolated after various co-treatments (1 μM PMA, 30 μM AD4-015, 100 μM CHL), electrophoresed via SDS-PAGE and immunodetection performed of (A) COX-2 and of ERK proteins, or (B) IκB performed as described in the Methods section. (C) Dose-response of PMA treatment was performed and COX-2/ERK expression was quantified by scanning densitometry of the autoradiograms (Vehicle, closed circles; CHL, open circles, AD4-015, closed triangles). Data are expressed as the x-fold induction over untreated basal conditions.

## Article II

### Low intracellular ATP levels exacerbate carcinogen-induced inflammatory stress response and inhibit *in vitro* tubulogenesis in human brain endothelial cells

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Description de la contribution des auteurs de cet article :

Tahanian : Expérimentation essais biologiques, analyse des résultats, montage des figures, rédaction

Peiro : Expérimentation essais biologiques, analyse des résultats

Annabi : Analyse des résultats, montage des figures, rédaction, financement

## Article II

# Low intracellular ATP levels exacerbate carcinogen-induced inflammatory stress response and inhibit *in vitro* tubulogenesis in human brain endothelial cells

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*Key words :* Endoplasmic reticulum stress, MMP-9, COX-2, 2-deoxy-D-glucose, endothelial cells

*The abbreviations used are :* ATP, adenosine triphosphate; BBB, blood-brain barrier; COX-2, cyclooxygenase-2; 2-DG, 2-Deoxy-D-glucose; EC, endothelial cells; ECM, extracellular matrix; ER, endoplasmic reticulum; GRP78, glucose-related protein 78; HBMEC, human brain microvascular endothelial cells; MMP, matrix metalloproteinase; PMA, phorbol 12-myristate 13-acetate

## Abstract

Solid tumour development requires angiogenesis and is correlated to the expression of inflammatory markers through cellular metabolic and energetic adaptation. While high glycolysis rates enable the cancer cell compartment to generate adenosine trisphosphate (ATP), very little is known about the impact of low intracellular ATP concentrations within the vascular endothelial cell compartment which is responsible for tumour angiogenesis. Here, we investigated the effect of 2-deoxy-D-glucose (2-DG), a glucose analog that inhibits glycolysis through intracellular ATP depletion, on human brain microvascular endothelial cell (HBMEC) angiogenic properties. While pre-formed capillaries remained unaffected, we found that *in vitro* tubulogenesis was dose-dependently decreased by 2-DG and that this correlated with reduced intracellular ATP levels. Pro-carcinogenic signalling was induced with phorbol 12-myristate 13-acetate (PMA) and found to trigger the pro-inflammatory marker cyclooxygenase (COX)-2 and endoplasmic reticulum (ER) stress marker GRP78 expression, whose inductions were potentiated when PMA was combined with 2-DG treatment. Inversely, PMA-induced matrix-metalloproteinase (MMP)-9 gene expression and protein secretion were abrogated in the presence of 2-DG, and this can be partially explained by reduced NF-κB signalling. Collectively, we provide evidence for an intracellular ATP requirement in order for tubulogenesis to occur, and we link increases in ER stress to inflammation. A better understanding of the metabolic adaptations of the vascular endothelial cells that mediate tumor vascularization will help the development of new drugs and therapies.

## Introduction

Tumour-associated angiogenesis, a fundamental process in tumour growth and metastasis, consists of recruiting endothelial cells (EC) toward an angiogenic stimulus.[1] The cells subsequently proliferate and differentiate to form endothelial tubes and capillary-like structures in order to deliver nutrients and oxygen to the tumour and to remove the products of its metabolism. In recent years several pathways have, in addition to stimulation of tumor angiogenesis, been suggested to contribute to the cell metabolic adaptations required for carcinogenesis, which include decreased tumoural apoptosis, increased invasion and metastasis, immune suppression and tumour-associated inflammation.[2,3]

An interesting link between overexpression of the pro-inflammatory marker cyclooxygenase (COX)-2 and tumour angiogenesis was recently described as one such metabolic adaptative phenotype.[4-6] This is supported by the fact that in normal cerebral cortex COX-2 is only present in neurons but absent from vascular EC.[7,8] It is however still unknown whether the EC-associated COX-2 correlates with high malignancy. Furthermore, little is known about the molecular events that dictate metabolic adaptation of EC in response to pro-carcinogenic stimuli. It is tempting to suggest that specific inhibition of metabolic pathways may offer a novel therapeutic approach that would simultaneously inhibit tumor-induced angiogenesis and inflammatory phenotypes.[9,10]

While human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components of the blood-brain barrier (BBB), its disruption by the brain tumor-secreted matrix metalloproteinase-9 (MMP-9) is believed to favor tumor invasion.[11,12] Recent studies delineated a unique brain endothelial phenotype in which MMP-9 secretion by HBMEC was increased upon treatment with the tumor-promoting agent phorbol 12-myristate 13-acetate (PMA).[13,14] Inhibition of MMP-9 secretion was demonstrated to reduce both *in vitro* invasion and angiogenesis in human microvascular EC.[15] In fact, adenoviral-mediated MMP-9 downregulation inhibited human dermal microvascular EC migration in cell wounding and spheroid migration assays, and reduced capillary-like tube formation, demonstrating the key role of MMP-9 in EC network organization.[15] Therefore,

among all MMP, the MMP-9 secreted from brain EC may be of importance in brain tumor-associated neovascularization.

Among the numerous signaling pathway candidates, NF-κB can regulate the expression of COX-2 through endoplasmic reticulum (ER) stress and, in part, through induction of the ER chaperone GRP78/BiP, which is expressed at high levels in a variety of tumors and which confers drug resistance to both proliferating and dormant cancer cells.[16] Importantly, it was recently demonstrated that partial reduction of GRP78 substantially reduced tumor microvessel density.[17] In the current study, we treated HBMEC with 2-DG which, once phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate, cannot be further metabolized and leads to a blockade of glycolysis and to depletion of intracellular adenosine triphosphate (ATP). *In vitro* tubulogenesis, expression of ER stress marker GRP78, PMA-induced MMP-9 secretion and COX-2 were also assessed in order to provide a metabolic and adaptative link between endothelial inflammation and angiogenesis.

## Materials and methods

### *2.1. Materials*

2-Deoxy-D-glucose, sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against IkB and phospho-IkB (Ser32/36) were purchased from Cell Signaling (Danvers, MA). The monoclonal antibodies against GRP78 and GAPDH were from Advanced Immunochemical Inc. (Long Beach, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Sigma-Aldrich Canada.

### *2.2. Cell culture*

Human brain microvascular endothelial cells (HBMEC) were characterized and generously provided by Dr Kwang Sik Kim of the Johns Hopkins University School of Medicine (Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV and Ulex Europeus Agglutinin I; they took up fluorescently labelled, acetylated low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain EC-specific phenotype.[18] HBMEC were immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages.[19] HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (iFBS) (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%) (Gibco), sodium pyruvate (1 mM) and EC growth supplement (30 μg/ml). Culture flasks were coated with 0.2% type-I collagen to support the growth of HBMEC monolayers. Cells were cultured at 37°C under a humidified atmosphere containing 5% $CO_2$. All experiments were performed using passages 3 to 28.

*2.3. Endothelial cell morphogenesis assay*

Tubulogenesis was assessed using Matrigel aliquots of 50 μL, plated into individual wells of 96-well tissue culture plates (Costar, Amherst, MA) and allowed to polymerize at 37°C for 30 minutes. After brief trypsinization, HBMEC were washed and resuspended at a concentration of $10^6$ cells/ml in serum-free medium. Twenty-five μL of cell suspension (25,000 cells/well) and 75 μL of medium with serum were added into each culture well. Cells were allowed to form capillary-like tubes at 37°C in 5% $CO_2$/95% air for 20 hours in the presence or absence of PMA or different 2-DG concentrations. The formation of capillary-like structures was examined microscopically and images (100x) were recorded using a Retiga 1300 camera (QImaging) and a Nikon Eclipse TE2000-U microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like network, using a commercially available image analysis program (Northern Eclipse) as described and validated previously.[20,21] For each experiment, four randomly chosen areas were quantified by counting the number of tubes formed. Tubulogenesis data are expressed as a mean value derived from at least three independent experiments.

*2.4. Gelatin zymography*

Gelatin zymography was used to assess the extent of proMMP-2 and proMMP-9 activity as previously described.[22] Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled $H_2O$. Gels were further incubated at 37°C for 20 hrs in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in $H_2O$. Gelatinolytic activity was detected as unstained bands on a blue background.

*2.5. Immunoblotting procedures*

Proteins or nuclear extracts were isolated from control and treated cells using the NE-PER Nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) and were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were

electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 hr at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1/2,500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

*2.6. Analysis of cell death by flow cytometry*

Cell death was assessed by flow cytometry as described previously.[23] Adherent and floating cells were harvested by trypsin digestion and gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with phosphate-buffered saline (PBS). Then, $10^5$ cells were pelleted, resuspended in 200 µL of buffer solution and stained with annexin-V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's protocol (BD Biosciences, Mississauga, ON). The cells were diluted by adding 300 µL of buffer solution and processed for data acquisition and analysis on a Becton Dickinson FACS Calibur flow cytometer using CellQuest Pro software.

*2.7. Analysis of autophagy by flurorescent microscopy and flow cytometry.*

HBMEC were seeded onto twelve-well plates and cultured for 18h to reach 80% confluence. Cells were then exposed to different concentrations of 2-DG in the presence or absence of PMA (1 µM) in serum free medium at 37°C in 5% $CO_2$/95% air. Acrydine orange was added to the culture medium at a final concentration of 0.5 µg/ml and incubated for 15 min in the dark at 37°C. The cells were then washed two times with medium lacking phenol red. Cells were immediately analyzed by flow cytometry or images taken with a fluorescent microscope using 510-560 nm for excitation and 590 nm for emission.

*2.8. Intracellular ATP levels assay.*

Cells were treated and 10X-100X-diluted lysates kept on ice as described by the manufacturer (SIGMA). One hundred (100) µl of ATP mix was added to the lysates and

incubated at room temperature for 3 min. Somatic cell ATP releasing reagent (1X) was prepared and added to the cell lysates. From this solution 100 µl was added to the ATP mix prior to analysis spectrofluorimetry.

*2.9. Statistical analysis.*

Statistical analyses were performed with Student's t-test when one group was compared with the control group. To compare two or more groups with the control group, one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used. All statistical analyses were performed using GraphPad Prism software. Differences with $P < 0.05$ were considered significant.

## Results

### *3.1. 2-Deoxy-D-glucose depletes intracellular ATP and inhibits in vitro capillary-like structure formation in HBMEC*

We first tested the effects of 2-deoxy-D-glucose (2-DG) against the angiogenic properties of HBMEC. Cells were seeded on top of Matrigel and left to adhere as described in the Methods section. Upon capillary-like structure formation, we then added 10-100 mM 2-DG or Mannose. No effect was found on the integrity of the pre-formed structures (Fig.1A). In contrast, when various concentrations of 2-DG were added at the very early time points (i.e. 30 minutes after cell seeding ontop of Matrigel), cell structure formation was significantly decreased with an $IC_{50}$ of 4.1 mM (Fig.1C). Cell survival was assessed with annexin-V-fluorescein isothiocyanate (apoptosis) and propidium iodide (necrosis) and was not significantly affected by 2-DG (Fig.1C). Mannose did not affect the capacity of the cells to form structures (not shown). We also validated 2-DG's ability to deplete intracellular ATP levels in HBMEC. While vehicle or 2-DG treatment did not affect total protein content levels (Fig.1D), we found that intracellular ATP levels decreased by ~20% in vehicle-treated cells during the 24 hours incubation prior to which tube formation was assessed (Fig.1D, left panel). When cells were treated with 100 mM 2-DG, >60% of intracellular ATP was depleted within the first hour of treatment, and >90% depletion was achieved at 4 hours of treatment (Fg.1D, right panel). ATP-dependent inhibition of tubulogenesis was next investigated to find out whether any extracellular matrix (ECM) degrading events were involved in this 2-DG effect.

### *3.2. 2-Deoxy-D-glucose inhibits PMA-induced MMP-9 secretion in HBMEC*

Among the secreted enzymes involved in ECM degradation, matrix metalloproteinases (MMP) are well-documented as being involved in cell migration and tubulogenesis.[13,24] More specifically, MMP-2 and MMP-9 are secreted by numerous cell types and their presence is often representative of angiogenesis.[25,26] HBMEC were serum-starved, treated for 18 hours with 2-DG and the conditioned media were harvested to measure the levels of MMP-2 and of MMP-9, in both control and in PMA-treated cells by gelatin

zymography. While MMP-2 extracellular levels were unaffected by 2-DG or by PMA (Fig.2A), MMP-9 levels were undetectable in basal conditions but were significantly increased in PMA-treated cells (Fig.2A, lower panel); the simultaneous presence of 2-DG significantly attenuated the MMP-9 levels seen in the presence of PMA with an $IC_{50}$ of ~18.1 mM (Fig.2B). Collectively, these results suggest that changes in intracellular ATP levels do not inhibit MMP vesicular trafficking since MMP-2 secretion was unaffected, but do affect signal transducing events such as those triggered by PMA and which control MMP-9 secretion.

### *3.3. 2-Deoxy-D-glucose inhibits PMA-induced IκB phosphorylation and nuclear translocation of the p65 subunit of NF-κB.*

Among MMP-9 expression regulators, the nuclear factor-kappaB (NF-κB) signalling pathway has been suggested.[27] We therefore first assessed whether this signalling pathway was activated upon PMA treatment and whether it was reflected in IkappaB (IκB) degradation. PMA-mediated phosphorylation of IκB was assessed. HBMEC were treated for 45 min with 1 μM PMA following preincubation with either vehicle or with 100 mM 2-DG. Preincubation with vehicle followed by PMA treatment rapidly led to IκB phosphorylation and to a concomitant decrease in IκB (Fig.3A, upper panels). When cells were preincubated with 2-DG (Fig.3A, lower panels), PMA-mediated IκB phosphorylation was significantly reduced (Fig.3B) as quantified by scanning densitometry. Nuclear translocation of the p65 and p50 subunits of NF-κB is among the direct consequences of IκB phosphorylation. When nuclear extracts were isolated, we found that lower p65 nuclear translocation (Fig.4A) occurred when cells were treated both with PMA and 2-DG as compared to PMA alone (Fig.4B). This is in agreement with the reduced phosphorylation of IκB observed in Fig.3.

### *3.4. 2-Deoxy-D-glucose potentiates PMA-induced cyclooxygenase-2 and GRP78 expression.*

As PMA is also a well documented inducer of cyclooxygenase (COX)-2,[28] we determined whether 2-DG also inhibited a possible NF-κB signalling pathway that leads to COX-2 induction. Cells were first treated with PMA or a combination of PMA/2-DG or

PMA/Man (mannose served as an osmotic control). PMA induced COX-2 expression, which expression was exacerbated in PMA/2-DG-treated cells which further had increased GRP78 expression (Fig.5A, left panel). These effects were not observed in PMA/Man-terated cells (Fig.5A, right panel). To investigate the dose-dependent effect, cells were incubated with vehicle or PMA in the presence of various 2-DG concentrations for 24 hours. No COX-2 expression was observed in the absence of PMA, while a slight basal increase in the endoplasmic reticulum (ER) stress marker GRP78 was noticed with increasing 2-DG treatment (Fig.5B, left panel). As expected, COX-2 expression was significantly induced by PMA (Fig.5B, right panel), and this induction was dose-dependently potentiated by 2-DG (Fig.5C). This 2-DG-mediated potentiation is in contrast to the results seen with MMP-9 and with the reduced NF-κB signalling described above (Fig.3 and Fig.4). Interestingly, we also found that the 2-DG-mediated expression of the ER stress marker GRP78 was potentiated upon PMA treatment (Fig.5D). Altogether, our data suggest ATP depletion affects PMA-mediated signalling independent of NF-κB and results in both decreased MMP-9 levels and in increased COX-2 expression. The latter is possibly a consequence of ER stress induction.

### *3.5. Increased endoplasmic reticulum stress, rather than MMP-mediated ECM hydrolysis, correlates with cyclooxygenase-2 expression in HBMEC.*

As a consequence of the results above, we suspected a correlative relationship that would direct the molecular events observed following intracellular ATP depletion within HBMEC. Accordingly, we plotted GRP78 vs COX-2 as well as MMP-9 vs COX-2 and confirmed a positive correlation between ER stress and COX-2 expression (Fig.6A). As for MMP-9 and its possible involvement in the ECM degradation required for tubulogenesis to occur, we instead found an inverse correlation when plotted against COX-2 expression (Fig.6B). Collectively, this suggests that intracellular ATP needs are crucial for cell survival signalling through ER stress-mediated events that would lead to a proinflammatory phenotype.

### *3.6. 2-Deoxy-D-glucose-induced autophagy is abrogated in PMA-treated HBMEC.*

Given that 2-DG treatment of the cells did not result in any cytotoxic effect (Fig.1B), we tested the possible implication of autophagy, a crucial component of the cellular stress

adaptation response that maintains cell homeostasis and that may act as a survival mechanism that can rescue cells from apoptotic/necrotic death. HBMEC were treated in the presence of 100 mM 2-DG in combination or not with PMA. The acidic autophagic vesicles were visualized by supravital staining with a pH-sensitive dye acridine orange and fluorescent microscopy used to for visualization (Fig.7A). We observed that 2-DG, but more importantly PMA, triggered autophagic vacuoles formation which effect was slightly increased in the presence of combined 2-DG/PMA treatment. When flow cytometry was used to quantify the extent of autophagy, we found that 2-DG induced by 2.4-times autophagy (Fig.7B). PMA treatment alone triggered extensive autophagy, but when combined to 2-DG no increase (1.1-times over basal) in autophagy was observed.

## Discussion

The adaptive mechanisms responsible for EC survival under pro-carcinogenic and low energy conditions remain poorly documented. Although increasing interest has been manifested towards cancer therapies that target cell metabolism, very few studies have specifically assessed the impact of targeting the EC metabolic compartment. In fact, EC are believed to be metabolically robust and to adapt to pro-carcinogenic paracrine stimulation and conditions such as encountered within the hypoxic tumour microenvironment.[29,30] In our study, we induced *in vitro* pro-carcinogenic stimulation of brain microvascular endothelium using PMA in combination with ATP-depleting culture conditions in order to assess EC angiogenic and inflammatory responses. While preformed endothelial structures remained unaffected by 2-DG-mediated ATP depletion, we show that minimal supply of energy is required by HBMEC for tubulogenesis processes to occur and that the acquisition of a pro-inflammatory phenotype better correlates with induction of ER stress than with increased ECM degradation capacities. This is among the first demonstration linking molecular players such as GRP78 (ER stress) and COX-2 (inflammation) expression to the adaptative survival mechanisms of vascular EC's response to pro-carcinogenic and ATP depleting conditions. More importantly, this also in part evidences the crucial requirement for ATP in the early time points of tubulogenesis processes, while preformed vessels may adopt metabolic adaptative mechanisms enabling them to resist energy depletion. Alternate strategies, aside from using energy depletion agents, must therefore be envisioned in order to target preformed tumour-associated vessels, while optimization of anti-angiogenic therapies may be complemented with ATP-depleting strategies.

EC are the first cells to be exposed to decreases in $PO_2$ within a hypoxic tumor-associated environment. Accordingly, tight regulation of ATP and GTP turnover exists, which enables EC to maintain their high-energy phosphates during hypoxia.[31] In EC, ATP is also predominantly generated by glycolysis and $O_2$ consumption is comparatively low.[29] Although accelerated glycolysis is one of the biochemical characteristics of cancer cells metabolism, one can safely extrapolate that this metabolic shift alteration, in part consequent

to the hypoxic tumour microenvironment, must also occur within the tumour-associated vascular endothelium. Accordingly, it was demonstrated that low energy demand and high glycolytic activity may explain why the coronary endothelium is less severely injured than cardiomyocytes in ischemic and anoxic hearts.[29] Targeting glycolysis in the tumour-associated vascular compartment may become an attractive anti-angiogenic approach. As such, besides affecting cell death and stress signaling pathways, 2-DG was recently shown to activate autophagy via ER stress rather than ATP depletion in cancer cell lines.[32] Autophagy is a crucial component of the cellular stress adaptation response that maintains mammalian homeostasis, a process believed to protect against neurodegenerative and inflammatory conditions, aging, and cancer. Similar to tumor cells defense mechanisms, one can therefore hypothesize that the ER stress consequent to 2-DG treatment in tumour-associated EC may contribute to autophagy and promote cell survival (this study). While protective effects of autophagy were observed in the cancer cell compartment in both laser-induced cell death[33] and in resveratrol-induced cytotoxicity in glioma cells[34], inhibition of autophagy was on the other hand found to potentiate anti-angiogenic effects of sulforaphane in an EC model.[35] Our study provides evidence supporting the latter assumption whereas 2-DG-mediated inhibition of autophagy was only observed in carcinogen-treated HBMEC (Fig.7B). Deployment of therapeutic strategies to block autophagy for cancer and/or anti-angiogenic therapy may therefore be envisioned.

Our study documents the effects of limited intracellular ATP levels not only on the EC angiogenic properties but also on their capacity to express inflammatory markers. We showed that ATP depletion upon pro-carcinogenic stimulation with PMA exacerbates COX-2 expression in HBMEC, an effect correlated to ER stress-mediated mechanism. It has been established that COX-2 is usually undetectable in normal tissues, but can be induced through several stimuli, including mitogens, growth factors, hormones, and cytokines.[36] In recent years, many molecular pathways have been proposed to explain how increased COX-2, and the resulting prostaglandin over-production, may contribute to carcinogenesis.[37] COX-2 mediates this role through the specific production of PGE(2) that acts to inhibit apoptosis, promote cell proliferation, stimulate angiogenesis, and decrease immunity.[38] To date, only few reports document an association between COX-2 expression and survival in EC

consequent to tissue injury. In fact, it is believed that sepsis, consequent to the release of the endotoxin lipopolysaccharide (LPS) by Gram negative bacteria, is regulated trough induction of COX-2 expression. One can therefore envision a close parallel between pro-inflammatory molecular mechanisms involved in endotoxin-induced brain tissue injury and inflammation associated to brain tumour development. Interestingly, LPS-induced COX-2 expression was recently reported in bEnd.3 mouse brain endothelial cells.[39] This study actually provides strong support to our study as the contribution of NF-κB (p65) activation was reported to also regulate COX-2.[39] Noteworthy, LPS-induced adenosine was demonstrated to promote angiogenesis by the up-regulation of VEGF expression in macrophages.[40] Whether such a conceptual model impacts on the link between pro-inflammatory COX-2 and increased tumor angiogenesis remains to be further investigated.

On the other hand, moderate activity of the ER stress response system exerts an anti-apoptotic function and supports tumor cell survival and chemoresistance, whereas more severe aggravation may exceed the protective capacity of this system and turn on its pro-apoptotic module.[41] In our study we demonstrate *in vitro*, through the combination of two pharmacologic approaches, that increased COX-2 expression only occurs within those EC which possess low intracellular ATP levels and which are cultured under pro-carcinogenic conditions. 2-DG-mediated ATP depletion combined with PMA-induced ER stress synergize and inhibit autophagy potential of PMA-treated cells, an effect which ultimately impacts on EC angiogenic properties (i.e. low tubulogenesis and diminished MMP-9 secretion). Similar cytotoxic outcome was recently documented in human breast cancer cells where the HIV protease inhibitor nelfinavir (Viracept) and the COX-2 inhibitor celecoxib (Celebrex) were combined and produced aggravated ER stress, which caused pronounced toxicity.[38]

In summary, the present study has demonstrated that minimal ATP is required for EC-mediated tubulogenesis to occur. Moreover, we provide evidence that combined ATP depletion and pro-carcinogenic signalling trigger exacerbated ER stress that leads to the acquisition of a pro-inflammatory phenotype as reflected by increased COX-2 induction. Altogether, the combined output of those mechanisms may further impact on the cell survival

adaptative response and trigger anti-angiogenic effects within the tumour-associated vascular endothelium.

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Fig.1

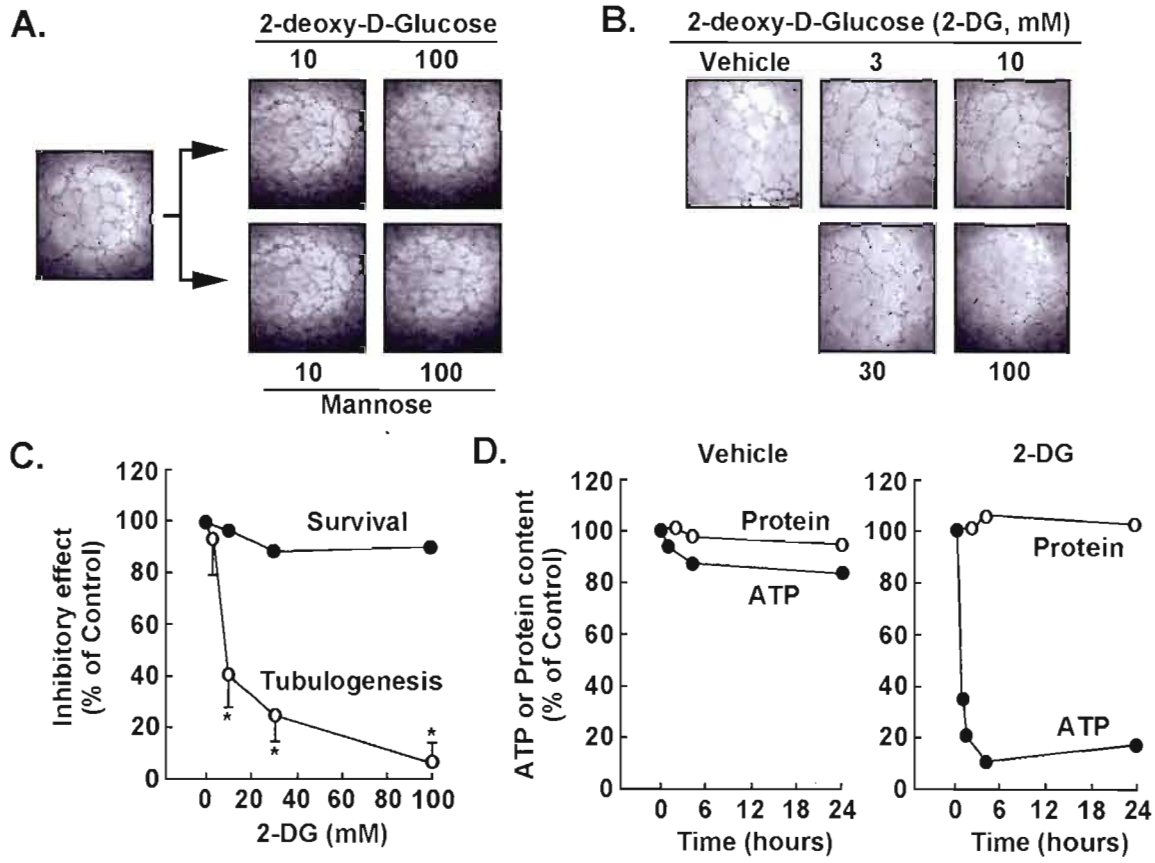


**Fig.1: 2-Deoxy-D-glucose depletes intracellular ATP and inhibits *in vitro* capillary-like structure formation in HBMEC.** In order to assess the impact of ATP requirement for *in vitro* tubulogenesis, HBMEC were seeded on top of Matrigel as described in the Methods section and left to form structures. (A) Capillaries were then treated with 2-deoxy-D-glucose (2-DG) or Mannose. (B) The impact of 2-DG was also monitored during the formation of the structures, where cells were treated with various concentrations of 2-DG 30 minutes after seeding of the cells ontop of Matrigel, and structure formation monitored after 18 hrs. Representative phase contrast pictures were taken. (C) The extent of three-dimensional capillary-like structure formation (tubulogenesis) and of cell survival was assessed as described in the Methods section. (D) Intracellular ATP as well as total protein content were assessed as described in the Methods section in vehicle- or 2-DG-treated cells. The length of the tube network was quantitated using Northern Eclipse software. Values are means of two independent experiments (*$p < 0.01$ versus control alone); bars, ±SD.

## Fig.2

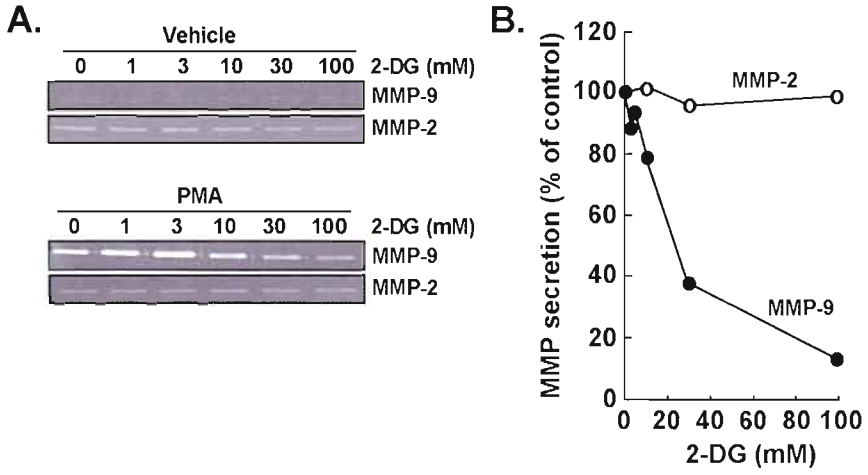


**Fig.2 : 2-Deoxy-D-glucose inhibits PMA-induced MMP-9 secretion in HBMEC.** HBMEC were serum-starved in the presence of various concentrations of 2-deoxy-D-glucose (2-DG) in combination with vehicle or 1 μM PMA for 18 hrs. (A) Conditioned media were then harvested and gelatin zymography was performed in order to detect proMMP-9 and proMMP-2 hydrolytic activity as described in the Methods section. (B) Scanning densitometry was used to quantify the extent of either basal proMMP-2 gelatin hydrolysis (open circles), or proMMP-9 (closed circles) in PMA-treated cells. Data shown is representative of two independent experiments.

## Fig.3

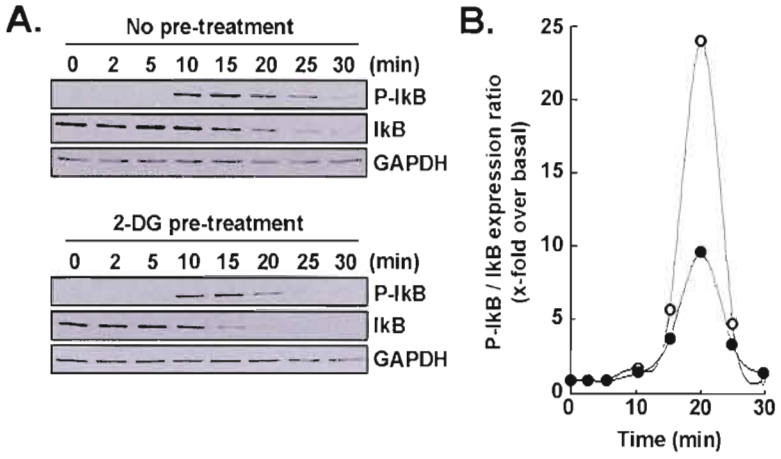


**Fig.3: 2-Deoxy-D-glucose inhibits PMA-induced IκB phosphorylation that leads to IκB degradation.** (A) HBMEC were serum-starved for 30 minutes in the presence of either vehicle or 100 mM 2-deoxy-D-glucose (2-DG). Cells were then incubated for the indicated time with 1 μM PMA. Lysates were isolated, electrophoresed via SDS-PAGE and immunodetection of phosphorylated IκB (P-IκB), IκB, and of GAPDH proteins was performed as described in the Methods section. (B) Quantification was performed by scanning densitometry of the autoradiograms. Data were expressed as x-fold induction over basal untreated cells of the P-IκB/IκB ratios in vehicle pre-treated cells (open circles) and 2-DG pre-treated cells (closed circles).

# Fig.4

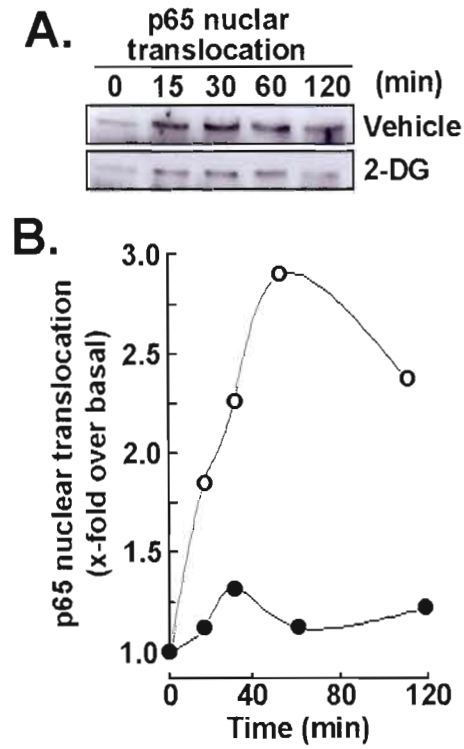


**Fig.4 : 2-Deoxy-D-glucose inhibits PMA-induced nuclear translocation of the p65 subunit of NF-κB.** (A) HBMEC were serum-starved for 30 minutes in the presence of either vehicle or 100 mM 2-deoxy-D-glucose (2-DG). Cells were then incubated for the indicated time with 1 μM PMA. Nuclear extracts were isolated, electrophoresed via SDS-PAGE and immunodetection of the p65 subunit of NF-κB protein was performed as described in the Methods section. (B) Quantification was performed by scanning densitometry of the autoradiograms. Data were expressed as x-fold induction over basal untreated cells of the vehicle pre-treated cells (open circles) and 2-DG pre-treated cells (closed circles).

## Fig.5

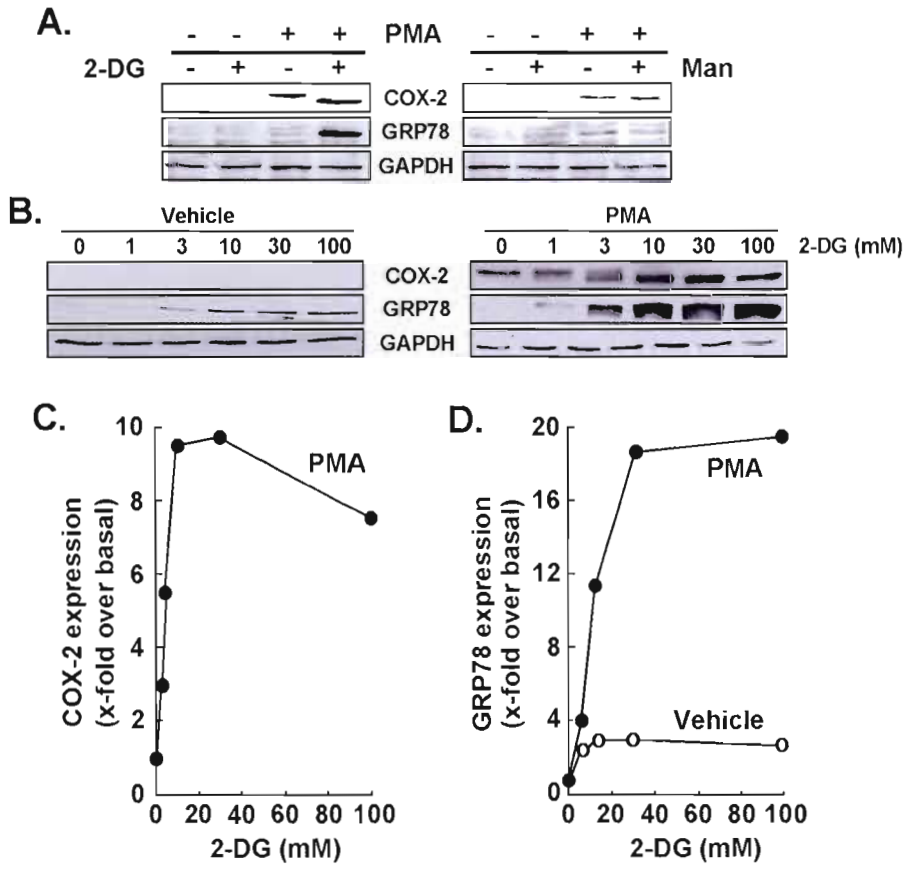


**Fig.5: 2-Deoxy-D-glucose potentiates PMA-induced cyclooxygenase-2 and GRP78 expression.** (A) HBMEC were serum starved for 18 hours in the presence or absence of 1 μM PMA and in combination with either 30mM 2-deoxy-D-glucose (2-DG) or 30 mM Mannose (Man). Lysates were isolated, electrophoresed via SDS-PAGE, and immunodetection of COX-2, GRP78, and GAPDH was performed as described in the Methods section. (B) HBMEC were treated as in (A) with various doses of 2-DG. Lysates were isolated, electrophoresed via SDS-PAGE, and immunodetection of COX-2, GRP78, and GAPDH was performed as described in the Methods section. (C) Scanning densitometry of COX-2 expression was only performed in PMA-treated cells since no COX-2 was detectable in vehicle-treated cells. (D) Scanning densitometry of GRP78 expression was performed in vehicle- and in PMA-treated cells.

## Fig.6

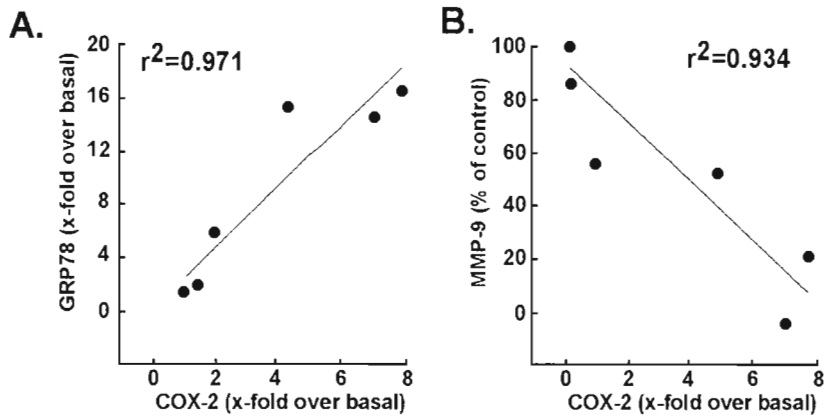


**Fig.6: Increased endoplasmic reticulum stress, rather than MMP-mediated ECM hydrolysis, correlates with cyclooxygenase-2 expression in HBMEC.** The effects of PMA treatments in the presence of increasing 2-deoxy-D-glucose (2-DG) concentrations were plotted in order to assess any correlation between (A) ER stress (GRP78 expression) and inflammation (COX-2 expression), and (B) between ECM hydrolysis (MMP-9 expression) and inflammation.

# Fig.7

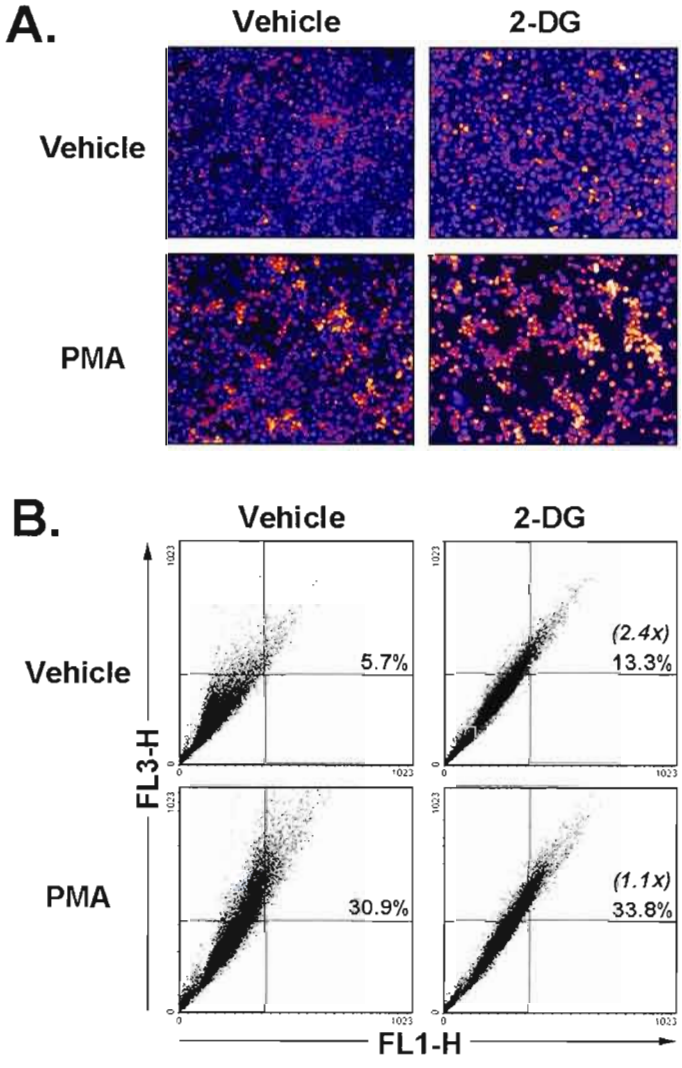


**Fig.7: 2-Deoxy-D-glucose-induced autophagy is abrogated in PMA-treated HBMEC.** Autophagy was assessed as described in the Methods section in vehicle, 100 mM 2-DG-, 1 μM PMA, and 100 mM 2-DG/ 1 μM PMA-treated HBMEC. (A) Representative microphotographs of the formation of acidic vesicular organelles were taken upon acridine orange staining. (B) The extent of autophagy was further confirmed by flow-cytometry of acridine orange stained cells and the percent of autophagy indicated for each condition. In between parenthesis, the extent of autophagy induction is indicated.

## Article III

# Flavonoids targeting of IκB phosphorylation abrogates carcinogen-induced MMP-9 and COX-2 expression in human brain endothelial cells

Elizabeth Tahanian[1], Luis Arguello Sanchez[1], Tze Chieh Shiao[2], René Roy[2*] and Borhane Annabi[1*]

Description de la contribution des auteurs de cet article :

Tahanian : Expérimentation essais biologiques, analyse des résultats, montage des figures, rédaction

Arguello-Sanchez : Expérimentation essais biologiques, analyse des résultats

Shiao : Expérimentations synthèse chimique, montage des figures, rédaction

Roy : Analyse des résultats, rédaction, financement

Annabi : Analyse des résultats, montage des figures, rédaction, financement

## Article III

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*The abbreviations used are :* BBB, blood-brain barrier; COX, cyclooxygenase; EC, endothelial cells; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; MMP-9, matrix metalloproteinase-9; NF-□B, nuclear factor-kappa B; PMA, phorbol 12-myristate 13-acetate

## Abstract

Brain endothelial cells play an essential role as structural and functional components of the blood-brain barrier (BBB). Increased BBB breakdown and brain injury are associated with neuroinflammation and are thought to trigger mechanisms involving matrix metalloproteinase up-regulation. Emerging evidence also indicates that cyclooxygenase (COX) inhibition limits blood-brain barrier disruption, but the mechanisms linking metalloproteinase to cyclooxygenase remain unknown. In this study, we sought to investigate the nuclear factor-kappa B (NF-κB) signalling pathway, a common pathway in both the regulation of matrix metalloproteinase-9 (MMP-9) and COX-2 expression, and the inhibitory properties of several chemopreventive flavonoids. Human brain microvascular endothelial cells were treated with a combination of phorbol 12-myristate 13-acetate (PMA), a carcinogen documented to increase MMP-9 and COX-2 through NF-κB, and several naturally occuring flavonoids. Among the molecules tested, we found that Fisetin, Apigenin and Luteolin specifically and dose-dependently antagonized PMA-induced COX-2 and MMP-9 gene and protein expressions as assessed by qRT-PCR, immunoblotting and zymography respectively. We further demonstrate that flavonoids impact on IκK-mediated phosphorylation activity as demonstrated by the inhibition of PMA-induced IκB phosphorylation levels. Our results suggest that BBB disruption during neuroinflammation could be pharmacologically reduced by a specific class of flavonoids acting as NF-κB signal transduction inhibitors.

## Introduction

Tumour-associated angiogenesis, a fundamental process in tumour growth and metastasis, consists of recruiting endothelial cells (EC) toward an angiogenic stimulus.[1] The cells subsequently proliferate and differentiate to form endothelial tubes and capillary-like structures in order to deliver nutrients and oxygen to the tumour and to remove the products of its metabolism. In recent years several pathways have, in addition to stimulation of tumor angiogenesis, been suggested to contribute to the cell metabolic adaptations required for carcinogenesis, which include decreased tumoural apoptosis, increased invasion and metastasis, immune suppression and tumour-associated inflammation.[2,3] An interesting link between overexpression of the pro-inflammatory marker cyclooxygenase (COX)-2 and tumour angiogenesis was recently described as one such metabolic adaptative phenotype.[4-6] This is supported by the fact that in normal cerebral cortex COX-2 is only present in neurons but absent from vascular EC.[7,8] It is however still unknown whether the EC-associated COX-2 correlates with high malignancy. Furthermore, little is known about the molecular events that dictate metabolic adaptation of EC in response to pro-carcinogenic stimuli. It is tempting to suggest that specific inhibition of metabolic pathways may offer a novel therapeutic approach that would simultaneously inhibit tumor-induced angiogenesis and inflammatory phenotypes.[9,10]

While human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components of the blood-brain barrier (BBB), its disruption by the brain tumor-secreted matrix metalloproteinase-9 (MMP-9) is believed to favor tumor invasion.[11,12] Recent studies delineated a unique brain endothelial phenotype in which MMP-9 secretion by HBMEC was increased upon treatment with the tumor-promoting agent phorbol 12-myristate 13-acetate (PMA).[13,14] Inhibition of MMP-9 secretion was demonstrated to reduce both *in vitro* invasion and angiogenesis in human microvascular EC.[15] Among strategies developed to inhibit extracellular matrix (ECM) degradation and inflammation processes, the design, synthesis and evaluation of flavonoid derivatives has recently emerged as a potent strategy to target neurodegenerative disorders including different forms of dementia, as well as Alzheimer's disease.[16] In fact, a large number of

mechanisms of action have been attributed to flavonoids commonly found in fruits, vegetables, wine, or tea as they can act as potent antioxidants and free radical scavengers.[17,18] The rationale underlying the selection of flavones and related compounds was herein dictated by the known activity of Apigenin against several forms of cancer, including leukemia, due in part to its action through JAK/STAT and PI3K/PKB signaling pathways.[19] Thus, Apigenin, Luteolin, and Fisetin were considered potential good candidates possessing polyphenolic functions capable of counteracting oxidative mechanisms. Indeed, the three compounds are lacking hydrophylic sugar or sugar-like moiety, consequently, they are more hydrophobic and drug-like candidates. Quercitrin was also chosen because it has a very similar structure to that of Fisetin and yet exposes a rhamnoside residue which renders it more closely related to the other family of tested compounds (Chlorogenic acid, Arbutin, Salicin, Phlorizin, and Coniferin), hence filling the gap for our QSAR profiles. These phenolic glycosides, while possessing some structuraly related features, do not have the key flavone backbone.

Among the signalling pathways, NF-κB signalling is the one that enables the control of both MMP-9 and COX-2 inflammation marker expression.[20, 21] This current study therefore focuses on flavonoids as potential signal transduction inhibitors of carcinogen-mediated induction of the NF-κB pathway in a brain EC model. Eight flavonoids were evaluated: flavonols - Fisetin; flavones – Apigenin, Luteolin, and flavonol-glycoside Quercetrin; as well as Chlorogenic acid and few phenolic glucosides - Arbutin Salicin, Phlorizin, and Coniferin. The aim of the study was to relate the structural differences of the flavonoids to their potency to inhibit PMA-induced MMP-9 and COX-2 expression in HBMEC.

## Materials and methods

*Reagents :* Sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The polyclonal antibodies against IκB and phospho-IκB were purchased from Cell Signaling (Danvers, MA). The monoclonal antibody against GAPDH was from Advanced Immunochemical Inc. (Long Beach, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). All other reagents were from Sigma-Aldrich Canada. Eight flavonoids were evaluated : flavonol – Fisetin (2-(3,4-dihydroxyphenyl)-3,7-dihydroxychromen-4-one); flavones: Apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) and Luteolin (2-(3,4-dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone), the flavonol-glycoside Quercitrin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyl-2-tetrahydro-pyranyl]oxy]-4-chromenone), Chlorogenic acid ((1*S*,3*R*,4*R*,5*R*)-3-{[(2*Z*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid), as well as a few phenolic glucosides - Arbutin ((*2R,3S,4S,5R,6S*)-2-hydroxymethyl-6-(4-hydroxyphenoxy)oxane-3,4,5-triol), Salicin ((2*R*,3*S*,4*S*,5*R*,6*S*)-2-(hydroxymethyl)-6-[2-(hydroxymethyl)phenoxy]oxane-3,4,5-triol), Phlorizin (1-[2,4-dihydroxy-6-[(2*S*,3*R*,4*R*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-phenyl]-3-(4-hydroxyphenyl) propan-1-one), and Coniferin (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(hydroxymethyl)-6-[4-[(*E*)-3-hydroxyprop-1-enyl]-2-methoxyphenoxy]oxane-3,4,5-triol. The phenolic flavonoid derivatives were either purchased from Sigma-Aldrich (USA) or donated from the personal collection of Prof. Ragai K. Ibrahim from the University of Concordia (Montreal, QC).

*Cell culture*: Human brain microvascular endothelial cells (HBMEC) were characterized and generously provided by Dr Kwang Sik Kim of the Johns Hopkins University School of Medicine (Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV and Ulex Europeus Agglutinin I; they took up fluorescently

labelled, acetylated low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain EC-specific phenotype.[18] HBMEC were immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages.[22] HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (iFBS) (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%) (Gibco), sodium pyruvate (1 mM) and EC growth supplement (30 μg/ml). Culture flasks were coated with 0.2% type-I collagen to support the growth of HBMEC monolayers. Cells were cultured at 37°C under a humidified atmosphere containing 5% $CO_2$. All experiments were performed using passages 3 to 28.

*Gelatin zymography:* Gelatin zymography was used to assess the extent of proMMP-9 activity as previously described.[23] Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled $H_2O$. Gels were further incubated at 37°C for 20 hrs in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in $H_2O$. Gelatinolytic activity was detected as unstained bands on a blue background.

*Immunoblotting procedures:* Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 hr at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin and 0.1% sodium azide, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1/2,500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

*Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR:* Total RNA was extracted from cell monolayers using TriZol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 2 μg of total RNA were reverse-transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at -80°C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). DNA amplification was carried out using an Icycler iQ5 (Bio-Rad, Hercules, CA) and product detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-stranded DNA. The QuantiTect primer sets were provided by Qiagen (Valencia, CA): MMP-9 (QT00040040), COX-2 (QT00040586), β-Actin (QT01136772). GAPDH primer sets were synthesized by Biocorp (Dollard-des-Ormeaux, QC) with the following sequences: forward CCATCACCATCTTCCAGGAG and reverse CCTGCTTCACCACCTTCTTG. The relative quantities of target gene mRNA compared against two internal controls, GAPDH and β-Actin mRNA, were measured by following a $\Delta C_T$ method employing an amplification plot (fluorescence signal *vs*. cycle number). The difference ($\Delta C_T$) between the mean values in the triplicate samples of target gene and those of GAPDH and β-actin mRNAs were calculated by iQ5 Optical System Software version 2.0 (Bio-Rad, Hercules, CA) and the relative quantified value (RQV) was expressed as $2^{-\Delta C}{}_T$.

*Endothelial cell morphogenesis assay:* Tubulogenesis was assessed using Matrigel aliquots of 50 μL, plated into individual wells of 96-well tissue culture plates (Costar, Amherst, MA) and allowed to polymerize at 37°C for 30 minutes. After brief trypsinization, HBMEC were washed and resuspended at a concentration of $10^6$ cells/ml in serum-free medium. Twenty-five μL of cell suspension (25,000 cells/well) and 75 μL of medium with serum were added into each culture well. Cells were allowed to form capillary-like tubes at 37°C in 5% $CO_2$/95% air for 20 hours in the presence or absence of 30 μM of the tested molecules. The formation of capillary-like structures was examined microscopically and images (10x) were recorded using a Retiga 1300 camera (QImaging) and a Nikon Eclipse TE2000-U microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like

network, using a commercially available image analysis program (Northern Eclipse) as described and validated previously.[24,25] For each experiment, four randomly chosen areas were quantified by counting the number of tubes formed. Tubulogenesis data are expressed as a mean value derived from at least three independent experiments.

*Statistical data analysis :* Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of less than 0.05 were considered significant and an asterisk identifies such significance in the figures.

## Results

*Fisetin, Apigenin, and Luteolin inhibit HBMEC in vitro capillary-like structure formation.* The effects of chlorogenic acid and of eight structurally-related phenolic derivatives (Fig.1), all natural molecules present in plants, were tested on HBMEC. Matrigel induced tubulogenesis assay was used to assess the effect of flavonoids and phenolic derivatives on capillary-like structure formation in HBMEC. As described in the Methods section, cells were seeded on top of Matrigel and left to adhere. Several tested compounds were then added and capillary formation left to proceed for 18h. We found that, in vehicule-treated cells as well as in Chlorogenic acid-, Arbutin-, Salicin-, Phlorizin-, Coniferin-, and Quercitrin-treated cells capillary-like formation was well-defined (Fig.2A) in comparison to cells exposed to 30 □M of the non-glycosidic flavonoids Apigenin, Luteolin, or Fisetin which had their structures significantly disrupted (Fig.2B).

*Flavonoids inhibition of carcinogen-induced MMP-9 gene expression and protein secretion.* Among the secreted enzymes involved in ECM degradation, matrix metalloproteinases (MMP) are well-documented as being involved in cell migration and tubulogenesis.[13,26] MMP-9, an enzyme involved in the degradation of the extracellular matrix (ECM), is secreted by a variety of cells and its presence was shown to be increased upon carcinogen promoting agents such as the phorbol ester PMA.[27-29] HBMEC were treated for 18h with the above mentioned flavonoids in serum-free medium. Gelatin zymography (Fig.3A) was then used to measure MMP-9 levels, which were significantly increased upon PMA treatment in comparison to vehicule-treated cells (Fig.3B). Addition of the non-glycosidic flavonoids Fisetin, Apigenin, or Luteolin to PMA-treated cells resulted in inhibition of MMP-9 activity (Fig.3B). It was found that PMA also increased MMP-9 gene expression while the presence of Fisetin, Apigenin, or Luteolin inhibited this increase suggesting transcriptional regulation of the MMP-9 gene (Fig.3C). The anti-MMP-9 effects of Fisetin, Apigenin, and Luteolin were also found to be dose-dependent as assessed by zymography (Fig.4A), with a Ki of 1,6 μM, 2,2 μM, and 8,3 μM respectively for Fisetin, Luteolin, and Apigenin (Fig.4B).

*Flavonoids inhibition of carcinogen-induced COX-2 gene and protein expression.* Various molecular mechanisms mediate inflammatory processes and angiogenesis, one of which is reflected by increased expression of the inflammatory biomarker COX-2.[30] In order to investigate the effect of flavonoids on HBMEC-associated inflammation, we tested the effects of the flavonoids on PMA-induced cell signaling in HBMEC by Western blotting. Cells were therefore treated with 1 μM of PMA in the presence of 30 μM of the flavonoid for 18h and COX-2 expression was evaluated in cell lysates by Western Blotting (Fig.5A). We found that the non-glycosidic derivatives Fisetin, Apigenin, and Luteolin significantly inhibited COX-2 protein (Fig.5B) and gene (Fig.5C) expression in the presence of PMA, whereas PMA-induced COX-2 expression was not affected by the other molecules. Further experiments were performed by treating HBMEC with various concentrations of Fisetin, Apigenin, or Luteolin in the presence of PMA for 18h (Fig.6A). These molecules were found to inhibit the COX-2 protein expression in a dose-dependent manner (Fig.6B).

*Carcinogen-induced IκB phosphorylation is inhibited by Fisetin, Luteolin, and Apigenin.* Among MMP-9 expression regulators, the nuclear factor-kappaB (NF-κB) signalling pathway has been demonstrated to link cancer to inflammatory diseases.[31] We therefore assessed whether this signalling was activated upon PMA treatment and whether it was reflected in IkappaB (IκB) degradation. HBMEC were serum-starved then treated with 1 μM PMA up to 25 minutes, lysates were isolated and IκB phosphorylation was assessed through Western Blotting (Fig.7A, upper panel). PMA signalling led to the phosphorylation of IκB at 15 minutes, followed by a decrease in IκB expression (Fig.7A, lower panel).[32] Inhibition of PMA-mediated phosphorylation of IκB was next assessed in order to demonstrate whether this mechanism contributes to the anti-MMP-9 and anti-COX-2 inhibitory activities of best three flavonoids identified above. Preincubation with Fisetin, Luteolin, or Apigenin followed by a 15 min PMA treatment led to IκB phosphorylation and to a concomitant dose-dependent decrease in IκB for Apigenin only (Fig.7B). Inhibition of IκB phosphorylation by Fisetin and Luteolin led to reappearance of IκB. The ratios of phosphorylated IκB over total IκB expression were quantified by scanning densitometry and represented (Fig.7C).

## Discussion

The adaptive mechanisms responsible for EC survival under pro-carcinogenic conditions remain poorly documented. EC are believed to be metabolically robust and to adapt to pro-carcinogenic paracrine stimulation and conditions such as encountered within the hypoxic tumour microenvironment.[33,34] Although increasing interest has been manifested towards cancer therapies that target cell metabolism, very few studies have specifically assessed the combined impact of targeting the EC angiogenic and inflammatory phenotype. In our study, we induced *in vitro* pro-carcinogenic stimulation of brain microvascular endothelium using PMA in combination with naturally-occurring flavonoids and phenolic glycosides in order to assess their anti-angiogenic and anti-inflammatory properties. *In vitro* tubulogenesis, PMA-induced MMP-9 secretion and expression of COX-2 were therefore assessed in order to provide a metabolic and adaptative link between endothelial inflammation and angiogenesis.

Our study highlights the combined anti-angiogenic and anti-inflammatory effects of non-glycosidic flavonoids against carcinogen-stimulated HBMEC, as demonstrated by decreased MMP-9 and COX-2 expression biomarkers. We showed that Fisetin, Apigenin, and Luteolin, upon pro-carcinogenic stimulation with PMA, efficiently inhibited both MMP-9 secretion and COX-2 expression in HBMEC, an inhibitory effect that we believe to be mediated through the common NF-κB signaling pathway that regulates both biomarkers' gene and protein expression. Our findings support those obtained in several other cell models. In fact, Fisetin's anti-inflammatory effects were found to suppress lipopolysaccharide-induced NF-κB activation in macrophage and dendritic cell maturation,[35] and in tumor necrosis factor-induced NF-κB activation in human lung adenocarcinoma cells.[36] Since Fisetin and Quercitrin have analogous aglyconic structures, the lack of activity of the later points toward the aglycons as the pharmacophoric entity. This is somewhat clearly illustrated in comparison the the phenolic glycosides which have phenols in common but are missing the flavonoid skeleton. As for Apigenin, several studies reported suppression of PMA-induced tumor cell invasion,[37] of PMA-induced COX-2 transcriptional activity,[38] and inhibition of inflammatory mediators release in human mast cell lines.[39] Finally, Luteolin

was found to suppress phorbol ester TPA (a mimic of diacylglycerol and PKC activator)-induced MMP-9 activation in a glioblastoma cell line model.[40] Altogether, our findings not only support the potential anti-inflammatory properties of those 3 molecules reported in tumoral and immune compartments, they now further highlight important anti-angiogenic effects on the vascular compartment as reflected through the inhibition of *in vitro* tubulogenesis and of carcinogen-induced MMP-9.

To date, only few reports documented an association between COX-2 expression and ECM degradation consequent to pro-carcinogenic stimulation. Among the numerous signaling pathways triggered by pro-carcinogenic culture conditions, NF-κB is at the crossroads of both MMP-9 and COX-2 regulation by PMA, but the intracellular players still remain undefined at least within the anti-angiogenic effects we report herein. Among the intracellular events that could link PMA-induced signaling to COX-2 induction, NF-κB can contribute to regulate the expression of COX-2 through endoplasmic reticulum (ER) stress and, in part, through induction of the ER chaperone GRP78/BiP, which is expressed at high levels in a variety of tumors and which confers drug resistance to both proliferating and dormant cancer cells.[16] Importantly, it was recently demonstrated that partial reduction of GRP78 substantially reduced tumor microvessel density.[17] On the other hand, moderate activity of the ER stress response system exerts an anti-apoptotic function and supports tumor cell survival and chemoresistance, whereas more severe aggravation may exceed the protective capacity of this system and turn on its pro-apoptotic module.[41] In a recent study, we further demonstrated *in vitro* through the combination of two pharmacologic approaches that increased COX-2 expression only occurs within those EC which possess low intracellular ATP levels and which are cultured under pro-carcinogenic conditions.[42] Noteworthy, Luteolin was shown to change ATP levels and trigger ER stress-induced cell death.[43]

Several flavonoids have also been reported to interfere with the oxidative damages activity of inducible nitric-oxide synthase activity, and to play a nitric oxide scavenging role in the therapeutic effects of flavonoids.[44] Nitric oxide is produced by several different types of cells, including endothelial cells and macrophages. When flavonoids are used as

antioxidants, free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage. Selected phenolic compounds were also shown to inhibit both the COX and 5-lipoxygenase pathways.[45] Moreover, the antiinflammatory ability of flavonoids to inhibit eicosanoid biosynthesis, such as prostaglandins which are the end products of the COX and lipoxygenase pathways, has also been reported.[46] The exact mechanism by which flavonoids inhibit these enzymes is not clear.

In summary, the present study has allowed the identification and molecular characterization of three specific flavonoids to act as inhibitors of EC-mediated tubulogenesis, and as signal transduction inhibitors against carcinogen-mediated induction of COX-2 and MMP-9, while phenolic glycosides were shown to be inactive including Quercitrin, a closely related rhamnosylated analog of the above flavonoids. Since Fisetin and Quercitrin have analogous aglyconic structures, the lack of activity of the later points toward the aglycons as the pharmacophoric entity. This is somewhat clearly illustrated in comparison the the phenolic glycosides which have phenols in common but are missing the flavonoid skeleton. Moreover, we provide evidence that the NF-κB pathway may be inhibited through the targeting of IκK phosphorylation capacity that ultimately may reduce both the acquisition of a pro-inflammatory phenotype, as reflected by decreased COX-2 expression, and the acquisition of pro-angiogenic phenotype, as reflected by a decrease in MMP-9. Our results therefore suggest that BBB disruption during neuroinflammation could be pharmacologically reduced by a specific class of flavonoids acting as NF-κB signal transduction inhibitors.

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# Fig.1

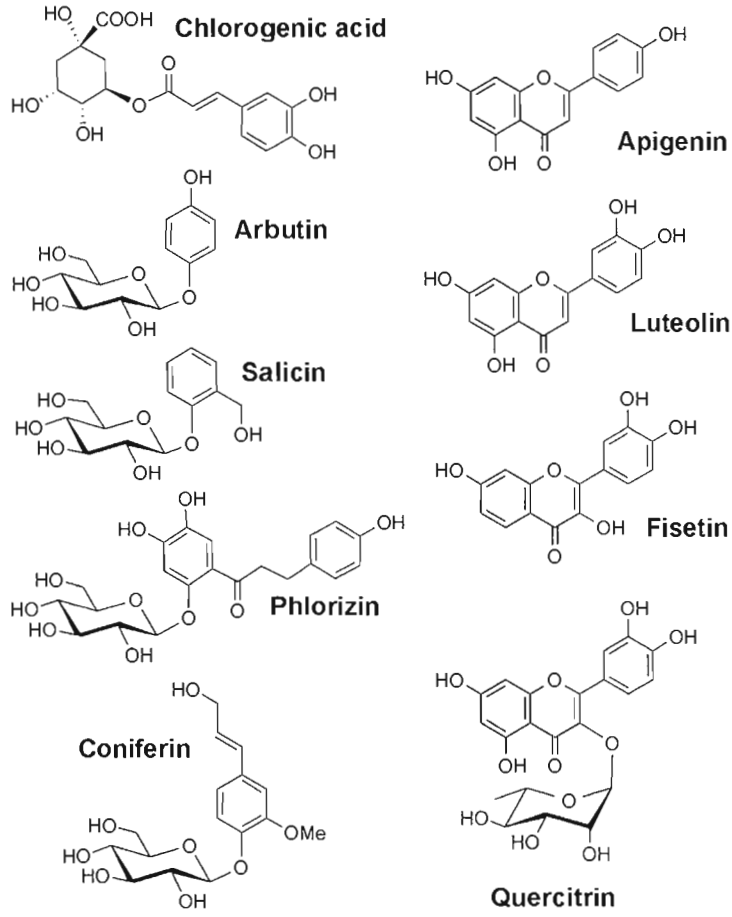


Fig.1 : **Chemical structures of the flavonoids used to antagonize carcinogen-induced MMP-9 and COX-2 expressions.** The different moieties of these compounds are indicated.

# Fig.2

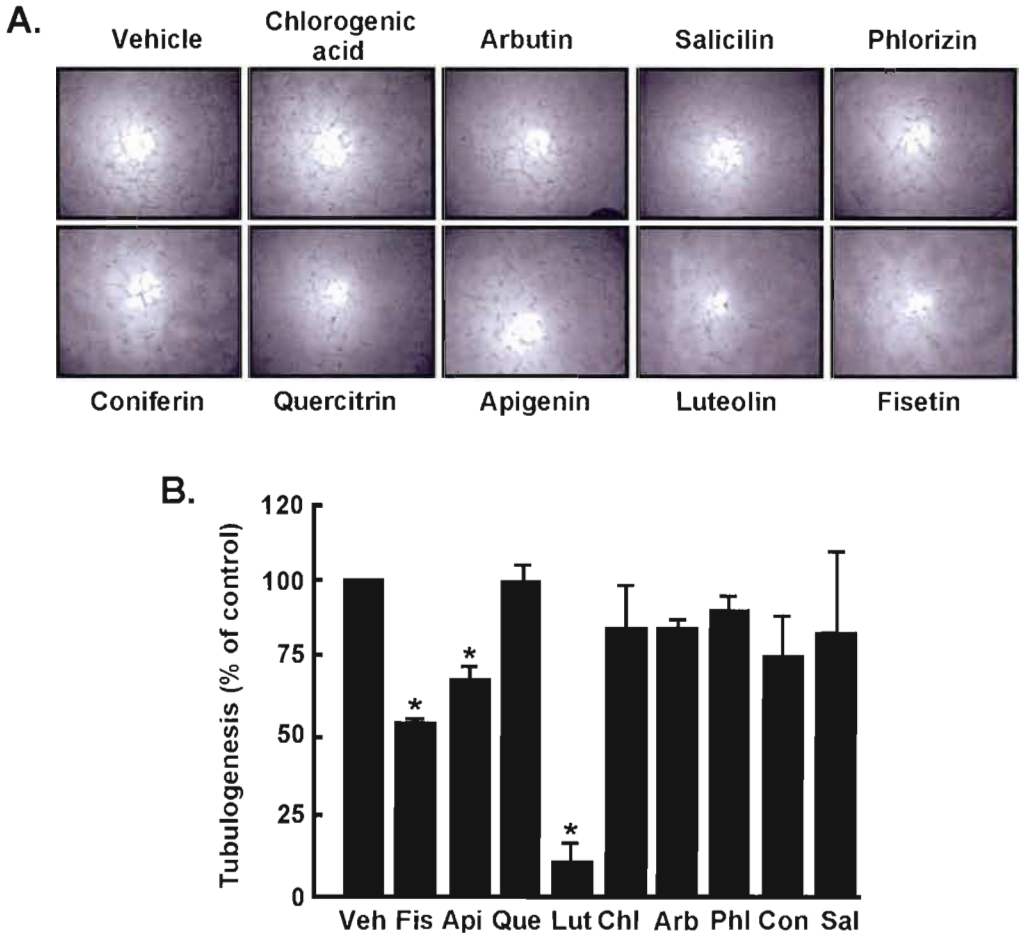


**Fig.2 : Inhibition of *in vitro* capillary-like structure formation by flavonoids in HBMEC.** In order to assess the potential antiangiogenic properties of flavonoids, *in vitro* tubulogenesis assay was performed with HBMEC seeded on top of Matrigel as described in the Methods section. (A) Molecules (30 μM) were added 30 minutes after seeding of the cells ontop of Matrigel. Structure formation was monitored after 18 hours. Representative phase contrast pictures were taken. (B) The extent of three-dimensional capillary-like structure formation (tubulogenesis) was assessed as described in the Methods section. The length of the tube network was quantitated using Northern Eclipse software. Data are representative of three independent experiments. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance to the respective vehicle treatment.

Fig.3

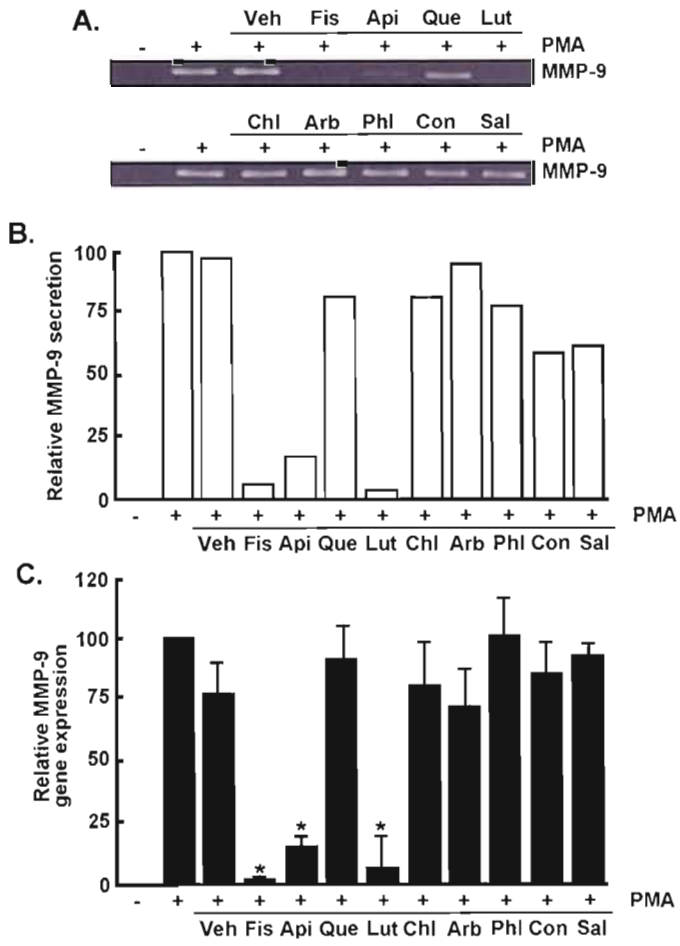


**Fig.3: Flavonoids inhibition of carcinogen-induced MMP-9 gene expression and protein secretion.** HBMEC were serum-starved in the presence of various flavonoids (30 μM) in combination with vehicle or 1 μM PMA for 18 hours. (A) Conditioned media were then harvested and gelatin zymography was performed in order to detect PMA-induced proMMP-9 and hydrolytic activity as described in the Methods section. (B) Scanning densitometry was used to quantify the extent of proMMP-9 gelatinolytic activity in treated cells. Data shown is representative of two independent experiments. (C) Total RNA isolation and qRT-PCR were performed as described in the Methods section to assess MMP-9 gene expression in the above-described conditions. Data are representative of three independent qPCR experiments. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance to the respective PMA treatment.

# Fig.4

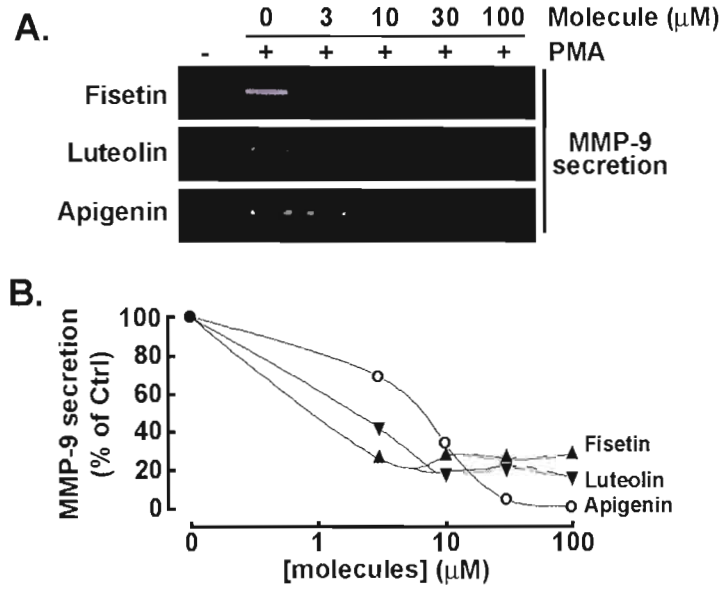


**Fig.4: Dose-dependent inhibition of MMP-9 secretion by Fisetin, Luteolin, and Apigenin.** HBMEC were serum-starved in the presence of various concentrations of Fisetin, Luteolin, and Apigenin in combination with vehicle or 1 μM PMA for 18 hours. (A) Conditioned media were then harvested and gelatin zymography was performed in order to detect PMA-induced proMMP-9 and hydrolytic activity as described in the Methods section. (B) Scanning densitometry was used to quantify the extent of proMMP-9 gelatinolytic activity in treated cells. Data shown is representative of two independent experiments.

Fig.5

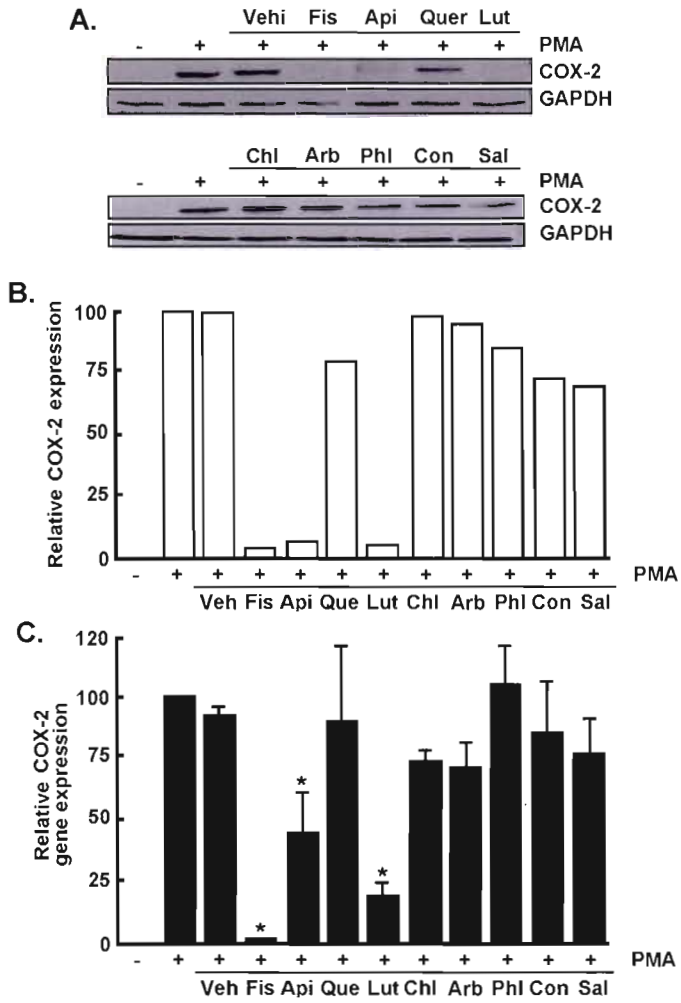


**Fig.5: Flavonoids inhibition of carcinogen-induced COX-2 gene and protein expression.** (A) HBMEC were serum-starved in the presence of various flavonoids (30 μM) in combination with vehicle or 1 μM PMA for 18 hours. Lysates were isolated, electrophoresed via SDS-PAGE, and immunodetection of COX-2 and GAPDH performed as described in the Methods section. (B) Scanning densitometry of COX-2 expression was only performed in PMA-treated cells since no COX-2 was detectable in vehicle-treated cells. Densitometric data of a representative blot is shown. (C) Total RNA isolation, RT-PCR, and qPCR were performed as described in the Methods section to assess COX-2 gene expression in the above-described conditions. Data are representative of three independent qPCR experiments. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance to the respective PMA treatment.

## Fig.6

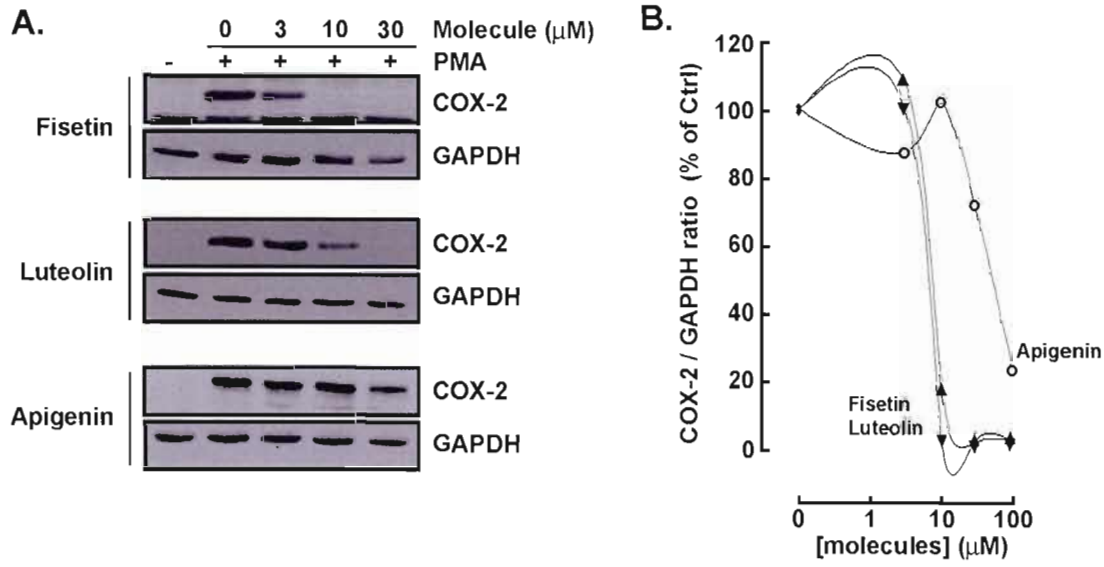


**Fig.6: Dose-dependent inhibition of COX-2 expression by Fisetin, Luteolin, and Apigenin.** HBMEC were serum-starved in the presence of various concentrations of Fisetin, Luteolin, and Apigenin in combination with vehicle or 1 μM PMA for 18 hours. (A) Lysates were isolated, electrophoresed via SDS-PAGE, and immunodetection of COX-2 and GAPDH performed as described in the Methods section. (B) Scanning densitometry of COX-2 expression was only performed in PMA-treated cells since no COX-2 was detectable in vehicle-treated cells. Densitometric data of a representative blot out of three is shown.

# Fig.7

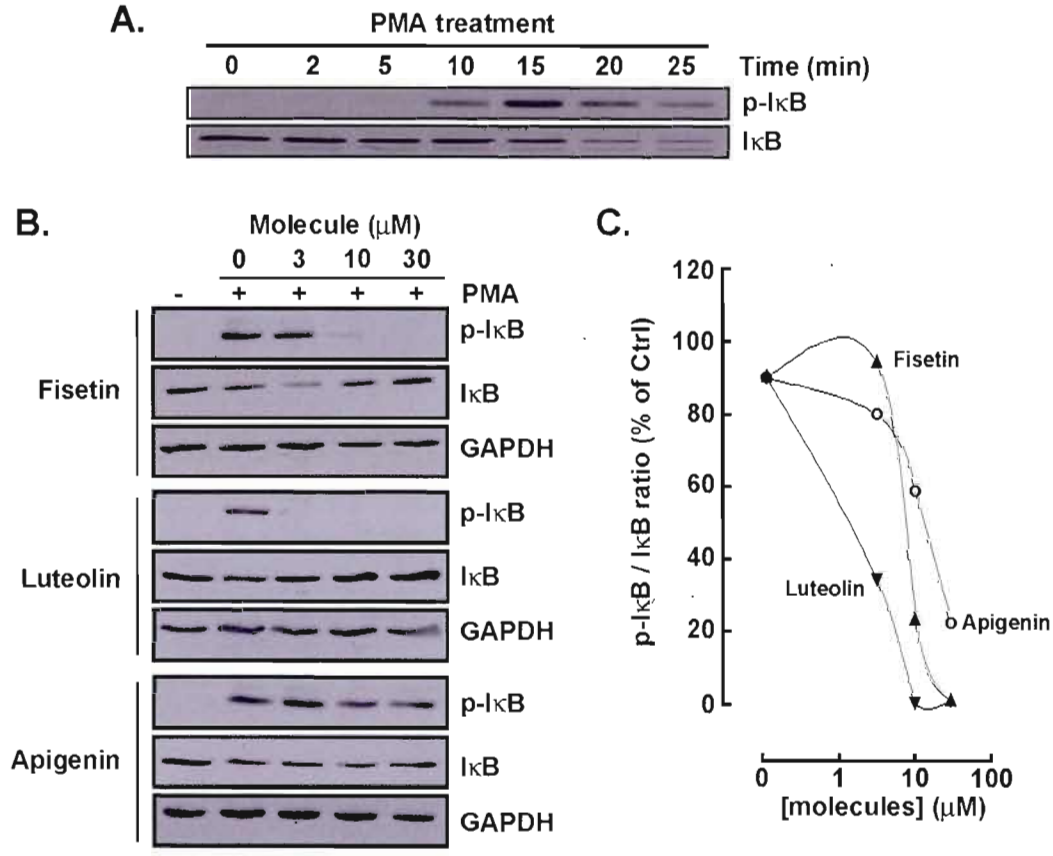


**Fig.7: Carcinogen-induced IκB phosphorylation is inhibited by Fisetin, Luteolin, and Apigenin.** (A) HBMEC were serum-starved for 30 minutes then treated with 1 μM PMA for the indicated time. Lysates were isolated, electrophoresed via SDS-PAGE and immunodetection of phosphorylated IκB (P-IκB) and IκB proteins was performed as described in the Methods section. (B) HBMEC were serum-starved for 30 minutes in the presence of either vehicle or 30 μM Fisetin, Luteolin, and Apigenin. Cells were then incubated for 15 minutes with 1 μM PMA. Lysates were isolated, electrophoresed via SDS-PAGE and immunodetection of phosphorylated IκB (P-IκB), IκB, and of GAPDH proteins was performed as described in the Methods section. (C) Quantification was performed by scanning densitometry of the autoradiograms. Data were expressed as the percent of basal P-IκB/IκB ratios in vehicle pre-treated cells. Densitometric data of a representative blot out of three is shown.

# CHAPITRE IV

# DISCUSSION

Les études présentées dans ce mémoire ont pavé la voie à l'identification de molécules d'intérêt thérapeutique et à une meilleure compréhension des mécanismes d'adaptation métabolique des CE régulant la vascularisation tumorale. Le développement tumoral est dépendant de la prolifération des CE et de l'angiogénèse afin d'assurer un apport adéquat en oxygène et en nutriments. Il est donc stratégique de cibler les vaisseaux sanguins en formation comme approche thérapeutique anti-angiogénique. À cet effet, le ciblage de la vascularisation tumorale en combinaison à la radiation ionisante est déjà, par exemple, un nouveau traitement utilisé contre le cancer (Annabi et *al.*, 2003). Les travaux rapportés dans ce mémoire ont permis de mettre à jour l'impact pharmacologique de molécules avec des fonctions inhibitrices des voies de signalisation impliquées dans l'angiogenèse tumorale cérébrale. Les CE vasculaires cérébrales que nous avons utilisées représentent d'un point de vue conceptuel, le modèle qui se rapproche le plus du phénotype et des fonctions des CE dérivées de tumeurs cérébrales (McLaughlin et *al.*, 2006; Stins et *al.*, 1997). En effet, la disponibilité d'un modèle humain stable de CE cérébrales permet de faire des études originales donnant une meilleure représentation de la microvasculature cérébrale.

En premier lieu, nous avons investigué les effets de différentes molécules sur la formation de structures capillaires *in vitro* par les CE. Nous avons montré que l'analogue de la mumbaistatine, l'AD4-015, un puissant inhibiteur du G6PT, inhibait d'une manière dépendante de la dose la tubulogenèse *in vitro*. Cette inhibition est également observée lorsque les HBMEC sont traitées avec un analogue du glucose le 2DG, et avec certains flavonoides, tels l'apigenine, la lutéoline et la fisetine. Pour que le processus de la tubulogenèse puisse se produire nous démontrons également que les HBMEC requièrent un minimum d'énergie. Nos résultats suggèrent en effet que l'ATP est essentielle dès les premiers stades de la formation de la tubulogenèse.

La migration des CE a aussi été analysée en comparant l'effet de l'AD4-015 avec celui de l'acide chlorogénique, qui est lui aussi un inhibiteur du G6PT. Nous avons observé une inhibition de la migration cellulaire *in vitro* des CE lorsque traitées à l'AD4-015 tandis que l'acide chlorogénique n'avait pas cet effet, démontrant ainsi de façon différentielle le potentiel anti-angiogénique de cet analogue. En effet, la migration des cellules est une étape importante dans l'induction de l'angiogenèse tumorale et son inhibition permettrait de ralentir la croissance tumorale.

Phénotype invasif et inflammatoire de l'endothélium vasculaire cérébral

Pour qu'il puisse y avoir migration des CE et formation de structures de type capillaire, il faut qu'il y ait dégradation de la MEC. Les protéases sont nécessaires pour que les cellules malignes puissent envahir les tissus. En effet des études *in vitro* et *in vivo* ont démontré une corrélation entre l'expression des gélatinases et le processus métastasique (John et Tuszynski, 2001). Une activité protéolytique est donc nécessaire durant la formation de capillaires pour que les CE puissent migrer au travers de la MEC. Les MMP sont capables de dégrader le collagène de type I, qui est un des constituants principaux de la MEC. Parmi les différents enzymes impliqués dans la dégradation de la matrice extracellulaire, les MMP sont impliquées dans l'induction de la migration cellulaire et de la tubulogenèse (Annabi et *al.*, 2008 ;Abécassis et *al.*, 2003). Plusieurs études ont démontré que les cellules endothéliales expriment et activent les MMP. MMP-2 et MMP-9, plus particulièrement, sont sécrétées par plusieurs types cellulaires et leur présence est souvent indicatrice d'une angiogenèse active (Handsley et *al.*, 2005; Noel et *al.*, 2008). Des études *in vitro* ont démontré que les CE microvasculaire ne sécrètent pas constitutivement la MMP-9, mais que lorsqu'exposées à des stimuli angiogéniques leur production est régulée à la hausse. Nous avons donc étudié les effets de différentes molécules sur le niveau des MMP secrétées par les HBMEC. Nous avons observé une induction de MMP-9 lorsque les HBMEC étaient traitées au phorbol 12-myristate 13-acétate (PMA). Cette induction est diminuée significativement par l'AD4-015, le 2DG ou par certains flavonoïdes tels la fisetine, l'apigénine et la lutéoline. Il existe plusieurs voies régulant l'expression de la MMP-9, dont la voie NF-κB. En analysant cette voie, nous avons déterminé que la phosphorylation d'IkB était réduite significativement en

présence de 2DG et de PMA ce qui corrèle avec la diminution de la translocation de p65 dans le noyau. La diminution de la phosphorylation d'IkB est aussi observée lors du traitement des HBMEC avec la fisetine, l'apigénine ou la lutéoline. L'utilisation de molécules inhibitrices de la sécrétion et de l'expression de MMP-9 peut servir de stratégies thérapeutiques pour le traitement de l'angiogenèse tumorale cérébrale. Des études ont démontré que l'épigallocatechine gallate, un polyphénol du thé vert, inhibe la sécrétion de MMP-9 en diminuant l'expression du facteur nucléaire HuR, le facteur stabilisant de l'ARNm de MMP-9 (Cao et *al.*, 2002; Lamy et *al.*, 2002). Dans le cancer, le niveau de HuR est élevé et celui-ci joue un rôle important dans l'angiogenèse tumorale. L'inhibition de MMP-9 par l'inhibiteur du G6PT, l'analogue de glucose et les flavonoïdes est donc importante due au fait que MMP-9 soit augmentée significativement durant la progression tumorale et est associé à l'ouverture de la barrière hémato-encéphalique.

La cyclooxygénase-2 est exprimée à de faibles niveaux dans l'épithélium gastrique, intestinal et du colon, mais est induite dans les macrophages et d'autres types cellulaires au niveau de sites inflammatoires, par des cytokines pro-inflammatoires. La surexpression de cox-2 a été retrouvée dans plusieurs types de cancers et a été associée à la résistance à certains médicaments. Sa surexpression est suffisante pour l'induction de la tumorigenèse, et est régulée par l'intermédiaire de différentes voies métaboliques incluant la voie de NF-κB, la voie des MAPK et le facteur de transcription C/EBP (Caivano et *al.*, 2001; Charalambous et *al.*, 2003). Dans les macrophages, l'induction de l'ARNm de cox-2 est régulée par NF-kB. L'expression de cox2 est reliée à l'augmentation de l'activité de NF-kB et l'induction de cox-2 par l'interleukine-1 est dépendante partiellement de NF-kB dans les cellules tumorales dans le cancer du colon (Liu et *al.*, 2003; Charalambous et *al.*, 2003). Plusieurs mécanismes moléculaires sont proposés pour expliquer l'implication de l'augmentation de cox-2 dans la carcinogenèse. Cox-2 est impliquée dans le développement du cancer et se reflète par la présence de haut niveau de PGE2 dans les tissus cancéreux, par l'inhibition de l'apoptose, par la diminution du système immunitaire et par la promotion de la prolifération cellulaire. Il a aussi été rapporté que cox-2 induit l'angiogenèse qui est essentielle à la croissance tumorale (Tsujii et *al.*, 1998). Plusieurs études testent les effets de la combinaison d'inhibiteurs de cox-2 à la radiothérapie pour avoir de meilleurs résultats au niveau du traitement. Il a été

démontré *in vitro* que l'utilisation d'inhibiteurs spécifiques de cox2 augmente la mort des cellules tumorales dans un modèle de tumeurs murines mammaires (Lanza-Jacoby et *al.*, 2004), des carcinomes, des tumeurs pulmonaires et des fibrosarcomes.

Importance de la voie NF-κB dans l'endothélium vasculaire cérébral

Pour mieux comprendre l'effet de l'AD4-015, du 2DG et des flavonoïdes nous avons évalué l'expression de cox-2 dans les HBMEC. Cox-2 n'est pas détectable dans les tissus normaux mais peut être induit par plusieurs stimuli comme les mitogènes, les facteurs de croissance, les hormones et les cytokines. Le traitement au PMA induit l'expression de cox-2, mais avec l'ajout de l'AD4-015, il y a inhibition significative de son expression. L'effet de l'AD4-015 est possiblement régulé en empêchant la dissociation de l'inhibiteur du facteur nucléaire kappaB (IkB) du complexe NF-κB. Des résultats similaires ont aussi été observés avec le traitement des HBMEC à la fisetine, l'apigenine et la lutéoline en présence de PMA. Ces observations peuvent êtres corrélées encore une fois à la diminution de la phosphorylation d'IkB.

L'importance de NF-kB dans l'induction de cox-2 a été démontrée dans plusieurs études. En effet, NF-κB joue un rôle important dans la carcinogenèse et il a un rôle essentiel dans la croissance tumorale de plusieurs types cellulaires cancéreux (Chiao et *al.*, 2002). Ainsi, le ciblage de l'activité de NF-κB s'avère être une importante avenue thérapeutique pour le traitement des cancers (Hung et *al.*, 2004). La voie de NF-κB peut réguler l'expression de cox-2 par le stress du RE et aussi par l'induction du chaperon du RE, le GRP78/BiP, qui est surexprimé dans une variété de tumeurs et qui confère une résistance aux cellules cancéreuses dormantes et prolifératives (Verfaillie et *al.*, 2010). Des études ont démontré que l'inhibition de GRP78 contribuait à une réduction de la densité des microvaisseaux des tumeurs (Dong et *al.*, 2008).

Adaptation métabolique et survie au stress

L'ajout du 2DG au PMA a potentialisé l'expression de cox-2 et l'expression de GRP78. Cette potentialisation est en contraste avec les résultats observés au niveau de MMP-9, dont l'expression était diminuée en présence de 2DG, et l'expression de l'unité p65 de NF-κB, qui était aussi réduite. Ceci pourrait indiquer que les effets observés sont indépendants de la voie de NF-κB et que la diminution de MMP-9 et la hausse de l'expression de cox-2 puissent être associées à l'augmentation du stress du RE. L'altération de l'intégrité du RE déclenche une série d'événements, incluant la synthèse de chaperons et la diminution de la traduction (Qu et *al.*, 2004). Il a été démontré que les cellules cancéreuses mammaires peuvent sécréter des facteurs de croissance endothéliaux vasculaires en réponse au stress du RE (Marjon et *al.*, 2004). Le stress du RE induit par les médicaments mène à la dégradation d'IkB et à la translocation de NF-κB dans les cellules cancéreuses du foie et du sein (Hung et *al.*, 2004). La dégradation d'IkB n'est pas observée dans l'activation de NF-kB induite par le stress du RE dans les fibroblastes des embryons de souris, ce phénomène pourrait donc être spécifique au type cellulaire (Jiang et *al.*, 2003).

Le 2DG interfère dans la synthèse des oligosaccharides menant à une N-glycosylation anormale des protéines et conduisent au stress du RE. En premier lieu, les cellules font face au stress du RE par le UPR, qui est un mécanisme permettant de contrer ce stress en diminuant l'entrée de protéines dans le RE, en augmentant la dégradation de protéines immatures et en augmentant les protéines chaperonnes. En plus de l'UPR, le stress du RE induit le processus d'autophagie, qui est un mécanisme de survie cellulaire activé lorsqu'il y a privation de nutriments (Bernales et *al.*, 2006; Ogata et *al.*, 2006; Yorimitsu et *al.*, 2006; Ding et *al.*, 2007). Il a été démontré que l'autophagie est activée par différentes conditions environnementales et par des médicaments qui diminuent le niveau d'ATP ou qui induisent le stress du RE (Lum et *al.*, 2005; Meley et *al.*, 2006; Xu et *al.*, 2007; Wei et *al.*, 2010). Il n'est cependant pas clair lequel de ces processus est responsable de l'induction de l'autophagie lorsque les cellules sont traitées au 2DG. Notre étude révèle aussi que l'inhibition de l'autophagie médiée par le 2DG a été observée dans les CE traitées par un carcinogène. Des

thérapies anti-angiogéniques et contre le cancer peuvent êtres envisagées par le bloquage de l'autophagie.

# CONCLUSION ET PERSPECTIVES

Nous avons démontré que l'inhibition des fonctions du G6PT dans les CE dérivées de tumeurs cérébrales résulte en la diminution de l'invasion. Elle pourrait donc diminuer la progression tumorale. En effet, l'inhibition des fonctions de G6PT aura un impact sur le phénotype invasif et métastatique des cellules cancéreuses (Belkaid, 2006). En ciblant l'expression de G6PT, nous pouvons mener au développement de nouvelles stratégies anti-cancéreuses. De plus, en étudiant l'analogue AD4-015 de la mumbaistatine, nous avons amené des preuves pharmacologiques sur le rôle de G6PT dans la néovascularisation médiée par les CE. L'AD4-015 pourrait donc être utilisé comme molécule ciblant les processus angiogéniques au niveau des tumeurs cérébrales.

Des études *in vivo* ont démontré que le 2DG augmente l'efficacité des agents chimio-thérapeutiques, tels l'adriamycine et le cisplatine, dans différentes tumeurs humaines. Ces résultats et d'autres résultats *in vitro* permettent d'améliorer les stratégies chimio-thérapeutiques utilisées chez les humains en proposant un essai clinique de phase I. Dans notre étude *in vitro,* nous avons découvert que l'expression de cox-2 est induite dans les CE en corrélation à de faibles niveaux d'ATP. La déplétion de l'ATP, en combinaison avec le stress du RE, inhibe l'autophagie dans les cellules traitées aux carcinogènes, ce qui pourrait ultimement affecter les capacités de survie des CE et représente donc une nouvelle stratégie thérapeutique anti-cancéreuse potentielle.

Il a été démontré que les effets anti-inflammatoires de la fisétine inhibent l'activation de NF-kB par le lypopolysaccharide dans les macrophages et dans les cellules dendritiques et l'activation de NF-κB par le TNF dans les cellules adénocarcinomes des poumons humains. Plusieurs études ont démontré que l'apigénine inhibe l'invasion tumorale induite par le PMA. Notre étude sur les flavonoïdes non seulement supporte les propriétés anti-inflammatoires de l'apigenine, de la lutéoline et de la fisétine, mais met surtout l'accent sur leurs effets anti-angiogéniques dans le compartiment vasculaire.

En conclusion, l'utilisation de molécules naturelles comme la mumbaistatine et les flavonoides, et du 2DG, qui ont des propriétés anticancéreuses, peuvent êtres utiles comme agents pharmacologiques en combinaison avec les traitements présentement utilisés pour optinmiser les résultats visés.

Perspectives 1 : L'activité de l'AD4-015 représente un premier pas vers le ciblage des processus angiogèniques au niveau des tumeurs cérébrales. Le dosage de l'activité de G6PT par l'isolation de microsomes permettrait de démontrer que les effets d'inhibition observés sont dus à l'inhibition de l'activité de la G6PT. De plus, en transfectant les CE il est possible de valider que l'AD4-015 affecte ces processus de manière G6PT-dépendante. Les fonctions de G6PT pourraient donc être liées à la régulation de la néovascularisation par les cellules endothéliales.

Perspective 2 : L'ATP est nécessaire pour que le processus de la tubulogenèse se produise. Il serait intéressant de vérifier dans le futur si le 2DG, qui est un dépleteur de l'ATP, affecte l'expression de TIMP-1, l'inhibiteur principal de MMP-9, sachant que l'activité protéolytique dans la matrice extracellulaire dérive de la balance entre les protéases et leurs inhibiteurs. Une meilleure compréhension de la régulation de la vascularisation tumorale par les CE permettrait le développement et l'utilisation de nouveaux médicaments et thérapies.

Perspective 3 : Différentes molécules sont testées pour l'identification et la caractérisation d'inhibiteurs potentiels de l'angiogenèse tumorale cérébrale. Il faudrait dans le futur, envisager de déterminer l'impact des flavonoides au niveau de la prolifération et de la viabilité des CE. De plus, sachant que le PMA induit l'autophagie dans ces cellules, il serait intéressant de déterminer si les flavonoides pourraient renverser cet effet. L'utilisation de molécules naturelles ayant des effets anti-inflammatoires permettrait donc de cibler l'angiogenèse tumorale.

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