INRS – Centre Armand Frappier Santé Biotechnologie

THE USE OF MELATONIN AS AN ADJUVANT TO CHEMOTHERAPEUTIC AGENTS IN THE TREATMENT OF HUMAN PLACENTAL CHORIOCARCINOMAS

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Siempre para ti, mam

ABSTRACT

Human placental choriocarcinomas are rare, but potentially lethal and their ability to resist chemotherapy has shed light on a need for novel treatment options. Meanwhile, studies have documented an anti-tumoral role for melatonin. Thus, we hypothesize that the hormone is a potent adjuvant to antineoplastics. ***Methodology and Results:*** MTT assays were performed to determine that BeWo choriocarcinoma cells are sensitive to 75 μM 5-FU, 1 μM ETO, 80 μM MTX, 1 μM OXA and 1 nM PTX. The CM-H2DCFDA probe revealed that 1 mM melatonin induces a transient increase in ROS production (relative to the drug alone) which, in some cases, correlates with decreased cell viability, assessed via flow cytometry. DNA gel electrophoresis and immunocytochemistry showed that this reduction in viability is independent of the cytotoxic mechanism of the antineoplastics. Finally, using the XCELLigence apparatus, a solid tumor model was developed, on which melatonin retains its antitumoral potential when coupled to some antineoplastics. ***Conclusion:*** Melatonin thus increases ROS production, which may induce cell death independently of the cytotoxic mechanism of the antineoplastic in monoloayed and multilayered BeWo cell cultures. Our data suggests that melatonin may be used as an adjuvant to some antineoplastics in the treatment of human placental choriocarcinomas.

Key words: cancer; choriocarcinoma; placenta; antineoplastic; melatonin; adjuvant

RÉSUMÉ

Le choriocarcinome placentaire humain, rare, mais létal, peut résister à la chimiothérapie, soulignant un besoin pour de nouvelles thérapies. Alors que des études ont attribué un effet antitumoral à la mélatonine, nous émettons l'hypothèse que cette indolamine peut être utilisée comme adjuvant aux antinéoplasiques. ***Méthodologie et Résultats :*** Des essais MTT ont démontré que les cellules de choriocarcinome placentaire BeWo sont sensibles à 75 µM 5-FU, 1 µM ETO, 80 µM MTX, 1 µM OXA et 1 nM PTX. La sonde CM-H2DCFDA a montré que la mélatonine (1 mM) induit une surproduction transitoire d'EROs (relativement à l'antinéoplasique seul) liée, dans certains cas, à une mort cellulaire, démontrée par cytométrie en flux. L'électrophorèse et l'immunocytochimie ont vérifié que cette mort est indépendante du mécanisme cytotoxique des agents chiomiothérapeutiques. Finalement, un modèle de tumeur solide, mis en place avec l'appareil XCELLigence, a montré que la mélatonine peut, lorsque couplée à certains antinéoplasiques, garder son potentiel antitumoral. ***Conclusions*** : La mélatonine induit une production d'EROs qui peut entrainer la mort de cellules cancéreuses BeWo indépendamment du mécanisme cytotoxique de l'agent auquel elle est couplée. Ceci indique que la mélatonine peut être utilisée comme adjuvant à certains antinéoplasiques dans le traitement du choriocarcinome placentaire.

Mots-clés : cancer; choriocarcinome; placenta; antinéoplasique; mélatonine; adjuvant

SYNOPSIS (FRENCH)

I. RÉSUMÉ DES CONNAISSANCES

a. Le Développement Placentaire et le Cancer

Le placenta est un organe critique lors de l'embryogenèse, bien connu pour son implication dans le transport fœto-maternel des gaz (O_2, CO_2), des nutriments et des déchets métaboliques. Cet organe est aussi impliqué dans les fonctions endocrines et immunologiques importantes dans le maintien d'une grossesse normale. La complexité des fonctions occupées par le placenta témoigne de la complexité de sa formation, la placentogenèse. En effet, la formation du placenta dépend d'une communication sophistiquée entre le système maternel et fœtal, par le biais de divers hormones, cytokines et facteurs de croissances. Après la fécondation, le zygote devient une structure sphérique et creuse, appelée le blastocyste. Cette structure migre vers l'utérus maternel et les cellules se trouvant en périphérie du blastocyste, les cellules trophoblastiques, vont par la suite proliférer et se différentier pour débuter un processus d'invasion. Cette invasion, ainsi que les autres évènements qui en découlent, va permettre la formation complète du placenta. Étant donné l'importance du placenta lors du développement fœtal, ces évènements de migration, de différentiation et d'invasion se doivent d'être rigoureusement contrôlés, et ce, par les molécules communicatrices décrites ci-dessus.

Cette communication est si critique que des altérations entrainent souvent des grossesses aberrantes, dites pathologiques, et peuvent même mener au développement d'un certain type de cancer : le choriocarcinome placentaire, caractérisé par une croissance incontrôlée des trophoblastes. Dans une revue de littérature menée par Hanahan and Weinberg (2011), les auteurs définissent dix caractéristiques communes à tous types de cancers, le choriocarcinome placentaire inclus. Ces caractéristiques incluent une croissance incontrôlée, une capacité d'invasion et une aptitude à s'évader de la détection par le système immunitaire ainsi qu'une angiogenèse accrue et une dérégulation des sources d'énergie cellulaires. Étonnamment, il s'avère que toutes ces propriétés sont, d'une façon ou d'une autre, aussi impliquées dans le processus normal de placentogenèse. Les similarités sont inouïes en considérant que ce processus et celui de la tumorigenèse ont recours aux mêmes voies de signalisation, la nuance étant qu'elles sont contrôlées ou pas, respectivement, comme indiqué dans le tableau 1.1 (section

1.1.2, page 13). Ceci explique pourquoi le processus du développement placentaire est souvent nommé comme étant *pseudotumorigène.* Il est donc sans surprise qu'en Amérique du Nord, environ 1 grossesse sur 40,000 se voit développer en choriocarcinome placentaire, une incidence qui est augmentée dans d'autres parties du monde, comme en Asie.

b. Implication des Espèces Réactives de l'Oxygène

Qui dit cancer, dit souvent espèces réactives de l'oxygène (EROs), et ce, étant donné leur implication dans le développement et dans le traitement des tumeurs. Les EROs (des exemples sont listés dans le tableau 1.2 (section 1.6, page 39) sont des composés qui contiennent de l'oxygène et qui, par leur structure, s'avèrent être extrêmement réactifs. Ils sont issus de procédés biochimiques intracellulaires normaux, produits par les réactions de la chaine respiratoire respiration ou par l'activité de certaines enzymes (ex : NADPH oxydase (NOX) et xanthine oxydase (XO)). Leur réactivité leur permet de réguler plusieurs voies de signalisation. Étant donné leur implication dans cette régulation, leur présence dans le milieu intracellulaire se doit d'être étroitement contrôlée. Ce contrôle est exécuté par diverses molécules et enzymes antioxydantes (ex : catalase (CAT), superoxyde dismutase (SOD) et glutathion réductase). Ces mécanismes (décrits dans la section 1.6.2) permettent de limiter le potentiel réactif des EROs et de préserver l'homéostasie.

Un problème survient lorsque les niveaux d'EROs deviennent élevés. Ceci peut être dû à une dérégulation des voies impliquées dans leur production ou à une source exogène. Dans tous les cas, laissées à elles-mêmes, un plus grand nombre de ces espèces dans les cellules risquent d'induire des réactions altérant l'homéostasie. Les EROs peuvent notamment induire la formation de ponts disulfure et induire une conformation tertiaire des protéines aberrantes. En prenant *Phosphatase and TENsin homolog* (PTEN) comme exemple, une telle altération mènerait à son inactivation ce qui entrainerait une accumulation de PIP_3 au niveau de la membrane cellulaire. Cette accumulation entraine une prolifération cellulaire constitutive menant au cancer. Cet exemple n'est qu'un parmi tant d'autres qui décrivent comment les EROs sont impliqués dans la progression du cancer.

Les EROs peuvent donc participer à une dérégulation des processus intracellulaires ou induire la tumorigenèse en fonction de leur abondance. Cependant, à des niveaux encore plus élevés, ces espèces peuvent aussi entraîner la mort cellulaire. Encore une fois dû à leur réactivité, elles peuvent entraîner une conformation protéique aberrante (comme décrit ci-dessus), mais

risquent davantage de s'attaquer à plusieurs composantes cellulaires (ADN, protéines, lipides) pour ainsi forcer les cellules à activer des mécanismes de mort cellulaire contrôlés (ex. : apoptose et autophagie) ou induire des dommages si sévères qu'ils entraînent une mort spontanée (ex. : nécrose).

c. Les Agents Antinéoplasiques

La surproduction d'EROs est un mécanisme par lequel les agents antinéoplasiques vont, au moins en partie, conduire à la mort des cellules cancéreuses. Ces agents sont aussi utilisés parce qu'ils interfèrent avec des voies de signalisation régulée à la hausse par les cellules hautement prolifératives, comme les cellules tumorales. Par exemple, le méthotrexate (MTX), couramment utilisé dans le traitement des choriocarcinomes placentaires, agit comme un inhibiteur compétitif du dihydrofolate réductase (DHFR), une enzyme critique dans la production de tétrahydrofurane (THF) essentiel à la synthèse de nucléotides. Une baisse de l'activité du DHFR entraine une diminution de l'aptitude à produire des nucléotides. Conséquemment, les cellules en division ne peuvent pas dupliquer leur bagage génétique de façon fiable, entrainant ainsi des aberrations et potentiellement une mort cellulaire. Le MTX interfère donc avec les activités métaboliques de la cellulaire et est classifié comme un antimétabolite.

Le développement d'une résistance au MTX n'est pas inhabituel. Dans ces cas-là, les médecins ont souvent recours à un traitement plus robuste qui implique plusieurs agents antinéoplasiques administrés séquentiellement. Dans le cas du choriocarcinome placentaire, cette alternative se définit par le régime étoposide (ETO), MTX, Dactinomycine/ Cyclophosphamide, Vincristine (EMA/CO). Cependant, une résistance à ce traitement a elle aussi été observée. Dans ces cas-là, il faut avoir recours à d'autres agents, tels que le 5-fluorouracil (5-FU), l'ETO, l'oxaliplatine (OXA) ou le paclitaxel (PTX), parmi tant d'autres.

Le 5-FU est un autre exemple d'antimétabolite. Il altère la capacité des cellules hautement prolifératives à synthétiser des nucléotides. Contrairement au MTX, le 5-FU inhibe l'activité de la thymydylate synthase (son rôle étant décrite en détail à la section 1.4.2.1) pour générer des lésions au niveau de l'ADN et l'ARN causant l'arrêt de procédés physiologiques importants (ex. : synthèse de l'ADN et la traduction). L'ETO fait partie de la classe d'inhibiteurs de la topoisomérase II. Ladite enzyme génère des bris transitoires de l'ADN pour moduler l'accessibilité au code génétique lors de procédés critiques tels que la duplication ou la transcription. L'ETO altère cette fonction en bloquant l'habileté de l'enzyme à réparer les bris censés être transitoires

qu'elle a elle-même introduits. Par conséquent, les cellules se retrouvent avec un taux de lésions génotoxiques si élevé que les cellules induisent leur mort plutôt que leur réparation. L'induction de lésions d'ADN est aussi employée par l'OXA dans son mécanisme cytotoxique. Cet agent va directement se lier à l'ADN et ainsi ajouter des groupes alkyles. L'ajout de ces groupes suffit pour générer des altérations qui compromettent l'intégrité de la molécule d'ADN. L'OXA est donc classifié comme un agent alkylant. Le PTX fait partie des alcaloïdes végétaux puisque, comme son nom l'indique, il est un alcaloïde dérivé de plantes. Sa structure permet au PTX de se lier à la tubuline et, ainsi, altérer la dynamique des microtubules. Cette dynamique est centrale à une division cellulaire propice puisque les microtubules sont hautement impliqués dans la séparation des deux copies du bagage génétique pendant la mitose.

Bien que les agents antinéoplasiques exploitent une panoplie de mécanismes pour induire la mort des cellules tumorales, ils ont tout de même quelques points en commun. D'une part, comme décrit précédemment, ils induisent tous, d'une façon ou d'une autre, le surproduction d'EROs qui peut directement entrainer la mort cellulaire. D'autre part, par leurs mécanismes de cytotoxicité, ces agents s'attaquent à toutes les cellules en division, qu'elles soient cancéreuses ou pas. Donc, biens que ces agents s'attaquent principalement aux cellules tumorales, une mort indésirable de cellules saines (mais prolifératives) est inévitable. Ceci impose une limitation importante puisque les effets néfastes d'une mort non ciblée varient de purement esthétique (perte de cheveux) à potentiellement mortelles (l'état immunodéprimé qui en découle). Une quête pour le développement d'un traitement *parfait* pour le cancer se poursuit donc.

d. Les Divers Rôles de la Mélatonine

De plus en plus d'études décrivent des effets prometteurs de la mélatonine quant à son utilisation dans les traitements ou comme adjuvant au traitement existant contre le cancer. Bien connue comme étant l'hormone du sommeil, la mélatonine est produite par la glande pinéale par un mécanisme complexe qui implique la détection des périodes d'obscurité par des récepteurs au niveau des yeux. Par la suite, cette indolamine régule une panoplie d'effets physiologiques en se liant principalement à ces récepteurs (MT1 et MT2).

Bien plus qu'être simplement l'hormone du sommeil, la mélatonine occupe plusieurs autres rôles physiologiques. En effet, dans les cellules saines, elle agit comme une molécule antioxydante. Par sa structure, elle interagit avec plusieurs EROs, limitant ainsi leur potentiel réactif et leur habilité de causer les effets néfastes discutés ci-dessus et à la section 1.7.4.

Cependant, le potentiel antioxydant de la mélatonine n'est pas limité à son interaction directe avec les EROs puisqu'il a été démontré qu'elle peut aussi induire la production d'enzyme antioxydante telle que la SOD, la CAT et le glutathion réductase. De plus, la mélatonine a la capacité de diminuer l'activité d'enzymes responsables de la production endogène d'EROs comme la NOX et XO. La mélatonine, dans les cellules saines, contribue donc à maintenir les EROs à des niveaux homéostatiques.

Étonnamment, la mélatonine joue un rôle opposé dans les cellules cancéreuses, où elle induit la surproduction d'EROs. Bien que les mécanismes par lesquels cet effet paradoxal est engendré demeurent mal compris, il a été proposé qu'il serait lié, au moins en partie, à la voie phospholipase A2 calcium-dépendante (iPLA2)/ 5-lypoxygénase (5-LOX). Dans les cellules saines et cancéreuses, la iPLA2 hydrolyse les phospholipides présents dans l'environnement intracellulaire pour générer de l'acide arachidonique (AA). La 5-LOX va par la suite catalyser la conversion de l'AA pour produire des eicosanoïdes impliqués dans divers procédés physiologiques, et des EROs. Dans cette voie de signalisation, la calmoduline (CaM) interagit avec iPLA2 pour engendrer une conformation inactive de cette dernière. La mélatonine, quant à elle, peut interagir et inactiver la CaM ce qui inhibe la répression de iPLA2 et permet la production d'EROs. Sachant que la iPLA2 et la 5-LOX sont régulées à la hausse dans les cellules cancéreuses, il s'ensuit que la production des eicosanoïdes est accentuée et que, par conséquent, la production d'EROs est accrue.

L'interaction entre la mélatonine et la CaM est aussi proposée comme étant la source de l'habilité de l'hormone de réguler une composante du cytosquelette : les microtubules. Une étude a montré que la mélatonine, à faibles doses, réussit à hausser le taux de polymérisation de ces structures dynamiques alors qu'à des doses plus fortes, elle à l'effet opposé, servant ainsi à causer une dépolymérisation. Les auteurs ont proposé que cet effet de la mélatonine soit dû à son interaction avec la CaM. Les filaments d'actine sont, eux aussi, régulés par la mélatonine. Elle altère la distribution et la localisation ainsi qu'un épaississement desdits filaments. Bien que le mécanisme impliqué demeure mal compris, il serait dû à la capacité de la mélatonine d'activer certains gènes (ex : palladin, filamin-C, et autres) plutôt qu'à sa liaison avec la CaM.

Une autre habilité de la mélatonine importante dans l'étude du cancer est celle de favoriser l'intégrité de l'ADN. Grâce à son implication directe vis-à-vis la protection contre le stress oxydatif dans les cellules saines, la mélatonine prévient les dommages génotoxiques qui pourraient être causés par les EROs. Inversement, elle peut induire ces mêmes lésions dans les cellules cancéreuses et en promouvoir leur mort. Son effet protecteur provient aussi de son

habilité d'induire la phosphorylation récepteur-dépendant de p53. La protéine p53 est impliquée dans l'induction de l'apoptose ou la réparation de l'ADN, dépendamment des signaux intracellulaires reçus. L'implication de p53 dans ces deux procédés est décrite en détail dans la section 1.3.

Les rôles paradoxaux de la mélatonine décrits ci-dessus en fonction de sa présence au sein de cellules saines ou cancéreuses font de celle-ci une molécule intéressante à examiner comme potentiel traitement de divers cancers. En effet, non seulement la mélatonine serait néfaste pour les cellules cancéreuses, mais elle aurait l'habilité de protéger les cellules saines et ainsi contrer les effets hors cible des agents antinéoplasiques. Ces effets sont si prometteurs que des essais cliniques sont déjà en cours. Cependant, ce potentiel ne semble pas encore avoir été étudié en dans les cas de choriocarcinome placentaire.

II. Problématique

L'incidence du choriocarcinome placentaire humain est rare et les stratégies thérapeutiques impliquant des agents antinéoplasiques, couramment utilisées, se sont avérées être plutôt efficaces, limitant d'autant plus les complications potentielles. Cependant, de nombreux cas où les patients affligés finissent par développer une résistance auxdites stratégies thérapeutiques ont été documentés, et deviennent de plus en plus communs. La résistance aux agents antinéoplasiques est chose répandue dans plusieurs types de cancers tels que le cancer du sein et la leucémie myéloïde chronique, ce qui pourrait expliquer leur occurrence accrue. Dès lors, le développement de nouvelles thérapies visant à traiter le choriocarcinome placentaire, avant que celles mises en place actuellement commencent à faire défaut, devient critique dans une quête visant à prévenir une autre épidémie potentielle, cette fois-ci liée au cancer.

Des études précédemment menées au sein de notre équipe de recherche ont démontré que la mélatonine régule positivement des mécanismes de survie cellulaire dans les cellules trophoblastiques saines soumises à un stress causé par une hypoxie/réoxygénation. La même étude a été démontrée que la mélatonine peut avoir un effet opposé dans des cellules trophoblastiques tumorales BeWo, en lien avec le rôle paradoxal de la mélatonine. D'autres études effectuées par notre équipe ont confirmé que la mélatonine a la capacité d'induire la surproduction d'EROs dans ces cellules tumorales. Bien que ces études soient prometteuses quant à l'utilisation de la mélatonine dans le traitement du choriocarcinome placentaire, un lien

direct entre lesdites habiletés de cette indolamine et son potentiel comme adjuvant avec divers agents antinéoplasiques demeure à être démontré.

III. HYPOTHÈSE

En prenant en compte la littérature scientifique et les résultats précédents de notre laboratoire, l'hypothèse de l'étude décrite ici est que la mélatonine combinée à divers agents antinéoplasiques, entraîne une surproduction d'EROs allant au-delà de ce que l'antinéoplasique seul peut, stimulant ainsi la mort des cellules tumorales.

IV. OBJECTIFS SPÉCIFIQUES

Pour vérifier si les cellules de choriocarcinome placentaire humain BeWo répondent à un traitement avec la mélatonine les objectifs spécifiques de cette étude sont de déterminer si la mélatonine induit:

1. une surproduction d'EROs lors d'un co-traitement avec divers agents antinéoplasiques;
2. la mort cellulaire lors d'un co-traitement avec divers agents antinéoplasiques;
3. un effet synergique lors d'un co-traitement avec divers agents antinéoplasiques dans un modèle qui s'apparente à une tumeur solide.

V. MÉTHODOLOGIE

Les cellules BeWo (clone CCL-98, *American Type Culture Collection (ATCC))* ont été mises en culture dans du milieu *Dulbecco's Modified Eagle Medium (DMEM)*/F-12 supplémenté avec 10% de sérum bovin fœtal (FBS). Les cellules ont été cultivées dans un incubateur à 37°C avec 5% de CO_2. Des passages ont été effectués à un ratio de 1 :10 lorsque les cellules ont atteint une confluence de 80%, jusqu'au passage 30.

Les détails des divers essais réalisés au courant de cette étude se trouvent à la section 3. Brièvement, pour déterminer la toxicité des divers agents antinéoplasiques (5-FU, ETO, MTX, OXA et PTX) et obtenir leurs EC50, des essais MTT ont été effectués pour des traitements de 24h, 48h et 96h.

Avec ces valeurs d'EC50, l'effet combinatoire de la mélatonine avec les agents antinéoplasiques sur la production d'EROs a été évalué avec la sonde CM-H2DCFDA selon les directives du fournisseur. L'effet sur viabilité cellulaire en réponse à l'ajout d'une dose de 1 mM de mélatonine a été déterminé à l'aide de la cytométrie en flux, avec la coloration à l'anexine 5/FITC et à l'iodure de propidium.

Par la suite, les effets cytotoxiques des antinéoplasiques a été déterminée, d'une part, par électrophorèse sur gel d'ADN en utilisant des gels de 1% d'agarose et d'autre part, par immunocytochimie visant à colorer les microtubules et les filaments d'actine.

Finalement, le potentiel synergique de la mélatonine a été évalué avec un modèle de tumeur solide, avec technologie XCELLigence. Pour ce faire, les cellules ont été laissées croitre pendant plusieurs jours, jusqu'à l'occurrence d'un indice cellulaire (CI) d'au moins 6.0, puis soumis aux mêmes conditions décrites ci-dessus (antinéoplasique $\pm$ mélatonine) pendant 96 h pour évaluer la prolifération cellulaire en temps réel.

VI. RÉSUMÉ DES RÉSULTATS

Les essais MTT ont démontré une diminution constante de la viabilité cellulaire et pour laquelle un EC50 a déterminé après un traitement de 48h pour tous les agents (Figure 4.1). Les concentrations et le temps d'exposition sont représentatif des traitements en milieu clinique, dans lequel les patients souvent se voient administrer plusieurs doses des médicaments. Pour ce temps d'exposition, les EC50 sont indiqués dans le tableau 4.1. Ces valeurs étaient de 72.65 µM, 1.126 µM, 78.14 µM, 1.046 µM et 0.8488 nM pour le 5-FU, l'ETO, le MTX, l'OXA et le PTX, respectivement.

En utilisant les concentrations indiquées ci-haut, la production d'EROs en fonction du temps d'exposition a été analysée. Nous avons démontré que la mélatonine, lorsque couplée aux agents antinéoplasiques induit une surproduction transitoire d'EROs qui débute à 48h et qui tend à se dissiper par la suite (Figure 4.2). Bien que des niveaux élevés d'EROs peuvent induire le stress oxydatif et, conséquemment, la mort cellulaire, la surproduction de ces espèces ne garantit pas un tel effet. Nous avons donc eu recours à la technique de cytométrie en flux pour valider la corrélation entre la production d'EROs et la mort cellulaire. Nous observons que la mélatonine induit une réduction de la viabilité cellulaire, lorsque couplée au 5-FU, l'ETO et l'OXA (Figure 4.3). Nous observons que les cellules se sont vues poussées vers le stage d'apoptose tardive alors que les proportions de cellules en phase précoce ne sont pas altérées de façon significative.

Ayant vérifié que la mélatonine a un effet sur la viabilité cellulaire, nous avons ensuite cherché à savoir si ces effets sont dépendants des mécanismes cytotoxiques des agents examinés dans cette étude. Les résultats sur gel d'agarose, nous observons que la mélatonine a eu des effets irréguliers quant à son habilité d'induire la réparation de l'ADN (Figure 4.4). En effet, la mélatonine a tantôt pu protéger des lésions génomiques, tantôt n'a pas eu d'effet quant à ce même paramètre, selon le répliqua biologique. Il est d'intérêt de souligner que cette variabilité n'est pas attribuable à un agent antinéoplasique ni à un répliqua spécifique, indiquant que cette variabilité est bel et bien due à une capacité de protection génomique variable de la mélatonine.

En considérant que le PTX s'attaque aux microtubules plutôt qu'à l'ADN, nous avons eu recours à l'immunocytochimie pour déterminer l'effet cytotoxique de la mélatonine en co-traitement avec le PTX. Les proportions ainsi que les phénotypes des fuseaux mitotiques ont été analysés. Alors que le PTX semble causer une diminution du nombre de fuseaux mitotiques observables et une élévation du phénotype multipolaire de ceux-ci, un co-traitement de PTX/mélatonine ramène ces deux paramètres vers des niveaux similaires à ceux des cellules non traitées (Figure 4.5 et 4.6).

Enfin, nous avons déterminé si l'effet synergique de la mélatonine continue à être observé dans un modèle de tumeur solide en utilisant la technologie xCELLigence. Nous avons démontré que le co-traitement de la mélatonine avec le 5-FU et l'ETO induit une diminution accrue de la viabilité cellulaire comparée au traitement avec ces agents antinéoplasiques seuls (Figure 4.7). Cet effet n'a pas été observé dans le cas de cellules traitées avec le MTX, l'OXA ou le PTX.

VII. CONCLUSION

Le développement du choriocarcinome placentaire chez l'humain est rare, mais ce cancer possède un grand potentiel métastatique. Ce cancer est couramment traité avec des agents antinéoplasiques seuls, ou en combinaison. Par contre, des cas de résistances à ces agents commencent à faire apparition, ce qui suggère un besoin de développer de nouvelles stratégies thérapeutiques. Tout d'abord en utilisant des cellules BeWo cultivées en monocouche, nous avons démontré que la mélatonine, à une dose de 1 mM, stimule la surproduction d'EROs administrée en combinaison avec le 5-FU, l'ETO, le MTX, l'OXA et le PTX. Cette surproduction est couplée à une baisse de la viabilité cellulaire pour les cellules traitées au 5-FU, ETO et OXA en promouvant la progression vers l'apoptose tardive. Dès lors, nous proposons qu'alors que ces agents antinéoplasiques fournissent un premier signal apoptotique, la mélatonine en fourni un

deuxième, ce qui expliquerait la progression accrue de ce mécanisme de mort cellulaire. En comprenant que la mélatonine régule un grand nombre de procédés intracellulaires, nous avons cherché à savoir si cet effet était dépendant du mécanisme cytotoxique de l'antinéoplasique auquel elle est couplée. Nous avons démontré que, pour les agents endommagent l'ADN, la mélatonine induit la réparation de ces bris, mais de façon variable. Nous avons aussi démontré que la mélatonine s'oppose au mécanisme du PTX, induisant plutôt des proportions et des phénotypes de fuseaux mitotiques qui s'apparentent à ceux des cellules non traitées. Étant donné que la capacité de la mélatonine à induire l'intégrité du bagage génétique et de la dynamique des microtubules ne concorde pas avec ses propriétés synergiques, nous déduisons que la mélatonine agit de façon indépendante des antinéoplasiques. Nous proposons ainsi une possible dépendance à son habilité d'interagir avec la CaM étant donné son implication dans la production d'EROs et dans la dynamique des microtubules. Finalement, nous avons eu recours à des cellules BeWo cultivées en multicouche pour modéliser une tumeur solide au sein de laquelle l'accessibilité des agents antinéoplasiques limite leur efficacité. Nous avons démontré que la mélatonine retient ses capacités synergiques uniquement lorsque couplée au 5-FU et à l'ETO. Dans ce cas-là, nous proposons que la distribution des agents antinéoplasiques, et non de la mélatonine, soit le facteur limitant dans l'efficacité de celle-ci. Dans cette étude, nous présentons un premier argument en faveur de la mélatonine, comme potentiellement adjuvant. Nous proposons que la mélatonine via une interaction avec la CaM puisse agir comme adjuvant à certains agents antinéoplasiques dans le traitement du choriocarcinome placentaire humain.

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4-OH-E2 : 4-hydroxyestradiol

5-FU : 5-Fluorouracil

5-LOX : 5-Lipoxygenase

A5 : Annexin 5

AA : Arachidonic Acid

AANAT : Aralkylamine N-Acetyltransferase

AC : Adenylyl Cyclase

Act D : Actinomycin D or Dactinomycin

APC : Anaphase-Promoting Complex

ATM : Ataxia Telangiectasia Mutated

ATR : Ataxia Telangiectasia and Rad3

ATRIP : ATR-Interacting Protein

BAD : Bcl-2-Associated Death Promoter

BAK : Bcl-2 Homologous Antagonist/Killer

BAX : Bcl-2 Associated X-protein

BCL-2 : B-cell lymphoma 2

BCL-X_L : B-cell Lymphoma-Extra Large

BER : Base-Excision Repair

(t)BID : (truncated) BH3 Interacting Domain Death Agonist

BUB(R)1/3 : Budding Uninhibited by Benzimidazoles (Related) 1 and 3 Homologs

c-FLIP : cellular FADD-like IL-1β-Converting Enzyme-Inhibitory Protein

CaM : Calmodulin

cAMP : cyclic Adenosine Monophosphate

CAT : Catalase

CDCA1 : Cell Division Cycle Associated 1

(n)CI$_{(avg)}$: (Normalized) Cell Index (average)

CREB : cAMP Response Element Binding Protein

CTB : Cytotrophoblast

(h)CTR1 : (human) Copper Transport Protein 1

D&C : Dilation and Curettage

DAPI : 4′,6-diamidino-2-phenylindole

DDR : DNA Damage Response

DED : Death Effector Domain

DHF : Dihydrofolate

DHFR : Dihydrofolate Reductase

DISC : Death Inducing Signalling Complex

DMSO : Dimethyl Sulfoxide

DNA-PK$_{(CS)}$: DNA-Dependent Protein Kinase (Catalytic Subunit)

DSB : Double-Strand Break

E2: Estradiol

(HB)-EGF(R) : (Heparin-Binding) Epidermal Growth Factor (Receptor)

EMA/CO : (Acronym) ETO, MTX, Act D, Cyclophosphamide, Vincristine

EMT : Epithelial-to-Mesenchymal Transition

ERK : Extracellular-Signal Regulated Kinase

ERα/β : Estrogen Receptor α/β

ETO : Etoposide

EVT : Extravillous Trophoblasts

FA : Folic Acid

FADD : Fas Associated Via Death Domain

FBS : Fetal Bovine Serum

(b)FGF : (Basic) Fibroblast Growth Factor

FITC : Fluorescein Isothiocyanate

GCPR : G-Protein Coupled Receptor

GLUT1 : Glucose Transporter 1

GPx : Glutathione Peroxidase

GSH : Glutathione

GSSG : Glutathione Disulfide

GST : Glutathione S-transferase

GTD : Gestational Trophoblastic Disease

hCG : Human Chorionic Gonadotropin

(γ)H2AX : (gamma) H2A Histone Family Member X

HEJ : Homologous End Joining

HIOMT : Hydroxyindole-O-Methyltransferase

HIPK2 : Homeodomain-Interacting Protein Kinase 2

HM : Hydratidiform Mole

ICC : Immunocytochemistry

IGF(BP) : Insulin-like Growth Factor (Binding Protein)

IHH : Indian Hedgehog

IL-1(β)/6: Interleukin-1(beta)/6

IP3 : Inositol Triphosphate

iPLA2 : Calcium-Independent Phospholipase A2

JNK : c-Jun N-terminal Kinase

KNL1 : Kinetochore Scaffold 1

LC3 : Microtubule-Associated Protein 1A/1B-Light Chain 3

LIF : Leukemia Inhibitory Factor

MAD2/3 : Mitotic Arrest Deficiency 2/3

MAP : Microtubule-Associated Protein

MAPK : Mitogen-Activated Protein Kinase

MCC : Mitotic Checkpoint Complex

MDC1 : Mediator of DNA Damage Checkpoint Protein 1

MDM2 : Mouse Double Minute 2 Homolog

MLKL : Mixed-lineage Kinase Domain-like Pseudokinase

MMR : Mismatch Repair

MPS1 : Monopolar Spindle 1

MRN : (a complex composed of) Double Srand Break Repair Nuclease 11- Double Strand Break Repair Protein RAD50-Nibrin 1

MT1/2 : Melatonin Receptor Type 1/2

MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

MTX : Methotrexate

NADPH : Nicotinamide Adenine Dinucleotide Phosphate

NDS : Normal Donkey Serum

NE : Norepinephrine

NER : Nucleotide Excision Repair

NFAT : Nuclear Factor of Activated T cells

NHEJ : Non-Homologous End Joining

NOX : NADPH Oxidase

OCT1/2/3 : Organic Cation Transporter 1/2/3

OXA : Oxaliplatin

p53 : Tumour Protein p53

PD-(L)1 : Programmed Cell Death Protein (Ligand) 1

PDK : Phosphoinositide-Dependent Kinase

P-gp : P-glycoprotein

PHD : Pleckstrin Homology Domain

PI : Propidium Iodide

PIF : Pre-Implantation Factor

PIP_2 : Phosphatidylinositol-4,5-bisphosphate

PIP_3 : Phosphatidylinositol-3,4,5-triphosphate

PKA/PKB : Protein Kinase A/B

PLCβ : Phospholipase Cβ

PPARγ : peroxisome Proliferator-activated Receptor Gamma

PS : Phosphatidylserine

PTEN : Phosphatase and Tensin Homolog

PTX : Paclitaxel

RAD50/51 : Double Strand Break Repair Protein RAD50/RAD51

RIPK1/3 : Receptor-interacting protein kinase 1/3

ROS : Reactive Oxygen Species

RPA : Replication Protein A

SAC : Spindle Assembly Checkpoint

SNAI/SNAIL : Zinc-Finger Protein SNAI1

SOD : Superoxide Dismutase

SSB : Single Strand Break

ssDNA : Single-Stranded DNA

STAT(3) : Signal Transducer and Activators of Transcription (3)

STB : Syncytiotrophoblast

THF : Tetrahydrofolate

(d)TMP : (deoxy)Thymidine Monophosphate

TNF-α : Tumour Necrosis Factor α

TopBP1 : DNA Topoisomerase II Binding Protein 1

Topo : Topoisomerase

TOX : Thymocyte Selection Associated HMG Box

TS : Thymidylate Synthase

(d)UMP(K) : (deoxy)Uridine Monophosphate (Kinase)

uPA(R) : Urokinase Plasmogen Activator (Receptor)

(f)(u)UTP : (Fluoro)(deoxy)Uridine Triphosphate

VEGF : Vascular Endothelial Growth Factor

VT : Villous Trophoblast

Wnt : Wingless-related Integration Site

WSB1 : WD Repeat And SOCS Box Containing 1

XO : Xanthine Oxidase

1 LITERATURE REVIEW

1.1 The human placenta

The placenta is an organ critical for successful embryogenesis. It is well adapted to allow transfer of vital components and disposal of waste products, to and from the fetus. Some compounds, such as molecular oxygen and carbon dioxide, are transported via passive diffusion while others (*e.g.,* free fatty acids) are broken down by placental enzymes, allowing the resulting moieties to diffuse (Griffiths & Campbell, 2015). Transporter proteins are also present on the placental membrane to allow the transfer of, notably but not exclusively, glucose (Illsley & Baumann, 2020b). Finally, receptors present in the placenta enables maternal immunoglobulin transport, providing passive immunity to the fetus (Palmeira *et al.*, 2012).

Aside from enabling efficient transport, placental cells also play an important role in endocrine signalling. They secrete a panoply of hormones (*e.g.,* human chorionic gonadotropin (hCG), human placental growth hormone and human placental lactogen) to regulate all things pregnancy-related: inducing insulin resistance, development of mammary glands, and so on (Griffiths & Campbell, 2015). Thus, the placenta participates in feto-maternal gaseous and metabolic transfer, while having immunological and endocrine and functions.

In mammals, initial placental classification is based on a structural basis and can be denoted as choriovitelline or chorioallantoic, the latter representing human placentas. Then, the chorioallantoic placentas can be further classified based on gross morphology and histological structure (Furukawa *et al.*, 2014). In terms of morphology, the human placenta is comprised of a single, disk-shaped structure and is thus denoted as discoidal, as shown in Figure 1A (Hafez, 2017). Histologically-speaking, it is hemonochorial in that the maternal blood is in direct contact with the chorion (i.e., hemochorial) and lined by a single layer of trophoblast cells (hence, *mono-*) (Figure 1B) (Furukawa *et al.*, 2014). The function and structure of the human placenta can be quite complex, as is its formation, discussed next.

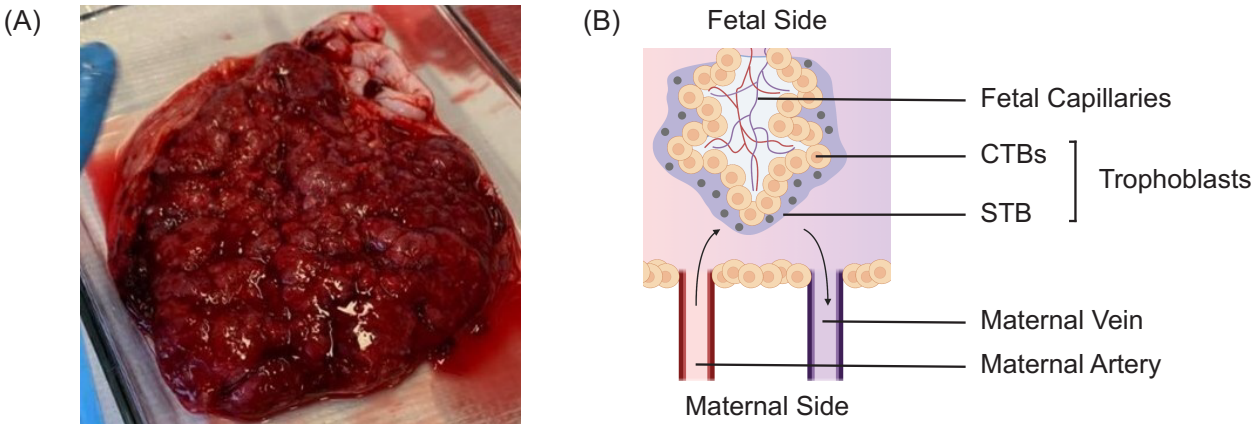


Figure 1.1: The Human Placenta is Discoidal and Hemonochorial

(A) A human placenta (fetal membrane removed) outlining its gross discoidal (disk-shaped) morphology. Image taken by Josianne Bienvenue-Pariseault in the laboratory of Dr. Cathy Vaillancourt. (B) Schematical representation of the human placenta outlining how maternal blood is separated from the fetus by a single layer of trophoblasts, hence its hemonochorial denotation. Abbreviations: CTBs, cytotrophoblasts; STB, syncytiotrophoblast.

1.1.1 Embryogenesis and mechanisms of human placentation

With an understanding of the physiological characteristics of the human placenta, it is important to understand the mechanisms of placentation such that an important parallels between normal placentation and tumorigenesis can be adequately drawn. The mechanisms of placentation are summarized in Figure 1.2. The process, which involves the formation of the zygote, acquisition of characteristics by maternal tissues and the blastocyst, implantation, invasion and finally the establishment of feto-maternal blood flow is discussed next.

1.1.1.1 Conception

Following copulation, sperm deposited in the vaginal canal find their way through the cervix and uterus, before attempting to reach a mature oocyte in the Fallopian tubes, aided by both the constituents of the surrounding fluids and uterine contractions (Georgadaki *et al.*, 2016). Ciliary beating and a series of muscle contractions then enable movement of the fertilized egg (*i.e.,* zygote) from the Fallopian tubes into the uterus (Coy *et al.*, 2012). Concurrently, a series of rapid cell divisions give rise to the morula and continue within the uterus to yield a hollow sphere of cells called the blastocyst, at roughly day 5 post-conception (Kim & Kim, 2017; Rossant & Tam, 2022). Conception is illustrated in Figure 1.2A.

1.1.1.2 Pre-implantation: decidualization and blastocyst activation

Next, a series of physiological changes in both the maternal uterus (termed *decidualization*) and the blastocyst (termed *blastocyst activation*) must occur to allow for the eventual implantation event. Decidualization is mainly characterized by the differentiation of endometrial stromal fibroblast cells into decidual stromal cells, a distinct cell type with increased proliferative potential, immune tolerance, and receptivity to invasion (Ng *et al.*, 2020). On the other hand, blastocyst activation involves a shift in gene expression leading to enhanced metabolism, biosynthesis and cell proliferation to name but a few (He *et al.*, 2019). Implantation itself can only occur between days 19 and 21 of a typical menstrual cycle, denoted as the window of implantation, and highlights how pre-implantation consists of tightly regulated events (Enciso *et al.*, 2021; Harper, 1992). Decidualization and blastocyst activation are the result of complex uterine/blastocyst crosstalk, largely enabled by hormones, cytokines, and growth factors, as discussed next and depicted in Figure 1.2B.

Hormones. A first major hormone involved in the pre-implantation processes is estrogen. Women synthesize three major types of estrogen throughout their lifetime, with estradiol (E2), synthesized in the ovaries, being the main estrogen produced in the reproductive years (Cui *et al.*, 2013). Estrogen signalling occurs via the estrogen receptors α and β (ERα/β) which, upon ligand binding, dimerize and translocate to the nucleus to assist in the transcription of a variety of genes (Reviewed by Fuentes and Silveyra (2019)). Using murine models, Pawar *et al.* (2015) suggest that the lack of ERα limits the expression of the leukemia inhibitory factor (LIF) which in turn prevented the proliferation and differentiation of stromal cells characteristic of decidualization. Meanwhile, the metabolic product of E2 (4-hydroxyestradiol; 4-OH-E2) is believed to mediates a series of changes characteristic of a competent blastocyst (Paria *et al.*, 1998). All in all, then, E2 secreted by uterine cells and its metabolite, 4-OH-E2, enable a myriad of signalling cascades that drive the acquisition of critical properties required for successful implantation, in both the uterus and blastocyst.

Progesterone is also secreted by the uterus in response to trophoblast-derived hCG secretions (Cole, 2010; Gridelet *et al.*, 2020). The former hormone is heavily involved in regulation of the pre-implantation process. Its canonical signalling pathway resembles that of estrogen: binding of the hormone to the progesterone receptor, followed by receptor dimerization and entry into the nucleus. There, the receptor dimer can bind to full or partial progesterone response elements to regulate transcription of key genes involved in endometrial receptivity (Reviewed by Wetendorf and DeMayo (2014)). Chi *et al.* (2020) have put the importance of this signalling

pathway into perspective by identifying over 2000 progesterone-responsive, differentially expressed genes during the pre-implantation period which included Insulin-like growth factor (IGF) Binding Protein 1 (IGFBP1), Indian hedgehog (IHH), and members of the Wingless-related integration site (Wnt) family. IGFBP1 can induce endometrial stromal cell decidualization and is secreted by the decidualized cells, thus making the protein a marker of said process (Kim & Fazleabas, 2004; Matsumoto *et al.*, 2008). Meanwhile, Wei *et al.* (2010) propose that IHH promotes decidual stromal cell proliferation and differentiation characteristic of decidualization, the mechanisms of which are reviewed by Qi and Li (2020). Furthermore, knockdown of IHH has revealed its role in regulating epidermal growth factor (EGF) expression (involved in pregnancy-related events, as discussed later) and estrogen signalling pathways, the role of which has already been discussed (Franco *et al.*, 2010). Unsurprisingly, then, deletion of IHH has been shown to induce defects in embryonic implantation (Bhurke *et al.*, 2016; Lee *et al.*, 2006). Finally, Wnt signalling has been extensively described as central in regulating many aspects of pre-implantation (Reviewed by Tepekoy *et al.* (2015)). Thus, progesterone is a key hormone in the processes preceding implantation.

Yet, hormones involved in pre-implantation are not solely produced by the uterus. Indeed, although the blastocyst mainly secretes hCG to mediate later events in pregnancies, its production by the growing embryo begins early on, before implantation occurs (Bonduelle *et al.*, 1988; Makrigiannakis *et al.*, 2017). Meanwhile, luteinizing hormone/choriogonadrotropin receptor (LHCGR), hCG's receptor, is expressed on endometrial cells (Sacchi *et al.*, 2018). Han *et al.* (1999) began to show the ability of hCG to induce the differentiation of uterine stromal cells into decidual cells, both morphologically and functionally. hCG's ability to induce decidualization was later corroborated by Koch *et al.* (2018).

Cytokines. Cytokines are also widely involved in the physiological changes that occur during decidualization and blastocyst activation. Interleukin-1β (IL-1β) constitutes a first example, as it is secreted by decidual cells and trophoblasts alike (Reviewed by Knöfler and Pollheimer (2012); and Salamonsen *et al.* (2000)). IL-1 mediates gonadotropin receptor expression on ovarian cells, as well as progesterone accumulation and prostaglandin secretion (Chimote *et al.*, 2010). Prostaglandins are involved in modulation of the maternal immune system to allow for fetal tolerance, amongst other functions (Mayoral Andrade *et al.*, 2020). The role of hCG and progesterone signalling have already been described. IL-6 is also reportedly heavily expressed by both endometrial and trophoblastic cells, particularly at the start of the window of implantation, and disruptions in its expression have led to dramatic decreases in viable implantations

(Guzeloglu-Kayisli *et al.*, 2009; Robertson *et al.*, 2000; Tabibzadeh & Babaknia, 1995). Within the same family of cytokines, LIF expression has also been shown to be induced at the time of blastocyst implantation (Charnock-Jones *et al.*, 1994). LIF is a known inducer of the Janus Kinase/Signal Transducer and Activators of Transcription (JAK/STAT), mitogen-activated protein kinase (MAPK) and phosphatidylinositol phosphate kinase (PIPK) pathways, involved in cell survival and differentiation (Duval *et al.*, 2000; Guzeloglu-Kayisli *et al.*, 2009). Expression of its receptor has been discovered in trophoblasts and in the pregnant (but not in the non-pregnant) endometrium, suggesting its importance in embryogenesis (Kojima *et al.*, 1995). Many other cytokines are involved in blastocyst pre-implantation, but the ones outlined here suffice to highlight to complexity of the process.

Growth factors. Multiple growth factors are also heavily involved in the pre-implantation process. EGF is mainly known to drive trophoblast invasion, differentiation, and proliferation (Bass *et al.*, 1994; Li & Zhuang, 1997). Yet, knock-out of its receptor leads to the degeneration of the blastocyst's inner cell mass and death at the pre-implantation phase, suggesting its involvement in the latter process as well (Threadgill *et al.*, 1995). Meanwhile, Chobotova *et al.* (2005) outline an important involvement for heparin-binding EGF (HB-EGF) in decidualization, inducing deep physiological changes in the decidua while Paria *et al.* (2001) indicate that IGF1 drives many of these same changes

1.1.1.3 Implantation and invasion of uterine wall

Decidualization and blastocyst activation provide suitable conditions for attachment of the blastocyst to the uterine wall (termed *implantation*), which can be divided into two events: apposition and adhesion. Then, invasion of the maternal uterus may begin, a process that involves uterine degradation, followed by the proliferation and migration of trophoblasts.

Implantation. Implantation (Figure 1.2C) occurs when the blastocyst attaches to the uterine wall lining the fundus, excluding ectopic pregnancies. The first stage in implantation is apposition, during which the microvilli on the outer surface of the blastocyst will interlock with the pinopodes of the uterine epithelium (upregulated by a variety of hormones), beginning to form a stable interaction between the two structures (Staun-Ram & Shalev, 2005). The following steps in implantation are still misunderstood but still involve critical crosstalk between the uterus and the blastocyst (*e.g.,* LIF-mediated STAT3 activation and hCG-derived progesterone production, discussed earlier in section 1.1.1.2). Next, comes adhesion, in which interactions between E-

cadherins found on both endometrial and trophoblastic cells or between integrin (expressed in both endometrial and trophoblastic cells) and its receptor (glycoprotein osteopontin, found in endometrial cells) allow for a more solid attachment. These mechanisms, and the many others are at play during implantation are reviewed in further detail by Massimiani *et al.* (2019). Uterine degradation may then proceed.

Uterine Degradation. Invasion (Figure 1.2C) of the maternal uterus is largely mediated by the urokinase plasmogen activator (uPA) and its receptor (uPAR). uPA can cleave plasminogen to yield its active form, plasmin (Lijnen *et al.*, 1986). Cells secrete uPA in its inactive for (pro-uPA), which can be cleaved by multiple proteases before or after binding to uPAR, another event required for activation of its catalytic activity Mahmood *et al.* (2018)). Active plasmin, a serine protease, can then degrade multiple targets, including components of the extracellular matrix, initiating the invasion process (Fay *et al.*, 2007). Nordengren *et al.* (2004) have suggested that uPA and uPAR are expressed by endometrial tissue, in a cyclic manner, and increasing throughout the secretory phase of the menstrual cycle. The increased expression of uPA and uPAR coincides with the window of implantation (from section 1.1.1.2) and provides yet another token of the tight regulatory control to which embryogenesis is subjected to. Endometrial cells are not alone in expressing these proteins. Indeed, another study has shown that trophoblasts, particularly those derived from first-trimester placenta, also express uPAR (Zini *et al.*, 1992). Meanwhile, Prutsch *et al.* (2012) suggest that trophoblasts may also express uPA in response to certain molecular cues (*e.g.,* IL-1β secreted by the endometrium, from section 1.1.1.2). Thus, both endometrial and trophoblastic cells contribute to plasminogen activation and, ultimately, degradation of the uterus, setting the stage for uterine invasion.

Trophoblast Proliferation and Differentiation. Shortly after implantation, the trophoblasts cells making up the outer layer of the blastocyst will begin to proliferate and differentiate. Undifferentiated trophoblasts are referred to as cytotrophoblasts (CTBs) whereas differentiated ones, resulting from the fusion of CTBs, are called syncytiotrophoblasts (STBs). Although the mechanisms of CTB proliferation are misunderstood, data stemming from cultured CTBs suggest a potential involvement of growth factors (specifically, EGF) to that effect (Johnstone *et al.*, 2005). Then, not only are CTBs hyperproliferative during early placentation, they also *die* less as indicated by a low incidence of apoptosis at this stage (Smith *et al.*, 2000). Thus, it is suggested that CTBs also respond to anti-apoptotic cues such as preimplantation factor (PIF) secreted by the embryo (Moindjie *et al.*, 2016).

Then, the large number of CTBs undergo fusional events, reportedly mediated by syncytins (1 and 2), inductors of fusional properties (Blond *et al.*, 2000; Mi *et al.*, 2000b; Wang *et al.*, 2018c). Syncytin-1 has been found to be almost exclusively expressed in STBs, and syncytin-2 is heavily expressed in CTBs (Malassiné *et al.*, 2008; Mi *et al.*, 2000a). Activation of the syncytins is believed to be, at least partially, dependent on the activation of the peroxisome proliferator-activated receptor gamma (PPARγ) as agonists of this receptor have been shown to induce trophoblast differentiation (Schaiff *et al.*, 2000). Data suggesting both the overexpression and enhanced activity of PPARγ in pregnant women, and the induction of syncytins by the receptor further supports this theory (Ruebner *et al.*, 2012; Waite *et al.*, 2000). To that effect, upon ligand binding, PPARγ dimerizes with retinoid X receptors and induce a downstream signalling cascade that triggers the activation of many genes (Reviewed by Berger and Moller (2002)). Prostaglandins (and other lipid derivatives) produced during pregnancy are known ligands of PPARγ and thus provide a link between pregnancy-related metabolism and trophoblast differentiation (Yu *et al.*, 1995). The complexity of the genetic network of syncytialization is further highlighted by Papuchova and Latos (2022), who, in their review, propose the involvement of hCG and EGF in the induction of syncytin-1 and 2 expression.

Invasion and Migration. Finally, trophoblast will undergo an epithelial-mesenchymal transition (EMT), thereby unlocking their migratory potential (Kalluri & Neilson, 2003). The process involves a progressive transition in expression of proteins involved in tighter junctions to those involved in motility and resistance to apoptosis (hence, *invasive*) (Reviewed by Kalluri and Weinberg (2009)). To that respect, DaSilva-Arnold *et al.* (2018) report that extra-villous trophoblasts (EVTs) downregulate many epithelial markers. More markers of EMT (*e.g.,* Zinc-Finger SNAI1 (SNAIL) and β-catenin) have also been described by others as having an involvement in placental development, their expression being often altered in placental pathologies (Blechschmidt *et al.*, 2007; Davies *et al.*, 2016; Han *et al.*, 2019). Expectedly, this pattern of expression was markedly more prominent in first trimester placentas, wherein the process of placentation is still in effect. Nonetheless, only a subset of the markers expected from an EMT are differentially expressed such that the process outlined here is characterized by many as a *partial* EMT (DaSilva-Arnold *et al.*, 2018; Davies *et al.*, 2016). Many cues such as growth factors (*e.g.,* Granulocyte colony stimulating factor) and Wnt proteins activate diverse and complex signalling cascades to induce the transcription factors that drive the transition (Bilyk *et al.*, 2017; Ding *et al.*, 2021; Li *et al.*, 2020). Enabled by these changes in genetic prolife, trophoblasts will invade the maternal decidua, as shown in Figure 1.2D.

1.1.1.4 Implementation of blood flow

We have described how conception leads to blastocyst formation, which then travels into the uterine cavity where decidualization and blastocyst activation will enable implantation and invasion. How these events lead to the formation of the full-fledged placenta (Figure 1A and 2E) and the implementation of blood flow to enable the critical functions of the placenta is discussed next.

During the invasion process, two groups of trophoblasts can be found: apical trophoblasts are directly involved in endometrial invasion (referred to as EVTs) while those closer to the base of the growing placenta form chorionic villi (tree-like projections that enable gaseous exchange) and are hence called villous trophoblasts (VTs). Thus, a subset of CTBs are also VTs, and some of these may differentiate into a STB layer, who lines the outer layer of the chorionic villi (Reviewed by Kojima *et al.* (2022)). STBs result from the fusion of CTBs, a process in which the latter subtype will lose its cell membrane to form a multinucleate mass (Renaud & Jeyarajah, 2022). Open-ended maternal blood vessels resulting from trophoblast invasion will leak into spaces (lacunae) within the STB layer (Wang & Zhao, 2010a). Eroded veins and arteries carry blood to and from the lacunae, providing a closed system of blood flow which now includes the placenta. However, throughout the first trimester, trophoblasts will form plugs in the maternal blood vessels, preventing blood supply and enabling a hypoxic environment that further promotes trophoblast differentiation. These plugs are removed after the first trimester to enable maternal-placental blood flow by mechanisms still misunderstood (Saghian *et al.*, 2019). Meanwhile, EVTs form a layer that contacts with the maternal decidua, forming a so-called cytotrophoblastic shell that surrounds the STB layer (Burton & Jauniaux, 2017).

Within the chorionic villi, vasculogenic and angiogenic events lead to the intrusion of fetal capillaries (Chen & Zheng, 2014; Kingdom *et al.*, 2000). These capillaries connect to the umbilical vein and a set of paired umbilical arteries who, respectively, carry oxygenated and deoxygenated blood to and from the fetus (Reviewed by Wang and Zhao (2010b)). Thus, maternal blood eventually flows into the space provided by the STB layer, enclosed by the CTB shell, and the proximity of the blood with fetal capillaries enables nutrient and gas exchange to and from the fetus.

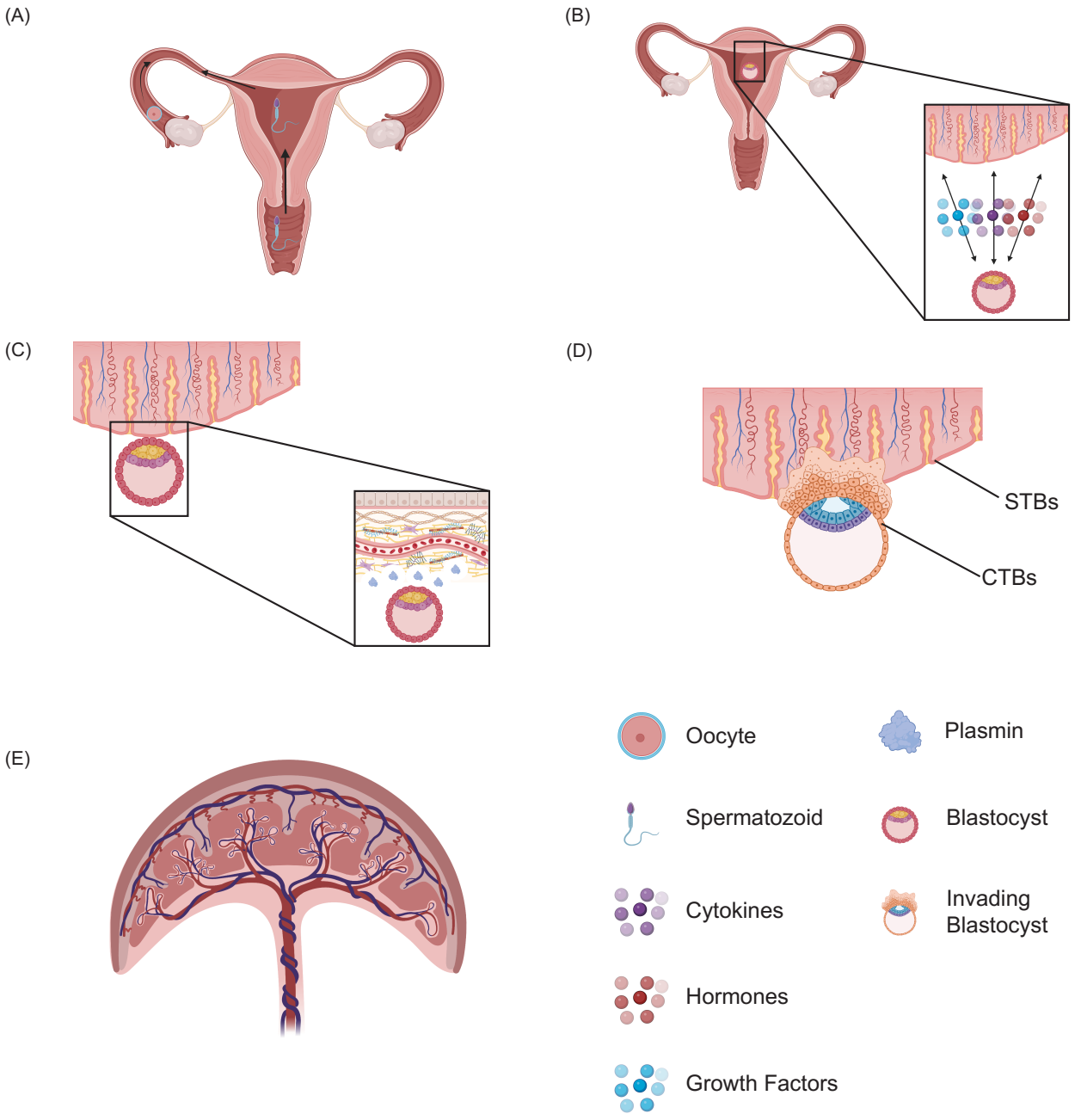


Figure 1.2: Overview of Human placentation

(A) Sperm deposited into the vagina will try to reach a mature oocyte in the Fallopian tubes. The then zygote will undergo cell divisions to yield the blastocyst and travel to the uterus, where it will (B) initiate crosstalk which endometrial tissue via cytokines, hormones, and growth factors. This will induce decidualization and blastocyst activation and enable (C) the implantation event. Activation of the uPA system will induce plasmin activation and subsequent uterine degradation, followed by (D) differentiation of CTBs into STBs and invasion of the endometrium. These events eventually lead to the implementation of blood flow to the placenta, eventually yielding (E) the full placenta. Abbreviations: uPA, urokinase plasmogen activator; CTBs, cytotrophoblasts; STBs, syncytiotrophoblasts. © Sanchez-Espinoza (2023)

1.1.2 Human placenta and cancer

Properties such as invasiveness, uncontrolled growth, immune evasion, angiogenesis and deregulating cellular energetics have become widely accepted as key properties of cancer progression (Hanahan & Weinberg, 2011). Yet, these same properties are key to proper placentation, albeit under tight regulatory control. Here, we highlight the resemblance between the molecular mechanisms of normal placentation and those of tumorigenesis, summarized in Table 1.1.

Invasiveness. Perhaps the most striking similarity between placentation and tumorigenesis is the inherit capacity to invade tissues. Indeed, EMT allows trophoblasts to invade the maternal uterus in normal pregnancies (section 1.1.1.3) but has also widely been accepted as a key property allowing cancer cells to enter the vascular system and reach distal sites (Reviewed by Mittal (2018)). The same biochemical markers of this transition are seen in both processes. β-catenin and SNAIL are examples of such, acting as a transcriptional co-activator of many EMT-related genes and as a repressor of E-cadherin (involved in tight cell adhesions), respectively (Lecarpentier *et al.*, 2019; Peinado *et al.*, 2004). β-catenin and SNAIL have been shown to be highly expressed in many cancers (Cheng *et al.*, 2019; Cui *et al.*, 2018; Shin *et al.*, 2012; Smith *et al.*, 2014; Yu *et al.*, 2020). Fan *et al.* (2016) demonstrate that these same proteins are readily expressed in the early phases of gestation, when trophoblast invasion, and EMT, occur (from section 1.1.1.3).

Uncontrolled growth. Invasiveness alone does not characterize cancers, instead defined by their basic property: uncontrolled cell growth. Uncontrolled cell growth relies on sustained proliferative signalling and evasion of growth suppressors. EGF appears to be a major regulator of cell proliferation in normal placentation (from sections 1.1.1.2 and 1.1.1.3). Yet, enhanced expression or activity of its corresponding receptor (epidermal growth factor receptor (EGFR)) is widely accepted as a driving factor in the incidence of many cancer types (Wee & Wang, 2017). Then, the evasion of growth suppressors is seen in both normal placentation and in cancer progression. We briefly touched on PIF and its ability to silence the apoptotic machinery preventing tumour protein p53 (p53) phosphorylation/activation and both inhibiting and inducing the expression of apoptotic effector proteins and apoptosis inhibitors, respectively (Moindjie *et al.*, 2016). Hanahan and Weinberg (2011) themselves refer to p53 as the *prototypical* tumor (growth) suppressor gene whose altered function is often, unsurprisingly, linked to tumor progression (Reviewed by Rivlin *et al.* (2011)).

Immune evasion. The semi-allogenic embryo is inherently recognized as non-self by the maternal immune system. As such, overzealous maternal immune systems tend to be involved in loss of pregnancies (Reviewed by Li *et al.* (2021)). Consequently, successful pregnancies necessitate the downregulation of the immune system, mainly driven by (1) immune tolerance or (2) T cell exhaustion. Immune tolerance is largely mediated by regulatory T (Treg) cells, whose function is to downplay the immune system in a variety of settings (Reviewed by Corthay (2009)). The observation that Treg cell levels increase during pregnancy, while declining *post-partum*, suggests their involvement in fetal protection (Somerset *et al.*, 2004). The immunosuppressive activity of Treg cells are mediated by the expression of Forkhead box P3 (FoxP3), which may be induced by IL-1β signalling (Ganesh *et al.*, 2011; Rudensky, 2011). Indeed, while IL-1β has pleiotropic effects in the immune system, it can activate Treg cells under certain conditions, such as the presence of dendritic cells (Brinster & Shevach, 2008). While IL-1β is secreted by the endometrium (from section 1.1.1.2), dendritic cells are readily to be found in the decidua during pregnancy (Reviewed by Wei *et al.* (2021)). Thus, secretions and cellular components during pregnancy may drive the immunosuppressive environment required for fetal development. Meanwhile, Apte *et al.* (2006) have demonstrated that IL-1β in the tumor microenvironment promotes cancer progression and Huang *et al.* (2019) have outlined its involvement in the induction and infiltration of Treg cells within these tumors. This is in line with the observation that Treg cell tend to be heavily present in patients afflicted with various cancer types (Liyanage *et al.*, 2002; Meloni *et al.*, 2006).

More recently, Lewis *et al.* (2022) suggested that T cell exhaustion is present in the feto-maternal interface, driven by the nuclear factor of activated T cells/thymocyte selection associated HMG Box/programmed cell death protein 1 (NFAT/TOX/PD-1) axis. NFAT is a transcription factor that appears to drive the expression of TOX and PD-1, as its constitutive activation enables overexpression of the latter proteins (Martinez *et al.*, 2015). TOX and PD-1 are heavily involved in the induction of T cell exhaustion (Reviewed by Blank and Mackensen (2007); and Liang *et al.* (2021)). Unsurprisingly, NFAT, TOX and the ligand of PD-1 (PD-L1) have been shown to be overexpressed in many cancers (Bardhan *et al.*, 2016; Kim *et al.*, 2020; Lobbardi *et al.*, 2017; Zhang *et al.*, 2012; Zhong *et al.*, 2022).

Angiogenesis. Generating new blood vessel *de novo* (vasculogenesis) or from previously formed ones (angiogenesis) are central, heavily regulated events in establishing blood flow to the developing fetus (Reviewed by Zygmunt *et al.* (2003)). When considering angiogenesis, molecular cues of the fibroblast growth factors (FGFs) and vascular endothelial growth factors

(VEGFs) families upregulate the migration, growth, and differentiation of endothelial cells during angiogenesis whereas thrombospondins inhibit these events (Good *et al.*, 1990; Ucuzian *et al.*, 2010). Unsurprisingly, normal term placental tissues have been shown to express high levels of the VEGF-A protein, and of the *VEGF-B* and *VEGF-C* mRNAs (Vuorela *et al.*, 1997). Meanwhile, another study showed that human VTs contribute to angiogenesis by releasing basic FGF (bFGF) (Hamai *et al.*, 1998). High levels of VEGFs and bFGF are also seen in many types of cancer (Birck *et al.*, 1999; Sobol-Milejska *et al.*, 2017; Trojan *et al.*, 2004).

Deregulated cellular energetics. Finally, we consider the similarities in cellular energetics between normal placental cells and cancerous ones. In the 1920s, Otto Warlberg coined the propensity of cancer cells to favour glycolysis over oxidative phosphorylation under aerobic conditions, despite its considerably lower ATP yield (Warburg *et al.*, 1927). To offset the lower ATP yield, it is believed that glucose transporters (especially glucose transporter 1 (GLUT1)) become overexpressed (Hanahan & Weinberg, 2011). Given that early placentation occurs in relatively anaerobic conditions, it is understandable that these same transporters are ubiquitously expressed throughout the placenta (Illsley & Baumann, 2020a; Rodesch *et al.*, 1992). Furthermore, *in vivo* studies have shown that CTBs upregulate glycolysis at specific times during placentation, particularly during STB differentiation (Bax & Bloxam, 1997; Kolahi *et al.*, 2017). Glycolysis in the placenta, however, does not only occur in anaerobic conditions but has instead been identified as a major energy source in aerobic conditions as well (Kolahi *et al.*, 2017).

1.2 Gestational trophoblastic disease and human placental choriocarcinoma

As discussed in section 1.1.1.2, normal human placentation relies on a complex network of hormones, cytokines, and growth factors to regulate key events such as trophoblast proliferation and uterine invasion. Nevertheless, these cells may escape the regulatory network and undergo abnormal growth and give rise to a collection of diseases termed gestational trophoblastic diseases (GTDs). Choriocarcinomas, a subset of GTDs, are tumours that arise from the outermost layer of the human embryo (hence, *chorio-*), that consists of trophoblast cells that line (i.e., *carcinoma*) the placenta.

Table 1.1: Overview of the Similarities Between Normal Placentation and Cancer Progression

Feature	Proteins Involved	Functional Role	Expression in placentation	Expression in cancerous cells
Invasiveness				
	β-catenin	Marker of EMT	+	+
	SNAIL	Marker of EMT	+	+
Uncontrolled Growth				
	EGF/EGFR	Proliferation	+	+
	P53	Induction of apoptosis	-	-
Immune Evasion				
	IL-1β	Treg cell induction	+	+
	NFAT	T cell exhaustion	+	+
	TOX	T cell exhaustion	+	+
	PD-1/PD-L1	T cell exhaustion	+	+
Angiogenesis				
	VEGF-A/B/C	Angiogenesis	+	+
	bFGF	Angiogenesis	+	+
Deregulated Cellular Energetics			+	+
	GLUT1	Glucose Transport	+	+

Normal placentation and tumorigenesis share many characteristics and patterns of gene expression. Expression patterns depicted here are generalized and may not be representative of specific cancer types. Plus signs (+) indicate expression, and minus signs (-) indicate lack thereof. Abbreviations: SNAIL, Zinc-Finger Protein SNAI1; EMT: Epithelial-Mesenchymal Transition; EGF(R): Epidermal Growth Factor (Receptor); IL-1β, Interleukin 1β; NFAT, Nuclear Factor of Activated T cells; TOX, Thymocyte Selection Associated HMG Box; PD-(L)1, Programmed Cell Death Protein (Ligand) 1; VEGF-A/B/C, Vascular Endothelial Growth Factor A/B/C; GLUT1, Glucose Transporter 1; Treg, Regulatory T cell. © Sanchez-Espinoza (2023)

1.2.1 Epidemiology and risk factors

The incidence of placental choriocarcinomas is rare. In Europe and North America, about 1 in 40,000 pregnancies result in its development, but increases in some parts of the world, such as Asia, in which its incidence is roughly 3 to 9 times higher (Lurain, 2010). Many factors drive its incidence, and it is widely accepted that cancer progression is driven by a combination of genetic and environmental components (the concept of *nature versus nurture*) and gestational choriocarcinoma is no exception.

Genetics. Genetic aberrations can be linked to the occurrence of choriocarcinomas. As an example, mutations in the nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing 7 (NLRP7) gene that lead to its upregulation have been shown to induce an immunosuppressive environment that leads to the progression of this type of cancer (Reynaud *et al.*, 2021). Yet, normal genetics may also contribute to the progression of choriocarcinoma in that data compiled from multiple case reports worldwide have outlined that crosses between maternal blood type A and paternal blood type O elevate the incidence of the disease (risk ratio of 10.4 relative to other groups) (Altieri *et al.*, 2003).

Environment. Environmental components are also believed to act as important risk factors for choriocarcinomas. Maternal age has been identified as a major one as retrospective studies from the UK and the Philippines have identified an increased likelihood of developing the disease in women over 40 years old (Baltazar, 1976; Savage *et al.*, 2020). Unsurprisingly, components of cigarette smoke have been shown to induce growth and migration of choriocarcinoma cells (Lee *et al.*, 2017). Meanwhile, the long-term use of oral contraceptives has also been proposed as a risk factor for the development of placental choriocarcinoma (Palmer *et al.*, 1999). However, this topic remains controversial as more recent studies seem to disprove it (Costa & Doyle, 2006).

Prior incidence of molar pregnancies (formation of a hydatidiform mole (HM)) appears to be linked to the incidence of choriocarcinomas. Indeed, reports indicate that approximately 3% of HMs develop into choriocarcinomas, and that roughly 25% of choriocarcinomas arise from HMs (Duffy *et al.*, 2015; Soper, 2021). Thus, it is important to consider risk factors of HMs as indirect risk factors of choriocarcinomas. To that effect, mutations in the *KH Domain Containing 3-Like* (*KHDC3L)* gene have been linked with the occurrence of HMs (Ngan *et al.*, 2021; Salehi *et al.*, 2011). The gene appears to be critical in repairing DNA lesions, perhaps explaining its involvement in cancer progression (Zhang *et al.*, 2019b). Dietary choices also appear to indirectly

affect the incidence of choriocarcinomas as diets low in carotene have been linked to the incidence of HMs (Berkowitz *et al.*, 1985).

1.2.2 Clinical manifestations

Major clinical manifestation of patients afflicted with choriocarcinoma include irregular vaginal bleeding and pelvic pain (Ryu *et al.*, 2015; Udare & Mondel, 2013). Masses felt in the abdominal or uterine region may also constitute an indicator of the disease (Yousefi *et al.*, 2016). The diagnosis, however, is often corroborated by testing for abnormally high levels of hCG levels as CTBs naturally produce the hormone (Cole & Butler, 2014). Under normal conditions, hCG levels rise as the pregnancy progresses until achieving a peak at roughly the 11^{th} week of gestation, at which point a decrease is observed until a plateau is reached (Korevaar *et al.*, 2015). Physicians often look for a steady increase in hCG levels (Pang & Ma, 2020).

The clinical manifestations of metastasis to other regions of the body are numerous and site-specific. As an example, complications involving the respiratory tract such as hemoptysis, angina, and respiratory failure have been documented in cases in which lung metastasis has occurred (Zhang *et al.*, 2017). In cases of brain metastases, cerebral hemorrhages and hematomas are common (Wang *et al.*, 2018a).

1.2.3 Experimental models

Left untreated, placental choriocarcinoma can be highly metastatic, and deadly. However, efficient treatment requires a complete understanding of the complex mechanisms of the disease and, as such, highlights the requirements for proper models. Here, models for both normal placentation and choriocarcinomas are outlined.

1.2.3.1 In vivo

Ethical constraints and long gestation period render the use of human placentas as models impractical. As such, those from non-human primates (*i.e.,* old-world monkeys) may be used instead as they have a similar villous placental structure, do not exhibit progesterone withdrawal during labour and have comparable feto-maternal exchange mechanisms. Furthermore, these monkeys have longer gestational periods that approximate those of humans more accurately than

other models, but shorter gestational (*e.g.,* 165 days for the rhesus macaque, 170 days for the baboon) than humans, making it an attractive model (Tardif *et al.*, 2012). The use of this model does have its limitations, an example being a less pronounced trophoblast invasion (Reviewed by Grigsby (2016)).

Depending on the gestational mechanism being studied, guinea pigs, sheep and mice may also be used. Indeed, whereas guinea pigs have an overall different placental structure, they too do not undergo progesterone withdrawal. On the other hand, sheep exhibit a different placental structure as well as a different rate of angiogenesis (amongst other differences), but have an elaborate period of trophoblast invasion, which also form structures mimicking STBs. Finally, mice have similar placental structure and feto-maternal gas exchange mechanisms, but differ in the types of trophoblasts found and, in their organization (Reviewed by Costa *et al.* (2021); and Grigsby (2016)). Thus, the use of a particular *in vivo* model is dependent on the mechanism under scrutiny.

1.2.3.2 Ex vivo

Placental explant cultures provide an *ex vivo* model of the organ in which a portion of a placenta is cut out and grown in culture media. The use of this model can be dated back to at least the mid 1960s (Miller *et al.*, 2005; Sybulski & Tremblay, 1967). Since then, explants have been used in studies assessing viability, hormone secretion, and many more in response to various agents (Eliesen *et al.*, 2021; Wójtowicz *et al.*, 2007). Explant cultures provide insight as to the organ as a whole since they contain all the cell types, organized as they would be *in vivo*. However, obtaining *ex vivo* models may cause stress on the tissues of interest, which ought to be considered during analysis.

Later on, Panigel *et al.* (1967) described the *ex vivo* placental perfusion model, for which Conings *et al.* (2017) have provided a complete, updated protocol. Briefly, the full placenta is kept alive *post-partum* in appropriate media. Catheters are inserted in the maternal and foetal sides, and various solutions of interest can be flowed to analyze various placental parameters (glucose consumption, drug transfer percentage, etc.). Of course, the technique comes with its limitations in that perfusions can only be sustained for up to 48 hours, with a success rate of about 50%. Furthermore, these perfusions are usually only carried out in full-term placentas and thus do not provide insight into the characteristics of first and second trimester placentas, in which the

pharmacokinetics may largely differ (Myllynen & Vähäkangas, 2013). Thus, the use of this technique is particular to certain studies.

1.2.3.3 In vitro

In vitro studies of choriocarcinomas are largely mediated by 3 cell lines (BeWo, JEG-3 and JAR) which are extracted from the same type of tumour, but with different origins. The behavior of each cell type varies, an example being the well-characterized ability of BeWo cells to form STBs in response to forskolin, which is not observed in JEG-3 cells (Al-Nasiry *et al.*, 2006; Wice *et al.*, 1990). Differences are also observed between cell types in their ability to form tight junctions (BeWo form tigher junctions, faster) and in their metabolic activity (lowest in BeWo) (Rothbauer *et al.*, 2017). Differences are even observed between syncytialized and non-syncytialized BeWo cells, as shown by varying responses to antineoplastics (Eliesen *et al.*, 2021). Comparisons between BeWo and JEG-3 cells alone suffice to put these differences into perspective, as roughly 2700 genes involved in many cellular processes were found to be differentially expressed (Burleigh *et al.*, 2007). Although data for a complete profiling of the three above-mentioned cell lines lack, some molecular differences between the three cell types have been identified, making each cell type more or less ideal for the study of specific processes: JAR and JEG-3 cells are used as a model of invasiveness whereas BeWo cells are used to study syncytialization and act as a third trimester model (Drwal *et al.*, 2018; Reviewed in Hannan *et al.*, 2010).

On the other hand, studies on healthy trophoblasts are enabled by elaborate methods for cellular extraction from placental tissue (An example of which is provided by Sagrillo-Fagundes *et al.* (2016)). The use of these primary cells is scarce as they do not grow in culture, can become overgrown by contaminating cells, and have a limited lifespan of 7 to 30 days with conventional and novel techniques, respectively (Nursalim *et al.*, 2021). To overcome this limitation, trophoblasts are often immortalized via genetic transformation with *immortalization genes* (*e.g.,* HTR-8/SV_{neo} cells) or via fusion with another immortal cell line (*e.g.,* ACH-3P cells) (Graham *et al.*, 1993; Hiden *et al.*, 2007). Immortalized cells, however, sooner or later have distorted behaviour and data obtained using these models should be interpreted with caution (Gupta, 2017).

1.2.4 Treatment options

The models outlined previously have propelled the emergence of efficient treatment options for placental choriocarcinomas. Prior to the emergence of chemotherapy, the mortality rate was roughly 60% in the case of non-metastatic choriocarcinomas, and close to 100% once metastasis had occurred (Capobianco *et al.*, 2021). With current treatment options, the mortality rate has significantly decreased, down to 10% (Capobianco *et al.*, 2021; Kohorn, 2014). Radiation, surgery and, more commonly, chemotherapy are the main treatment options for the disease. The implementation of one form of therapy over the other is based on grading systems implemented by the International Federation of Gynecology and Obstetrics (FIGO) and/or the World Health Organisation (WHO). Scores consider multiple prognostic factors, with stage 4 representing advanced, highly metastatic tumors (Ngan *et al.*, 2018).

1.2.4.1 Radiation therapy

Radiation therapy employs localized, high-energy particles to directly kill cancer cells or induce irreparable DNA lesions, thus halting cancer cell growth (Baskar *et al.*, 2012). In the treatment of placental choriocarcinomas, radiotherapy is usually reserved for cases in which metastasis has occurred to the brain. In such cases, the risk of post-operative intracranial hemorrhage outweighs the side effects of radiation therapy (Hanna & Soper, 2010). On the other hand, it has been reported that most chemotherapeutic agents (also called antineoplastics) cannot efficiently enter the brain due to the physiological barriers imposed by the blood-brain barrier (Bhowmik *et al.*, 2015). Although these reports appear to have been disproven by some, radiation therapy is nonetheless still preferred over chemotherapy in the treatment of brain metastases (Lee *et al.*, 1989; Rosner *et al.*, 1986). Radiation therapy is not the primary form of treatment, however, and is given as an adjuvant to chemotherapy (reviewed in section 1.2.4.3).

1.2.4.2 Surgery

The use of surgery in the treatment of placental choriocarcinomas appears to be quite limited due to the high efficacy of chemotherapy (discussed in section 1.2.4.3). As such, surgery is often used as an adjuvant to chemotherapeutic treatment in high-risk neoplasms (Lurain, 2010). Nonetheless, the *Journal of Obstetrics and Gynaecology Canada (JOGC)* outlines two surgical

procedures that are to be considered in treating GTDs (choriocarcinomas included) as a whole: dilation and curettage (D&C) and hysterectomy (Eiriksson *et al.*, 2021).

A D&C is a minor surgical procedure in which the cervix is opened (*i.e.,* dilated) and tissue from the endometrium is scraped off (*i.e.,* curettage). The procedure is mainly used for diagnostic reasons as execution of the procedure often does not remove all the endometrial lining and has been linked to spreading of the cancer (Al-Talib *et al.*, 2010). Furthermore, clinical studies have demonstrated that the technique, as a treatment option, cures about 40% of patients affected with gestational trophoblastic neoplasia (which include choriocarcinomas), a success rate much lower than that of chemotherapy (Osborne *et al.*, 2016).

On the other hand, a hysterectomy is the removal of a female's uterus, of which a major drawback is that it leads to infertility. Furthermore, with recent advances in the medical field, patients undergoing hysterectomies and fertility-preserving treatment options have been shown to have comparable survival rates (Goto *et al.*, 2004). Again, the procedure is mainly used in conjunction with chemotherapy, in which cases it has been shown to be highly effective (Xiang *et al.*, 2000). Nonetheless, the procedure is usually prescribed in cases where the ability to conceive is no longer wanted.

Although D&C and hysterectomy may be suitable for treating primary site choriocarcinomas, the range of appropriate surgical procedures becomes quite large in cases where metastasis has occurred. To put things into perspective, a retrospective study from a single hospital has outlined the use of lung resections, craniotomy and adrenalectomy, to name but a few (Eoh *et al.*, 2015).

1.2.4.3 Chemotherapy

Here, we discuss the various chemotherapeutic regimens used in the treatment of choriocarcinomas whereas the mechanism of action of each major drug classes are explored later (section 1.4). Studies dating back to the late 1990s outline methotrexate's (MTX) high efficiency in curing low risk choriocarcinomas (Lurain & Elfstrand, 1995). More recent retrospective studies have consolidated this knowledge, while independent clinical trials have proposed Dactinomycin (Actinomycin D; Act D) as perhaps an even better alternative to MTX (Osborne *et al.*, 2011; Taylor *et al.*, 2015). Given their efficiency, administration of single-agent MTX or Act D have become the norm in treating low-risk choriocarcinomas.

High-risk choriocarcinomas do not respond as well to single-agent therapies and thus require more robust alternatives. To that effect, physicians have tested the efficacy of many multi-drug regimens: MFA (MTX, folic acid, Act D), MAC (MTX, Act D, Cyclophosphamide), and CHAMOCA (cyclophosphamide, hydroxycarbamide, doxorubicin, Act D, MTX, melphalan, and vincristine). However, EMA/CO (etoposide (ETO), MTX, Act D, cyclophosphamide, vincristine) stood out as the most effective (Kim *et al.*, 1998b). Although recent studies suggest that the EMA (ETO, MTX, Act D) regimen may be just as effective, the current standard for high-risk choriocarcinomas remains the EMA/CO regimen (Jareemit *et al.*, 2020).

Still, standard EMA/CO treatment has been shown to fail in some cases (Kim *et al.*, 1998a). Thus, many other regimens have been attempted throughout the years. The use of TP/TE (paclitaxel (PTX), cisplatin/ PTX, ETO) was reported in 2020 for an persistent, unresponsive tumor (Goldfarb *et al.*, 2020). In another case involving lung and brain metastases, a regimen revolving around 5-fluorouracil (5-FU) was administered (Yang *et al.*, 2015). In yet another case, a regimen involving oxaliplatin (OXA) to treat placental choriocarcinomas was documented (Lan *et al.*, 2010). Thus, although single-agent MTX or Act D, and the EMA/CO regimen are the common treatment options, many other alternatives are often used by physicians worldwide.

1.3 Fundamentals of error correction during mitosis

Understanding the mechanism of action of chemotherapeutic agents requires fundamental knowledge of how intracellular machinery detects and handles abnormalities throughout mitosis. Most antineoplastics function by (1) inducing high degrees of DNA damage or (2) altering the dynamicity of microtubules and inducing aberrant mitotic spindles during metaphase (further discussed in section 1.4). The end goal is to induce sufficient aberrations such the cancer cells undergo apoptosis.

1.3.1 DNA damage: converging towards p53

Since antineoplastics aim to kill cancerous cells, we begin by discussing how DNA damage induces cell death. Multiple DNA damage sensors exist in cells, ultimately leading to the activation of p53 (summarized in Figure 1.3). The first major player to this effect is homeodomain interacting protein kinase 2 (HIPK2). In the absence of genotoxic stress, HIPK2 is constitutively tagged from destruction by the E3 ubiquitin ligase WD Repeat and SOCS Box Containing 1 (WSB-1) (and others). However, when HIPK2 senses DNA damage, it undergoes auto-phosphorylation which

prevents ubiquitination by WSB-1 and induces its kinase activity. Activated HIPK2 can then phosphorylate many targets involved in cell cycle regulation and apoptosis (*e.g.,* p53), DNA repair (*e.g.,* wild-type p53 induced phosphatase 1 (WIP1)), and many others (Kuwano *et al.*, 2016).

Simultaneously, DNA-dependent protein kinase (DNA-PK) also monitor for DNA damage. Contrary to HIPK2, it is the activity of DNA-PK that is regulated, rather than its intracellular levels which remain fairly constant throughout the cell cycle (Lee *et al.*, 1997). The activity of DNA-PK is believed to be mediated by the recruitment of DNA-PK to the site of DNA damage. First, it is believed that the scaffold subunit of DNA-PK, K_u, through its high affinity for free ends of DNA, binds the DNA and recruits the catalytic subunit (DNA-PK_{cs}) (Lee *et al.*, 2015; Yang *et al.*, 2003). It is also believed that protein components of the nuclear matrix (*e.g.,* C1D, which is inducible by infrared light) are capable of binding damaged DNA and recruit DNA-PK in a similar fashion. Binding of DNA-PK to its partners then allows a conformational change, enabling its catalytic role (Yang *et al.*, 2003). DNA-PK can then phosphorylate many proteins downstream to modulate their activity, with a notable example being, once again, p53 and its negative regulator Mouse double minute 2 homolog (MDM2) (Mayo *et al.*, 1997; Sakaguchi *et al.*, 1998).

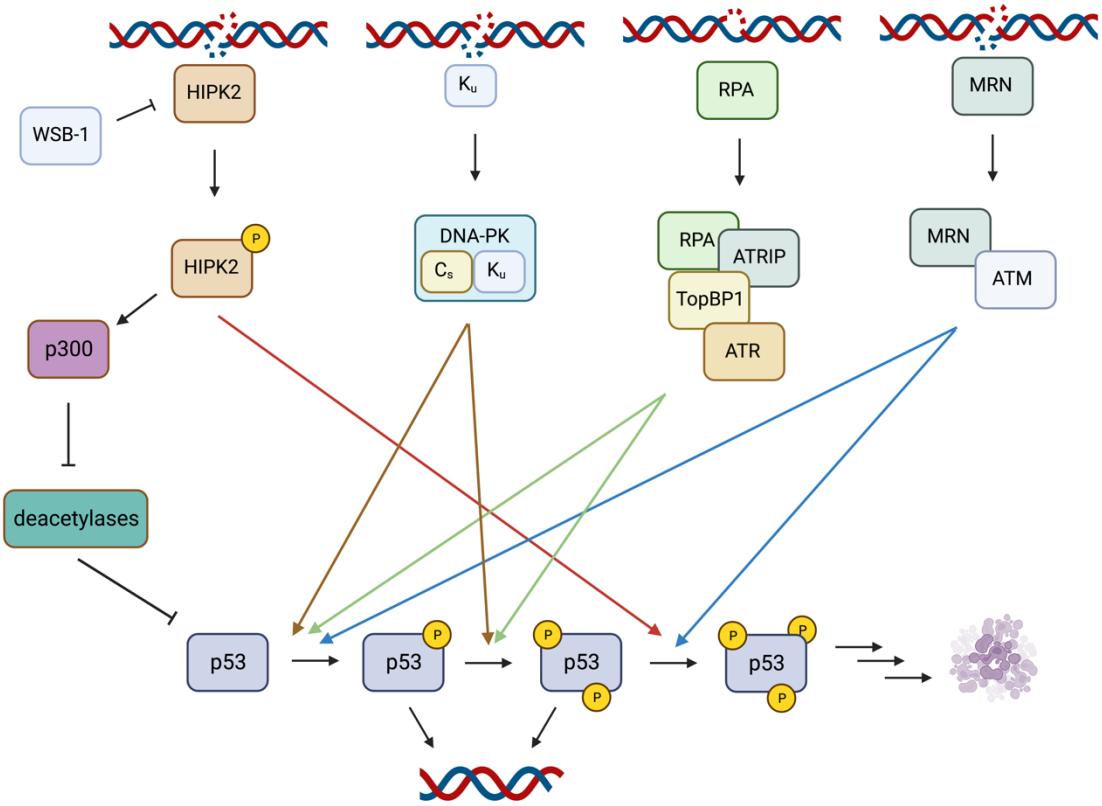


Figure 1.3: Multiple DNA Damage Sensors Ultimately Lead to the Activation of p53

HIPK2, DNA-PK, ATR and ATM systems detect DNA damage and induce DNA repair or apoptosis. HIPK2 can interact with breaks in DNA strands to induce its autophosphorylation and release from its inhibitor WSB-1. Phosphorylated HIPK2 can also activate p300 to repress the activity of many deacetylases that act on p53. A subunit of DNA-PK (K_u) is recruited to the site of DNA damage, which allows further recruitment of the catalytic subunit (C_s) and DNA-PK reconstitution. RPA binds to ssDNA, and sequentially recruits ATRIP, TopBP1 and ATR. The MRN complex can interact with the site of DNA damage and recruit ATM. All these pathways lead to the phosphorylation of p53 at multiple sites to either create pulses of active p53 (inducing DNA repair) or sustained p53 activation (inducing apoptosis). Red, brown, green and blue arrows indicate the phosphorylation of p53 induced by HIPK2, DNA-PK, ATR and ATM, respectively. © Sanchez-Espinoza (2023)

A third mechanism for sensing of DNA damage lies in the Ataxia Telangiectasia Mutated/Ataxia Telangiectasia Mutated and Rad 3 (ATM/ATR) pathway. In this pathway, the Double Strand Break Repair Nuclease 11- DNA repair protein Rad50- Nibrin 1 (MRE11-RAD50-NBS1; MRN) complex recognizes the presence of DSBs and recruits ATM to the site of DNA damage. Recruitment of ATM increases its catalytic activity, which in turn, enables the phosphorylation of many downstream effectors: Breast Cancer Type 1 Susceptibility Protein (BRCA1), Checkpoint Kinase 2 (CHK2), and p53, to name but a few (Maréchal & Zou, 2013). Conversely, the occurrence of single-stranded DNA (ssDNA) can also be quickly recognized by

replication protein A (RPA), a high-affinity and stable interaction (Chen & Wold, 2014). ATR-interacting protein (ATRIP) can then interact with RPA through multiple, redundant, binding sites (Namiki & Zou, 2006). ATRIP has then been shown to recruit DNA Topoisomerase II (TopoII) Binding Protein 1 (TopBP1) and ATR, which induces the activation of the latter kinase (Mordes *et al.*, 2008). ATR can then phosphorylate its downstream effector proteins, such as p53 (Tibbetts *et al.*, 1999).

Thus, the major mechanisms of DNA damage detection (HIPK2, DNA-PK and ATM/ATR) are all implicated in the direct or indirect activation of p53. Yet, p53, a transcription factor, is known to govern the activation of seemingly conflicting set of genes involved in cell cycle arrest, followed by either DNA repair and survival, or apoptosis under different stress conditions (Fischer, 2017). Understanding how p53 can make this switch requires that we acknowledge that the mechanisms of p53 activation discussed so far are quite simplistic. Indeed, different sensors of DNA damage phosphorylate p53, but they can do so at multiple sites: HIPK2 is known to phosphorylate Ser46, DNA-PK to phosphorylate Ser15 and Ser37, ATM to phosphorylate Ser15 and Ser46, and ATR to phosphorylate Ser15 and Ser37 (D'Orazi *et al.*, 2002; Saito *et al.*, 2002; Shieh *et al.*, 1997; Tibbetts *et al.*, 1999). Phosphorylation of Ser46, but not Ser15, is believed to be heavily involved in the progression of apoptosis, and to be linked to its ability to interact with a different set of binding partners, activating a different set of genes (Beckerman & Prives, 2010; Smeenk *et al.*, 2011). Furthermore, phosphorylation is not the only regulatory event involved in the activation of p53: acetylation also plays an important role and is mediated by HIPK2 who recruits p300 (an acetyltransferase) and inhibits deacetylases (Puca *et al.*, 2009). It is believed that the whole regulatory network of p53 can either induce pulses in p53 activation in response to mild DNA damage (in which cases the DNA repair machinery is enabled and cells eventually regain their growth potential), or induce a sustained p53 activation which leads to the activation of genes involved in a terminal fate in response to severe genotoxicity (Purvis *et al.*, 2012; Zhang *et al.*, 2009). Thus, it is evident that p53 plays a major role in maintaining the integrity of the cell's genome, and mutations which may cause reduced activity or loss of function have been described, to varying degrees, in almost every type of cancer. In such cases, the cell's ability to repair DNA is severely compromised, which contributes to cancer progression (Reviewed by Rivlin *et al.* (2011)).

1.3.2 Mechanisms of DNA damage repair

Damaged cells will first and foremost attempt to repair DNA insults. The same set of phosphoinositide 3-kinase-related protein kinases (PIKKs) described previously (ATR, ATM, and DNA-PK; section 1.3.1) are largely involved in the DNA damage response (DDR). Whereas DNA damage caused by irradiation activates all three of said kinases, the presence of ssDNA or replicative fork arrest, and apoptotic DNA fragmentation activate ATR and DNA-PK, respectively. p53 can the act as a transcription factor, inducing the expression of DDR proteins (Reviewed by Lieberman *et al.* (2017)).

Double stand breaks (DSBs) are often seen as the most dangerous types of genotoxic lesions possible. In such cases, activation of some of these kinases leads to the phosphorylation of H2A histone family member X (H2AX) at a specific serine residue (S139), yielding γH2AX (Reviewed in Podhorecka *et al.* (2010); (Rogakou *et al.*, 1998). H2AX is a component of the nucleosome and is thus already in proximity of DNA, which allows efficient recruitment of the DDR proteins to the site of DNA damage (Pan *et al.*, 2011). Peptide pull-down studies have revealed that γH2AX directly interacts with mediator of DNA damage checkpoint protein 1(MDC1) and, to a lesser degree, with components of the MRN complex (Stucki *et al.*, 2005). The MRN complex can singlehandedly interact with DNA (from section 1.3.1), but further accumulation to the site of DNA damage is mediated by the histone (Rupnik *et al.*, 2008). Interestingly, Salguero *et al.* (2019) have shown that MDC1 can also associate to the site of DNA damage in a γH2AX-independent manner via a : proline-serine-threonine rich (PST)-repeat region. Thus, again, we see intricate mechanisms by which cells improve the efficiency of crucial cellular processes.

Yet, phosphorylation of H2AX is not always involved in repairing DSBs. As an example, these same lesions can activate DNA-PK, which can then activate effector proteins X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor. Other types of lesions (*e.g.,* inter-strand cross-links, single strand breaks (SSBs), adduct formations, etc.) also appear to be largely resolved by pathways that do not involve H2AX. All in all, the concept is the same: DNA damage is detected by a set of sensor proteins, that relay (directly or indirectly via signal transducer and mediators) the need for DNA repair to effector proteins (Molinaro *et al.*, 2021).

Small-scale DNA lesions are repaired via the base excision (BER) or mismatch repair (MMR) mechanisms whereas larger lesions are repaired using nucleotide excision repair (NER), homologous recombination (HR) or non-homologous end joining (NHEJ) (Chatterjee & Walker, 2017). These repair mechanisms are summarized in Figure 1.4 and reviewed by Huang and Zhou

(2021). p53, ultimately activated in response to DNA damage (from section 1.3.1), has a major involvement in all these processes by regulating the transcription of DDR proteins, through its interaction with said proteins, and more (Reviewed by Williams and Schumacher (2016)).

1.3.3 Mechanisms of DNA damage-induced cell death

As previously discussed in section 1.3.1, cells may decide to induce cell death when the degree of DNA damage is deemed unrepairable and is heavily mediated by the activation of p53. This transcription factor then allows the transcription of proteins involved in both the intrinsic and extrinsic apoptotic pathway.

1.3.3.1 Intrinsic apoptosis and the role of p53

The intrinsic apoptotic pathway is largely mediated by the B-cell lymphoma 2 (Bcl-2)-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK), who oligomerize to induce permeabilization of the mitochondrial membrane. This permeabilization event allows the release of cytochrome C, which can then recruit Apaf-1 and dATP to form the apoptosome. This complex then cleaves and activates caspase-9 (from pro-caspase-9), followed by caspase-3 activation and induction of apoptosis via the caspase cascade (Reviewed by Elmore (2007)). BAX has a propensity to localize to the mitochondria and its inhibition is mediated by pro-survival Bcl-2 proteins (*e.g.,* B-cell lymphoma-extra large *(*Bcl-X_L)) who actively work to retro-translocate BAX back into the cytosol (Edlich *et al.*, 2011). The same retro-translocation mechanism is believed to be exerted on BAK, but to a lesser degree as it localizes primarily in the mitochondria (Edlich, 2015). The dormancy of BAK is then mainly attributed to an inhibitory interaction with some, but not all, pro-survival Bcl-2 proteins (*e.g.,* Bcl-X_L and Mcl-1) (Willis *et al.*, 2005). Thus, activation of the intrinsic apoptotic pathway requires the inhibition of Bcl-X_L and Mcl-1 (along with other proteins with similar function). Phorbol-12-myristate-13-acetate-induced protein (NOXA), p53 upregulated modulator of apoptosis (PUMA) and Bcl-2-associated death promoter (BAD) are amongst the well-characterized inhibitors of these proteins and it turns out that p53 induces their transcription (Jiang *et al.*, 2006; Nakano & Vousden, 2001; Oda *et al.*, 2000). Then, p53 further contributes to activation of the intrinsic apoptotic pathway by direct upregulation of BAX (Basu & Haldar, 1998; Chipuk *et al.*, 2004). Thus, p53 induces apoptosis in response to DNA damage by (1) direct induction of BAX and (2) by inhibition of the inhibitors of BAX and BAK.

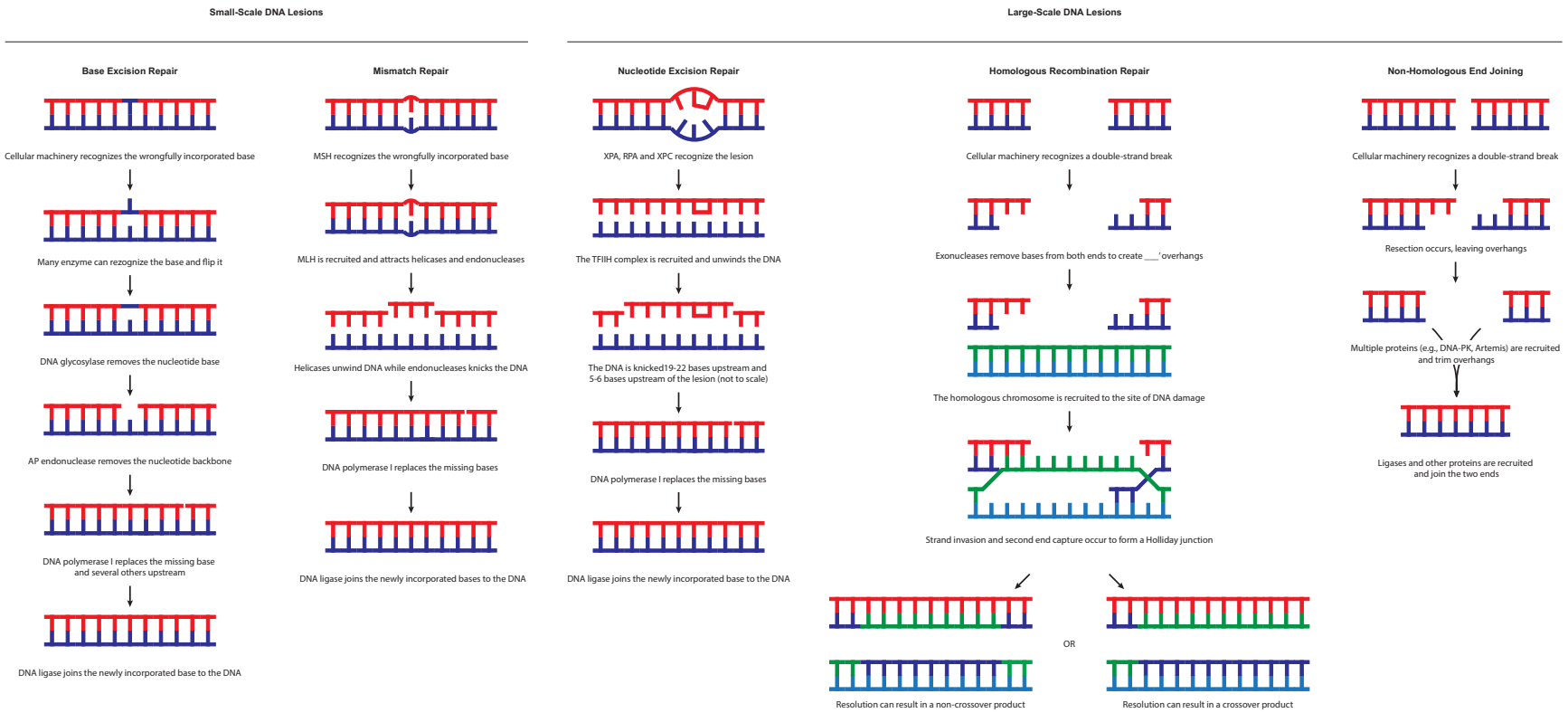


Figure 1.4: Overview of the Mechanisms of DNA Repair

Small-scale DNA lesions can be repaired by base excision repair (BER) or mismatch repair (MMR). BER involves the removal of nucleotide base, followed by the backbone while MMR involves the removal of nucleotides surrounding the lesion before the proper base(s) are incorporated. Large-scale DNA lesions are repaired via nucleotide excision repair (NER), homologous end joining (HEJ) or non-homologous end joining (NHEJ). NER involves the removal of large segments of DNA surrounding the lesion before proper bases are incorporated. HEJ involves the recruitment of the homologous strand of DNA to provide a template for the damaged one and may or may not result in a cross-over product. NHEJ trims bases surrounding the lesion and joins the strands which may result in loss of DNA segments. Use of one repair mechanism over another is lesion-specific. Figures not to scale. © Sanchez-Espinoza (2023)

1.3.3.2 Extrinsic apoptosis and the role of p53

In the extrinsic apoptotic pathway, cell death is mediated by the interaction between the cell surface protein Fas ligand (known as FasL, Apoptosis Ligand Antigen 1 (APO-1L) or cluster of differentiation 95 Ligand (CD95L)) and its receptor (known as Fas or CD95). It is believed that Fas oligomerizes prior to ligand interaction (Chan, 2000; Fu *et al.*, 2016). Upon ligation of FasL, Fas trimers form which can then recruit Fas associated via death domain (FADD) via an 80 amino acid sequence called the Death Domain (DD). FADD, now localized to Fas, also contains a death effector domain (DED) which can, in turn, allow the recruitment of many DED-containing proteins (*e.g.* cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP), caspase-8 and caspase-10) (Fu *et al.*, 2016). Through these DEDs, bridges of variable length depending on the strength of the apoptotic signal are formed between two Fas trimers (Schleich *et al.*, 2012). This connection between Fas trimers is believed to be required for apoptosis (Holler *et al.*, 2003). Fas, FADD, c-FLIP (a regulator of caspase-8) and the caspases form the death-inducing signalling complex (DISC) whose dynamics induce a conformational change in caspase-8, which unlocks its proteolytic activity and trigger its auto-proteolysis. Caspase-8 then dissociates from the DISC which can then cleave downstream effector procaspases (*e.g.,* procaspase-7 and procaspase-3, whose cleaving yields caspase-7 and caspase-3, respectively) as well as other targets. Altogether, a widespread cleavage of essential cellular components (proteins, and others) led by these cleavage-activated caspases ensues, leading to cell death. Caspase-10 is also heavily involved in this process, in a similar way to caspase-8 (Strasser *et al.*, 2009).

Some crosstalk exists between the intrinsic and extrinsic apoptotic pathway in that caspase-8 is known to cleave BH3-interacting domain death agonist (BID) to yield truncated BID (tBID) (Li *et al.*, 1998). tBID itself can singlehandedly induce mitochondrial membrane permeabilization characteristic of the intrinsic apoptotic pathway (section 1.3.3.1), even in the absence of BAX and BAK (Flores-Romero *et al.*, 2022).

The extrinsic apoptotic pathway can be triggered in response to DNA damage as ETO, teniposide, and UV light (known inducers of DNA damage) have been shown to upregulate the transcription of FasL in T lymphocytes and induce apoptosis requiring a FasL/FasR interaction (Kasibhatla *et al.*, 1998). Further studies with non-immune cells have shown that DNA lesions also induce the upregulation of Fas in a manner dependant on the presence of a wild-type copy of p53 (Müller *et al.*, 1998). Thus, p53 also induces apoptosis in response to DNA damage by upregulating the ligand and receptors involved in the extrinsic apoptotic pathway.

1.3.4 Mitotic spindle abnormalities and apoptosis

The dynamic instability of microtubules plays a major role during cell division. The mechanisms of mitosis are reviewed by McIntosh (2016). Briefly, some microtubules will interact with one another (non-kinetochore microtubules) or with the cell membrane (asters) to begin to form the mitotic spindle. Others, called kinetochore microtubules, polymerize from the centrioles found in opposite poles of the cell towards the middle, attaching to the kinetochore of duplicated chromosomes. A tug of war ensues until the chromosomes are aligned at the metaphase plate (Reece *et al.*, 2014). From there, before mitosis is allowed to continue, the spindle assembly checkpoint (SAC) monitors for improper chromosome alignment.

The SAC is yet another example outlining the intricacies of intracellular signalling in that the MAPK/extracellular-signal regulated kinase (MAPK/ERK) pathway (a major proliferative signalling cascade) also triggers activation of the SAC. Indeed, downstream effectors of MAPK/ERK signalling have been shown to activate the transcription of aurora kinase B (AurB) (Bonet *et al.*, 2012). AurB has many roles in mitosis, but in terms of the SAC specifically, it has been demonstrated to phosphorylate highly expressed in cancer 1 (Hec1), a component of the tetrameric Hec1 complex (also called the Ndc80 complex) (DeLuca *et al.*, 2006). Phosphorylated Hec1 allows the Hec1/Ndc80 complex to recruit monopolar spindle 1 (Mps1) to the outer kinetochore region through direct interaction with Hec1 and cell division cycle associated 1 (Cdca1; another component of the Ndc80 complex) (Zhu *et al.*, 2013). Mps1 is then activated by autophosphorylation, allowing it to phosphorylate kinetochore scaffold 1 (Knl1) (Ji *et al.*, 2017; Kang *et al.*, 2007). Phosphorylated Knl1 can then recruit Budding uninhibited by benzimidazoles 3 and 1 homologs (Bub3/Bub1) and budding uninhibited by benzimidazoles-related 1 (BubR1), who, in turn, recruit and activate cell division cycle protein 20 homolog (Cdc20) through two critical motifs (Diaz-Martinez *et al.*, 2015; Krenn *et al.*, 2014). Mitotic arrest deficiency 1 and 2 (MAD1/2) are then recruited through an independent mechanism, and MAD2 undergoes a conformational change while an association with Cdc20 allows an interaction with BubR1 and Bub3 to form the mitotic checkpoint complex (MCC) (Hara *et al.*, 2015; Kulukian *et al.*, 2009; Silió *et al.*, 2015). The MCC then interacts with another complex, the anaphase promoting complex (APC), sequestering it, and preventing it from ubiquitinating its targets. Without ubiquitination, the protease separase cannot cleave the link between sister chromatids, thus preventing chromosome separation critical in mitosis (Lawrence & Engebrecht, 2015). Thus, the default state of the SAC is to be activated

and is only repressed when proper spindle formation has been achieved, which includes attachment of all kinetochores and proper tension exerted on sister chromatids by the microtubules.

In cases where proper formation of the mitotic spindle cannot be achieved, the SAC is constitutively activated, followed by mitotic arrest and eventually apoptosis. As previously mentioned, the APC fails to tag some proteins for cleavage, one of them being cyclin B1 (Alfieri *et al.*, 2017; Clute & Pines, 1999). Accumulation of cyclin B1 allows its partner, cyclin-dependant kinase 1 (Cdk1), to remain enzymatically active and results in the phosphorylation of Bcl-XL and Bcl-2 (Terrano *et al.*, 2010). Phosphorylated Bcl-XL and Bcl-2 no longer interact with their binding partners (BAD and BAX, respectively). Thus, BAD and BAX un-repressed and free to perform their well-characterized pro-apoptotic roles (Reviewed by Elmore (2007)).

1.3.5 Other forms of cell death

Given the central role of p53 in apoptosis, mutations in this protein have been linked to resistance to various antineoplastics and poor prognostics (Reviewed by Hientz *et al.* (2017)). How, then, can chemotherapy lead to cancer cell death in cases where there is a loss-of-function p53 mutation? Mounting evidence has indicated that other forms of cell death are also triggered by antineoplastics mainly by their ability to induce ROS production (see sections 1.6 and 1.6.5). These forms of cell death include, but are not limited to autophagy, ferroptosis, necrosis, pyroptosis and necroptosis. The link between these mechanisms of cell death and treatment with antineoplastics are only briefly touched on next.

Autophagy. More than an intracellular recycling mechanism, autophagy has recently been described as a mechanism of cell death in itself (Galluzzi *et al.*, 2012). This type of cell death involves the formation of an autophagosome from an isolation membrane, which can then fuse with lysosomes and trigger digestion of cellular components, some of which can trigger cell death (Reviewed by Khandia *et al.* (2019)). Maturation the autophagosome is largely mediated by cleavage of Microtubule-associated protein 1A/1B-light chain 3 (LC3), followed by its conjugation deconjugated with phosphatidylethanolamine on the growing membrane. In that manner, LC3 is involved in membrane closure, transport, and many other processes (Lee & Lee, 2016; Zhou *et al.*, 2021). Cleavage of LC3 is mediated by Autophagy-Related 4A Cysteine Peptidase (ATG4), whose activity can be induced by ROS (He *et al.*, 2017). ROS, in turn, can be generated by antineoplastics.

Ferroptosis. This mechanism refers to an iron-dependent mode of programmed cell death. Cancer cells often have upregulated iron uptake and storage, and downregulated export. As a result, intracellular iron levels are often high in these cells (Brown *et al.*, 2020). Meanwhile, antineoplastics are known inductors of ROS, with H_2O_2 being able to participate in the iron-dependent Fenton's reaction to produce further ROS: hydroxide and the hydroxyl radical. Formation of these species can overwhelm the ROS homeostasis mechanisms, leaving the reactive potential of a subset unresolved. The ROS can then react with the lipids found on cellular membranes, a reaction catalyzed by lipoxygenases (Hajeyah *et al.*, 2020). As a result, cellular membranes exhibit altered structure, fluidity and integrity, which are toxic to cells (Juan *et al.*, 2021). Thus, the ability of antineoplastics to induce ROS production may also drive ferroptosis.

Necrosis. Necrosis an uncontrolled form of cell death characterized by a loss of membrane integrity resulting in an increase in cell volume, and eventual rupturing of the plasma membrane (Green & Llambi, 2015; Yuan & Kroemer, 2010). Controlled necrosis is herein referred to as *necroptosis*. Some antineoplastics are known to tamper with normal calcium homeostasis, leading to high cytosolic levels (Can *et al.*, 2013; Kidd *et al.*, 2002). Meanwhile, high calcium levels have been linked to necrosis (Orrenius *et al.*, 1989).

Necroptosis. Necroptosis is a controlled mechanism of necrotic cell death dependent on receptor interacting protein kinase 3 (RIPK3), and sometimes RIPK1 (Dhuriya & Sharma, 2018). Briefly, stress signals can lead to an interaction between RIPK1, RIPK3, and FADD to form the necrosome. The interaction induces the phosphorylation of RIPK1 and/or RIPK3 followed by the recruitment and activation of mixed-lineage kinase domain-like pseudokinase (MLKL) (Wu *et al.*, 2020). In RIPK1-independent necroptosis, RIPK3 is activated by other proteins ((Wang *et al.*, 2019). In all cases, MLKL units then polymerize and interact with the cell membrane to induce its permeabilization (Johnston & Wang, 2018).

Zhang *et al.* (2021) demonstrated that 5-FU can induce the overexpression of RIPK1 to induce cancer cell death, particularly when the apoptotic pathways are suppressed. Meanwhile, it was shown that reconstitution of RIPK1 leads to an increased sensitivity to many antineoplastics, further suggesting the involvement of necroptosis in the cytotoxicity of at least some chemotherapeutic agents (Ermine *et al.*, 2022). Induction of necroptosis, however, appears to be secondary to the mechanism of action of antineoplastics (Moriwaki *et al.*, 2015).

Pyroptosis. The mechanisms of pyroptosis are reviewed by (Man *et al.*, 2017). This mode of cell death can occur in a caspase-1-dependent or independent manner. Briefly, in caspase-1-

dependent pyroptosis, specific molecular patterns are recognized by membrane receptors which first drive the activation of inflammasomes, and associated caspase-1. In the caspase-1 independent mechanism, exogenous molecules (*e.g.,* lipopolysaccharide of bacteria) induce the cleavage and activation of caspase-4 and 5. Caspase-1, 4, and 5 can then cleave gasdermin D, the fragments of which induces pore formation in the plasma membrane. More recently, it has been proposed that cleavage of another gasdermin (gasdermin E) can be cleaved by caspase-3, outlining a role for the caspase in inducing pyroptosis instead of its canonical role in apoptosis (Wang *et al.*, 2017). Many antineoplastics have now been identified as being able to induce the cleavage of gasdermins, particularly gasdermin E (Wang *et al.*, 2020; Wang *et al.*, 2018d; Zhang *et al.*, 2019a).

1.4 Chemotherapeutic agents (antineoplastics)

The versatility of chemotherapeutic agents, as highlighted by the multiple regimens used in the treatment of placental choriocarcinoma (section 1.2.4.3), can be explained by their fundamental mode of action: exploitation of upregulated mechanisms of mitosis by inducing irreparable errors (section 1.3). Antineoplastics are classified according to their mechanism of action and the source from which they are derived. The National Cancer Institute (NCI) defines four major classes of chemotherapeutic agents: alkylating agents, antimetabolites, antitumor antibiotics and plant alkaloids (National Institute of Health, n.d.). However, the *Saunders Nursing Drug Handbook* adds another one: topoisomerase inhibitors (Kizior & Hodgson, 2020). In this section, we discuss these classes and provide common examples.

1.4.1 Alkylating agents

Alkylating agents are antineoplastics that owe their name to their ability to transfer alkyl groups (with chemical formula C_nH_{2n+1}; *e.g.,* methyl, ethyl, etc.) to DNA. They are classified based on the alkyl group transferred or the number of reactive sites, and include alkyl sulfonates, triazene, nitrosoureas, nitrogen mustards and aziridines. These agents can participate in mononuclear or bimolecular nucleophilic substitutions to induce N- and O-alkylation, or only N-alkylation, respectively (Peng & Pei, 2021). Although all deoxynucleotides can be subjected to alkylation, that of adenine and guanine appear to predominate (McQueen, 2010). Alkylation by these agents can result in (1) inter-strand cross-linkage (between two separate DNA strands), (2) intra-strand

cross-linkage (within the same strand of DNA) or (3) DNA-protein cross-linkage ((Ewig & Kohn, 1978); Reviewed by Noll *et al.* (2006); and Sreerama (2011)). Addition of these alkyl groups leads to distortions in structure of DNA and stereochemical alterations, inducing base pair opening (*i.e.,* dissociation of the H-bonds between 2 nucleotides of opposing strands) or alterations of the backbone (Kara *et al.*, 2015). Ultimately, these agents aim to induce strand breakages, and causing cancer cell death, as outlined in section 1.3.1.

1.4.1.1 Oxaliplatin (OXA)

As shown in Figure 1.5, OXA is a platinum-based compound complexed with oxalate and diaminocyclohexane. The cytotoxic mechanisms of OXA are reviewed by Alcindor and Beauger (2011). Briefly, despite its ability to induce inter-strand and DNA-protein crosslinks, OXA is believed to induce cell death mainly by introducing *intra*-strand crosslinks between two guanine nucleotides, resulting in strand breakage. Alcindor and Beauger (2011) also describe secondary mechanisms through which OXA inhibits transcription by interacting with, and sequestering, transcription factors and causing steric hindrance to prevent binding of RNA polymerase. This alkylating agent is also believed to induce cancer cell death, at least partly, by enhancing antitumoral immune responses (Tesniere *et al.*, 2010).

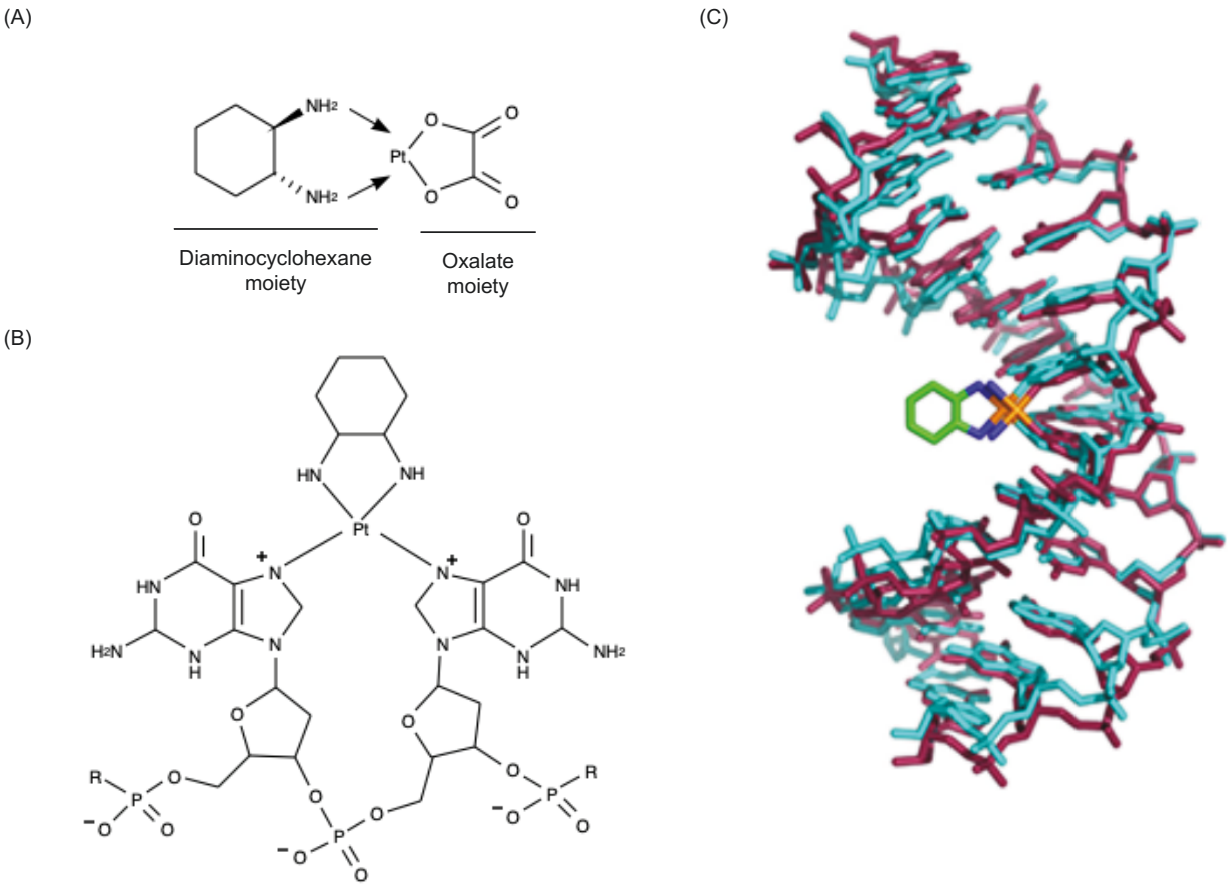


Figure 1.5: OXA is an alkylating chemotherapeutic agent whose antineoplastic activity is driven by inducing intra-strand cross-links between two DNA guanine residues

(A) OXA is a platinum-based compound with diaminocyclohexane and oxalate moieties. (B) OXA reacts with two guanine residues through the platinum element to induce an intra-strand cross-link. (C) Structural representation of the interaction between OXA and DNA. Structure taken from Alian *et al.* (2012).

1.4.2 Antimetabolites

Antimetabolites are a class of antineoplastics who function by interfering with the normal metabolism of nucleic acids. These can (1) prevent the synthesis of important metabolites in the nucleotide biosynthesis pathway or (2) act as direct analogues of nucleotides (Scholar, 2007). Ultimately, antimetabolites will seek to induce significant DNA damage, beyond what cellular DNA repair mechanisms can efficiently revert. Intracellular sensors can then trigger cell cycle arrest, followed by apoptosis (from section 1.3.1).

1.4.2.1 5-Fluouracil (5-FU)

5-FU is an analogue of uracil bearing a fluoride atom bound to the carbon structure of the pyrimidine ring (Figure 1.6). It's transport into the cell is mediated by the same transporter as uracil. Once inside the cell, 5-FU must be converted into its active forms fluorouridine triphosphate (FUTP), fluorouridine monophosphate (FUMP) or fluorodeoxyuridine triphosphate (FdUTP), to perform its antineoplastic functions (Figure 1.7).

The antineoplastic nature of 5-FU comes mainly from its ability to inhibit thymidylate synthase (TS). In the *de novo* nucleotide synthesis pathway, TS catalyzes the addition of a methyl group ($-CH_3$) to deoxyuridine monophosphate (dUMP) to yield deoxythymidine monophosphate (dTMP) ((Berg *et al.*, 2015)). However, 5-FU contains a fluoride atom at the same position the methyl group is added on the natural ligand of TS, preventing synthesis of dTMP. Furthermore, the fluoride moiety interacts with the active site of TS in such a way to induce a slowly reversible interaction, thereby sequestering TS and preventing the enzyme from performing normal cellular functions (Zhang *et al.*, 2008).

Early *in vitro* studies demonstrated that the cytotoxic effect of 5-FU may also driven by its incorporation into RNA and DNA (An *et al.*, 2007; Kufe & Major, 1981). However, while an *in vivo* study conducted by Noordhuis *et al.* (2004) confirmed that 5-FU is incorporated at detectable levels in nucleic acids, the efficacy of the antineoplastic was not a function of the degree of incorporation, but rather of the activity of TS.

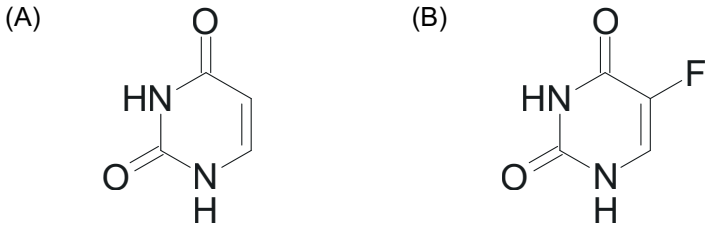


Figure 1.6: 5-Fluorouracil is an antimetabolite and an analogue of uracil

(A) The structure of uracil: a nitrogenous base found in RNA and a precursor of bases found in DNA. (B) The structure of 5-FU contains a fluorine atom attached to C5 of the pyrimidine ring which gives it its antineoplastic characteristics. © Sanchez-Espinoza (2023)

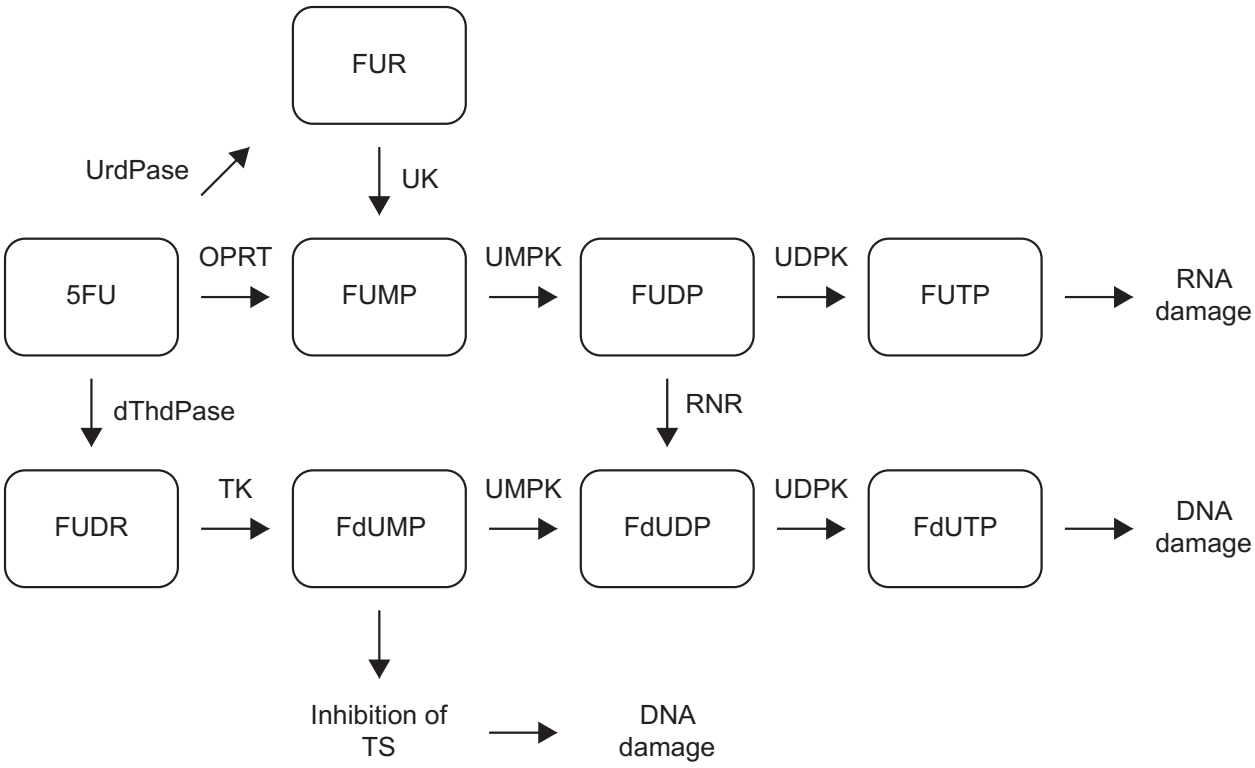


Figure 1.7: Anabolism of 5-FU to its active forms for its antineoplastic activity

Once inside the cell, 5-FU must be converted into its active forms FdUMP, FdUTP and FUTP by a series of enzymes to act as a suicide inhibitor of thymidylate synthase or be incorporated in DNA and RNA, respectively. These active forms each inhibit cell division in their own way. Figure adapted from Loganayagam *et al.* (2013) and Miura *et al.* (2010). Abbreviations: 5FU, 5-fluorouracil; FUR, Fluorouridine; FUDR, fluorodeoxyuridine; F(d)UMP, fluoro(deoxy)uracil monophosphate; F(d)UDP, fluoro(deoxy)uracil diphosphate; F(d)UTP, fluoro(deoxy)uracil triphosphate; UrdPase, uridine phosphorylase; UK, uridine kinase; OPRT, orotate phosphoribosyltransferase; dThdPase, thymidine phosphorylase; UMPK, uridine monophosphate kinase; UDPK, uridine disphosphate kinase. © Sanchez-Espinoza (2023)

1.4.2.2 Methotrexate (MTX)

MTX is an analogue of folic acid (FA, or vitamin B9), as shown in figure 1.8. Folic acid acts as a precursor to first dihydrofolate (DHF), then tetrahydrofolate (THF) and its derivative N^5,N^{10} – methylene THF. The latter molecule acts as a one-carbon donor, but more importantly, as a co-enzyme in reactions of both the *de novo* synthesis of purines and pyrimidines (Berg *et al.*, 2015). Thus, without THF, nucleotide synthesis would be severely hindered.

As important as THF is, it paradoxically cannot be synthesized from scratch by humans. Instead, it must be derived from FA or recycled from DHF following oxidation during nucleotide

metabolism, both reactions requiring the enzymatic activity of dihydrofolate reductase (DHFR) (Bailey & Ayling, 2009). Given the structural similarities with FA, MTX competitively inhibits DHFR to cause a depletion of intracellular THF pools. *De novo* nucleotide synthesis thus eventually halts, and the subsequent DNA lesions trigger the DDR. Thus, the replicative ability of cells – both cancerous and healthy – is dampened (Berg *et al.*, 2015).

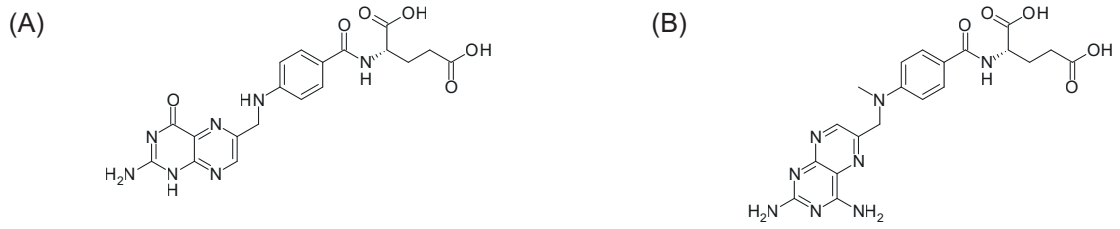


Figure 1.8: MTX is an antimetabolite, and an analogue and competitive inhibitor of folic acid

(A) The structure of folic acid. (B) The structure of MTX. (C) DHFR catalyzes the reduction of folic acid to DHF, and from DHF to THF. The enzyme is thus heavily involved in maintaining THF pools and allowing nucleotide biosynthesis. MTX competitively inhibits DHFR to induce DNA lesions, resulting in cell death. © Sanchez-Espinoza (2023)

1.4.3 Plant alkaloids

A formal definition of an alkaloid was provided by W. Pelletier to denote group of cyclic compounds in which a nitrogen atom is found in a negative oxidation state (Referenced in Roberts and Wink (1998)). Consequently, *plant alkaloids* refer to alkaloids naturally produced by plants, often as a defense mechanism (Ain *et al.*, 2016). Several thousand plant alkaloids have been discovered so far, and include narcotic analgesics (*e.g.,* morphine and codeine) and stimulants of the central nervous system (*e.g.,* cocaine and caffeine) (Singh *et al.*, 2017). More recently, antineoplastic properties have been identified for some of these plant alkaloids, with a notable example being PTX.

1.4.3.1 Paclitaxel (PTX)

PTX is a plant alkaloid and a microtubule-targeting agent that has been shown to promote the stability of microtubules by binding to the β-tubulin subunit within the polymer rather than the αβ

dimer (Rao *et al.*, 1995; Xiao *et al.*, 2006). Microtubules are highly dynamic intracellular structures characterized by alternating periods of depolymerization (termed *catastrophe*) and elongation (termed *rescue*), collectively called dynamic instability (Horio & Murata, 2014). This dynamicity of microtubules is essential for many processes such as transport, cell migration and cellular signalling (Garcin & Straube, 2019). As previously mentioned, microtubules are also critical in spindle formation during mitosis and PTX tampers with its proper formation, leading to the death of mitotic cells, cancerous or healthy, as outlined in section 1.3.4. Although microtubule stabilization is considered the major mechanism through which PTX induces cancer cell death, Lanni *et al.* (1997) begun outlining a mechanism through which the antineoplastic induces the release of Tumour Necrosis Factor α (TNF-α) to mediate p53-independent apoptosis.

1.4.4 Topoisomerase (Topo) inhibitors

The human genome extends over 2 meters long and needs to be tightly packed and twisted to fit in eukaryotic cells, themselves only a few micrometers in size (Piovesan *et al.*, 2019). This intertwining of DNA introduces the issue of topological stress (stress induced by the twists and turns of DNA) during essential processes such as transcription and DNA replication. The efficiency of these processes is partially mediated by the ability of the appropriate enzymes (*e.g.,* RNA polymerase for translation and helicase for replication) to access the DNA strands. DNA availability is, in turn, partially modulated by the torsional strain it is subjected to (Baranello *et al.*, 2012). Supercoiled DNA is not readily accessed while uncoiled DNA is.

Uncoiling of DNA molecules thus becomes a critical event, enabled by the activity of topoisomerases. In eukaryotic systems, two types of topoisomerases exist: type I (topo I; further subdivided into types IA, IB, and IC) and type II (topo II). Although they all function to relieve topological stress, they differ in the type of stress they relieve (negative *vs* positive supercoiling), in their co-factor requirements, and in the molecular mechanisms through which they perform their action (Reviewed in Baker *et al.* (2009)). Overall, the major difference between topo I and topo II is that they introduce topological alterations of DNA by cleaving one (type I) or both strands of DNA (type II) (Sinden, 1994).

The importance of these enzymes is highlighted by the observation that altered topoisomerase activity has been shown to modulate mRNA levels (Collins *et al.*, 2001). It is also believed to induce helicase arrest and impede further unwinding, distortion of the parental strands, chromosomal breaks and triggering the DNA damage checkpoints and repair mechanisms

(section 1.3.1 and 1.3.2) (Bermejo *et al.*, 2007; Keszthelyi *et al.*, 2016). As its name implies, this class of antineoplastics inhibits the activity of said mentioned topoisomerases. Examples of topoisomerase type I and type II inhibitors include irinotecan and ETO, respectively.

1.4.4.1 Etoposide (ETO)

ETO is a topo II inhibitor. Topo II is a homodimer, with each subunit containing a DNA-binding/cleavage domain (Vanden Broeck *et al.*, 2021). This enzyme cleaves both strands of a DNA molecule, and each end of the cleaved DNA is held by one of the two Topo II subunits. This allows a transient stabilization of the DSB, thus allowing another DNA double-strand to pass through the newly formed gap, and resealing of said gap shortly thereafter (Reviewed by McClendon and Osheroff (2007)).

ETO has some affinity for DNA alone but binds the DNA/topo II complex with much higher affinity, after the DSB has occurred (Burden *et al.*, 1996; Chow *et al.*, 1988; Wu *et al.*, 2011). The stoichiometry of the latter binding has been addressed by Bromberg *et al.* (2003), who proposed a *two-drug* model which wants that two molecule of ETO are involved for unit of topo II, each interacting with one or the other nicked DNA strands. In doing so, covalent bounds are formed within the enzyme's catalytic cores, which inhibits its ligation activity. As a result, the DSB induced by topo II, which is normally transient is further stabilized and, if left unresolved, can lead to genotoxicity detected by the cellular machinery, and cell death, as discussed in section 1.3.1 and 1.3.3. The complete mechanism of ETO is reviewed by Montecucco *et al.* (2015).

1.4.5 Antitumour (antineoplastic) antibiotics

Antitumour (antineoplastic) antibiotics are a class of chemotherapeutic agents that act by interfering with DNA synthesis through a variety of mechanisms (Hena & Znad, 2018). These agents are categorised so based on their origin: they are (derivates of) natural metabolites produced by microorganisms, with the bacterial genus *streptomyces* having the largest contribution. Antitumour antibiotics are then further characterized based on their structure as anthracyclines (*e.g.,* daunorubicin, mitoxantrone, etc.) or non-anthracyclines (*e.g.,* mitomycin, bleomycin, Act D, etc.) (Gao *et al.*, 2020).

A common example of an antitumour antibiotic is doxorubicin, which is believed to exert its antineoplastic activity via DNA intercalation and subsequent inhibition of topoisomerase II (Taymaz-Nikerel *et al.*, 2018). As such, doxorubicin is also considered a topoisomerase inhibitor. This dual classification is also observed for other antitumour antibiotics such as mitomycin (both an antitumour antibiotic and an alkylating agent) and outlines that the classification of antitumour antibiotics is anchored in their origin (Rockwell *et al.*, 1982).

1.5 Major concerns with antineoplastics

Given the complexities of intracellular signalling, the discovery of chemotherapeutic agents is a feat and have significantly contributed to a longer life expectancy in cancer patients. Administration of these agents is, however, linked to two major problems that contribute to a need for a seemingly ever-lasting chase to find the next best alternative: (1) antineoplastics are not tumour-cell specific, and (2) cells have developed mechanisms of resistance to these agents.

1.5.1 Specificity of antineoplastics to cancer cells

Antineoplastics employ a wide array of mechanisms (*e.g.,* 5-FU, ETO, MTX, or OXA-induced DNA damage; PTX-derived aberrant microtubule dynamics) to kill rapidly dividing cells. These agents, however, have one thing in common: they exploit biochemical pathways that are merely upregulated in cancer cells, but by no means inexistent in healthy cells. As a result, off-target effects are inevitable as normally hyperproliferative cell types are caught in the *crossfire* of the cytotoxicity of chemotherapy. Well-documented hyperproliferative cell types include hair follicle stem cells and cells of the immune system. Thus, adverse effects range from purely aesthetic (hair loss) to life-threatening (an immunocompromised state). Advances in targeted and/or localized therapy have made progress but are not yet a standard. Thus, the quest for a perfect cure to cancer continues.

1.5.2 Resistance to antineoplastics

A second major issue with conventional chemotherapy is the emergence of resistance to various chemotherapeutic agents. The resistance mechanism employed by the cancer cells is often antineoplastic-specific. In the case of MTX, gene amplification of the enzyme targeted by this

antineoplastic (*i.e.,* DHFR) has been long reported as a common mechanism of resistance in many cancers (Cowan *et al.*, 1982; Göker *et al.*, 1995; Trent *et al.*, 1984). Other proposed mechanisms of MTX resistance include activity-increasing mutations in the DHFR gene, and downregulation of the folate transporter (Chunduru *et al.*, 1994; Worm *et al.*, 2001). Similarly, gene amplification of TS, and increased activity of TS and ribonucleotide reductase are believed to confer 5-FU resistance (Fukushima *et al.*, 2001). Antineoplastic-specific resistance is also observed with other drugs: decreased uptake via the human copper transport protein 1 (CTR1) (OXA), altering of binding mechanisms in mutated topoisomerase II genes (ETO), overexpression of certain tubulin isoforms (PTXl) (Chan *et al.*, 1993; Plasencia *et al.*, 2006; Stengel *et al.*, 2010). Of course, these are but examples of the complex neoplastic-specific mechanisms of resistance cancers employ.

Resistance to antineoplastics can also be achieved in a general matter. A major mechanism lies in the upregulation of P-glycoprotein (P-gp; encoded by the *Multi-Drug Resistance 1* (*MDR1*) gene), an efflux pump involved in the export of a variety of drugs (Reviewed by Robinson and Tiriveedhi (2020)). The expression of P-gp has been shown to increase after exposure to antineoplastics, in a variety of cancers (Hille *et al.*, 2006; Kurimchak *et al.*, 2022; Trock *et al.*, 1997). Thus, the availability of the antineoplastics to reach its intracellular target can be severely reduced. Antineoplastic resistance has also been achieved via upregulation of components of the antioxidant system (Yen *et al.*, 2011). In response to the antineoplastics, cancer cells may enhance their antioxidant potential, leading to a game of cat-and-mouse in that, whereas classical chemotherapy often tries to elevate ROS to unbearable levels (discussed later, in section 1.6.5), cancer cells respond by further enhancing their antioxidant efficiency. The mechanisms of resistance outlined here are but examples of how cancer cells may achieve resistance but suffice to highlight the fact that the quest for an infallible cure against cancer is far from over.

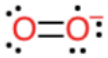
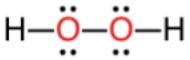
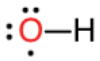
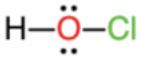
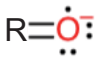
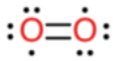
1.6 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) collectively denote highly reactive, oxygen-containing chemical species. Common examples are listed in Table 1.2 and include the superoxide anion (O_2^-), the hydroxyl radical ($OH^\bullet$), the alkoxyl radical ($R\text{-}O^\bullet$), hydrogen peroxide (H_2O_2), and many more (Nandi *et al.*, 2019). Under homeostatic conditions, ROS are a product of normal intracellular processes. They can originate from the electron transport chain during cellular respiration,

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase's (NOX) involvement in the immune response, the activity of xanthine oxidase (XO) during purine catabolism to produce uric acid and many more sources (Reviewed by Chen *et al.* (2018b); Furuhashi (2020); Segal *et al.* (2012); Snezhkina *et al.* (2019)).

ROS can also originate from exogenous sources such as antineoplastics who, through their cytotoxicity, affect many molecular pathways to induce the overproduction of these species (Reviewed by Yang *et al.* (2018)). Such a state is referred to as oxidative stress as it becomes detrimental to cells. Indeed, it may lead to lipid peroxidation, followed by apoptosis. They may also oxidize DNA and proteins, resulting in genotoxicity or aberrant activation (or lack thereof) of signalling pathways, which may lead to necrosis or apoptosis (Reviewed by Auten and Davis (2009)).

Table 1.2: Common examples of ROS

Radicals			Non Radicals		
Name	Molecular Formula	Lewis Structure	Name	Molecular Formula	Lewis Structure
Superoxide	$^{\bullet}O_2^-$		Hydrogen Peroxide	H_2O_2	
Hydroxyl Radical	$^{\bullet}OH$		Ozone	O_3	
Peroxyl	$ROO^{\bullet -}$		Hypoclorous Acid	HOCl	
Alkoxyl	$RO^{\bullet -}$		Singlet Oxygen	$^1O_2$	

Reactive oxygen species (ROS) can be classified as radicals or non-radicals. Radical ROS are classified as such because of the presence of an uneven number of valence electrons. The odd electron is unpaired and thus highly reactive. Non-radicals do not have an odd number of electrons, but the arrangement of atoms creates weak, unstable and thus highly reactive bonds. © Sanchez-Espinoza (2023)

1.6.1 Roles of ROS in healthy cells

In healthy cells, ROS play key roles in regulating signal transduction mechanisms. As an example, hydrogen peroxide (H_2O_2) can lead to the reversible oxidation of cysteine residues. Cysteine residues susceptible to this oxidation are commonly found in the active sites of certain protein tyrosine phosphatases such as Phosphatase and Tensin Homolog (PTEN), Cdc14B, Protein Tyrosine Phosphatase 1B (PTP1B) and MAPK phosphatases (Diebold & Chandel, 2016). As an example, formation of said disulfide bond on PTEN inhibits its activity (the mechanism of which is discussed later in section 1.6.3) (Lee *et al.*, 2002; Leslie & Downes, 2004). This inhibition can also contribute to the progression of many diseases such as cancers (Hollander *et al.*, 2011; Lee *et al.*, 2002; Milella *et al.*, 2015). Although the H_2O_2/PTEN interaction is only one of many examples of how ROS have dual roles to biological systems, it clearly highlights the need for maintaining homeostasis of these species, the intricacies of which are discussed next.

1.6.2 ROS homeostasis in healthy cells

The intracellular levels of ROS must be tightly regulated to prevent the adverse effects that high levels bring about. To maintain homeostasis, healthy cells rely on (1) so-called ROS scavengers and (2) a variety of antioxidant enzymes who catalyze a series of, often interconnected, reactions, to dampen the reactive potential of these species. In all cases, neutralization of ROS involves a redox reaction in which the ROS acts as the oxidizing agent and the ROS scavenger or antioxidant enzyme acts as the reducing agent (Lobo *et al.*, 2010).

ROS scavengers are molecular species that, by their chemical structure, can donate an electron to the ROS to yield stable, relatively unreactive products. As such, potent ROS scavengers must exhibit characteristics of a strong reducing agent (dependant on factors such as atom electronegativity and intramolecular binding; Reviewed in Zumdahl and Zumdahl (2010)). Perhaps the most commonly studied ROS scavenger is reduced glutathione (GSH), which can react non-enzymatically with many ROS to redirect the reactive potential into the formation of a disulfide bond with another GSH (abbreviated as GSSG). The disulfide bond within GSSG can then be cleaved by glutathione reductase to regenerate GSH in a NADPH-dependant manner (Sarma & Mugesh, 2008). Other examples of ROS scavengers include, uric acid, vitamin A, and many more (Ferreira *et al.*, 2018).

On the other hand, antioxidant enzymes are proteins that catalyze reactions involved in diminishing the reactive potential of ROS. A first example is superoxide dismutase (SOD), known to exist in three isoforms in humans. SOD1 is found in the cytosol whereas SOD3 is extracellular, but both require copper and zinc as co-factors. On the other hand, SOD2 is localized to the mitochondria and requires manganese (Mondola *et al.*, 2016). All isoforms participate in the dismutation (*i.e.,* simultaneous oxidation and reduction of the same substrate) of O_2^- to yield H_2O_2 and molecular oxygen (O_2).

As previously mentioned, H_2O_2 is itself a ROS. Its levels are, in turn, regulated by another antioxidant enzyme: catalase (CAT). Human CAT is a peroxisomal, tetrameric protein which each subunit containing a heme group and a tightly bound NADPH molecule. Whereas the heme group is directly involved in the oxidation of two equivalents of H_2O_2 to yield 2 molecules of water and O_2 in a 2-step reaction, the role of NAPDH is attributed to inhibiting the formation of inactive forms of CAT (Reviewed by Nandi *et al.* (2019)). Thus, SOD and CAT catalyze complementary reactions to neutralize the reactive potential of O_2^-.

Glutathione peroxidase (GPx) is another antioxidant enzyme, this time mitigating the reactive potential of organic peroxides (with chemical formulas R-O-O-H or R-O-O-R'). Many human isoforms of Gpx exist, and most are selenium-dependant in their multi-step reaction in which (1) the peroxide is released as water or alcohol as it oxidizes the selenium co-factor, followed by (2) an interaction between GSH and selenium to release water, and (3) interaction of a second unit of GSH to form GSSG, which is released from the enzyme (Bhowmick & Mugesh, 2015). As before, glutathione reductase can then regenerate the two units of GSH. Isoforms of GP_x differ in the number of subunits it is composed of, in their tissue localization, and dependency on selenium (Théophile Mbemba *et al.*, 2019). Overall, the activity of GPx goes hand in hand with that of glutathione and highlights the crosstalk between ROS *scavengers* and *antioxidants*.

1.6.3 ROS in cancerous cells

As discussed so far, ROS can be beneficial as they can act as transducers in normal cellular signalling (section 1.6.1), provided they are kept at relatively low levels by tight regulatory control (section 1.6.2). On the other hand, high intracellular ROS levels can quickly lead to cell death. At intermediary levels, however, tumour progression may occur. At this stage, the species alter key regulatory elements, thereby promoting cell cycle progression, survival, angiogenesis, and many more (Liou & Storz, 2010).

Previously, we mentioned that ROS can create a disulfide bond within cysteine residues of PTEN, thereby inactivating it (section 1.6.1). Inactivation of PTEN has been shown to have downstream effects that potentiate cancer progression (Takao *et al.*, 2018). Indeed, PTEN removes a phosphate from phosphatidylinositol (3,4,5)-triphosphate (PIP_3) to yield phosphatidylinositol (4,5)-bisphosphate (PIP_2), and its inactivation leads to an accumulation of PIP_3 at the cell membrane (Reviewed by Cantley and Neel (1999)). PIP_3 can then interact with phosphoinositide-dependent kinase 1 (PDK1) via the pleckstrin homology domain (PHD). Although PIP_2 also contains a PHD domain, PDK1 has higher affinity for PIP_3 and the discovery of molecules inhibiting molecular interactions with the PHD domain of PIP_3, but not PIP_2, confirm that different binding dynamics exist (Dannemann *et al.*, 2010; Miao *et al.*, 2010). The PIP_3/PDK1 interaction then enhances auto-phosphorylation and activation of PDK1 (Levina *et al.*, 2022). Activated PDK1, in turn, phosphorylates Protein kinase B (PKB; also known as Akt) at T308, which enables a second phosphorylation at S473 by mammalian target of rapamycin complex 2 (mTORC2). Fully activated PKB can then phosphorylate numerous targets to promote cell cycle progression (*e.g.,* CyclinD1) or survival (*e.g.,* BAD and forkhead box protein O1 (FOXO), inhibited by phosphorylation) (Reviewed in Carnero and Paramio (2014)). Thus, inactivation of PTEN induces PIP_3 accumulation, which contributes to constitutively active proliferative signalling. The mode of PTEN inactivation described here is merely an example of the profound effects that elevated ROS production can have on cancer progression but explains why cancer cells exhibit high ROS levels.

1.6.4 Antioxidant potential in cancer cells

Whereas cancer cells exhibit abnormal (elevated) ROS production, they also have aberrant antioxidant systems. As an example, Liu *et al.* (2020) have shown that SOD1 is overexpressed in tumoral lung cells relative to healthy ones while Papa *et al.* (2014) came to the same conclusion regarding breast cancer cells. Meanwhile, CAT expression tends to be downregulated in most cancer types (Glorieux *et al.*, 2015). Other studies have shown a decrease in expression of selenoproteins (selenium-containing proteins, often considered antioxidant) as a whole with varying expression of GPx in colorectal cancers (Al-Taie *et al.*, 2004). Of course, the pattern of expression of antioxidant enzymes is not *black-and-white* in that some enzymes will be differentially expressed according to the cancer type (inducing varying degrees of severities) and

stage (Gaya-Bover *et al.*, 2020; Leone *et al.*, 2017). Thus, the aberrant antioxidant system is often referred to as *redox imbalance* which tends to involve an overall elevation in ROS.

1.6.5 ROS in chemotherapy

The ability of chemotherapeutic agents to elevate ROS levels in cancer cells is believed to be linked to their ability to kill cancer cells. Yet, cancer cells are well adapted to surviving under conditions of elevated ROS levels (section 1.6.4). As such, it is believed that the anti-tumorigenic potential of antineoplastics lies in their ability to elevate ROS to a certain threshold which induces cell death. Different antineoplastics induce their cytotoxicity by tampering with a wide range of molecular mechanisms (*e.g.,* mitochondrial function) and, in doing so, generate ROS as a by-product (Yang *et al.*, 2018).

1.7 Melatonin

With an understanding of threats that ROS provide to the human body, we introduce melatonin, first an important hormone, then a strong antioxidant. Melatonin is produced by the pineal gland in response to darkness and is mainly known for its important role in inducing sleep and maintaining the circadian rhythm. The hormone, however, has also been shown to mitigate ROS levels, protect DNA from damage, and reorganize cytoskeletal components which may highlight its potential use as an antitumoral adjuvant. Melatonin's biosynthesis and said roles in cellular homeostasis are outlined next.

1.7.1 Production: from the retina to the brain

Melatonin's production in response to darkness and is primarily regulated by photoreceptors in the eyes. Yet, canonical photoreceptors (*i.e.,* cones and rods) have been shown to not be involved in its biosynthetic pathway, instead attributed to another type: melanopsin found in retinal ganglion cells (Hattar *et al.*, 2002; Lucas *et al.*, 1999).

Melanopsin is a transmembrane G-protein coupled receptor (GCPR) (Sexton *et al.*, 2012). It has been shown to interact with 11-*cis*-retinal under dark conditions (Walker *et al.*, 2008). When exposed to light, 11-*cis*-retinal is isomerized to all-*trans*-retinal which subsequently induces a

conformational change in the melanopsin receptor (Ebrey & Koutalos, 2001; Lei *et al.*, 2016). This induces the activation of the associated $G_{q/11}$ proteins, which then activates phospholipase Cβ (PLCβ) via its C-terminal (Hughes *et al.*, 2012; Mizuno & Itoh, 2009). Activated PLCβ can then cleave PIP_2, a component of the plasma membrane into inositol triphosphate (IP_3) and diaglycerol (DAG). IP_3 then diffuses into the cytoplasm and binds to its receptor found on the endoplasmic reticulum (ER) membrane, where it will trigger the release of Ca^{2+} into the cytoplasm (Bill & Vines, 2020; Streb *et al.*, 1983). The influx of Ca^{2+} can then induce the release of neurotransmitters to create an action potential that travels from the retinal ganglion cells to the suprachiasmatic nucleus, the paraventricular nucleus, and the superior cervical ganglion, and finally to the pineal gland (Aulinas, 2000; Karagas & Venkatachalam, 2019).

Norepinephrine (NE), one of such neurotransmitters, is released in proximity of the pineal gland (Scott, 2002). Pinealocytes, effector cells within the pineal gland, respond to NE via adrenergic receptors (α and β) to induce the production of cyclic adenosine monophosphate (cAMP) (Klein, 1985). cAMP then interacts with the regulatory subunit of protein kinase A (PKA) to unlock its catalytic activity (Sassone-Corsi, 2012). PKA can then phosphorylate cAMP response element binding protein (CREB), a transcription factor known to induce the expression of aralkylamine N-acetyltransferase (AANAT) (Reviewed by Ho and Chik (2010)). AANAT catalyzes the conversion of serotonin to N-acetylserotonin, which must then be converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT) (Mannino *et al.*, 2021). Ribelayga *et al.* (1999) demonstrate that the activity of HIOMT is enhanced at nighttime and suggest it is regulated similarly to AANAT or by the endogenous biological clock. Thus, periods of darkness allow the synthesis of melatonin to proceed by inducing the expression and activity of enzymes involved in the hormone's biosynthetic pathway.

1.7.2 Alternate sites of melatonin synthesis

Mounting evidence suggest that melatonin is not solely synthesized in the pineal gland. Indeed, Martin *et al.* (1992) reported production of the hormone in the ciliary epithelium, where its function appears to be other than in maintaining the circadian rhythm (Reviewed by Ostrin (2019)). Then, Conti *et al.* (2000) showed that bone marrow cells express the enzymes necessary to the hormone's biosynthesis, where melatonin is involved in bone maintenance (Zhou *et al.*, 2020). Later, Carrillo-Vico *et al.* (2004) obtained similar results, this time in human lymphocytes, also showing that the blockade of these enzyme led to reduction of melatonin levels in culture.

Melatonin stimulates lymphocytes in various ways to promote the activity of the immune system (Reviewed by Miller *et al.* (2006)). The synthesis of melatonin was also strongly suggested in the placenta by the work of Lanoix *et al.* (2008) where it is believed to protect against ROS while promoting survival and syncytialization of CTBs (Chuffa *et al.*, 2019). Many other sites of peripheral melatonin synthesis are being reported, where the hormone seems to have diverse roles (Konturek *et al.*, 2007; Maldonado *et al.*, 2010). It is evident, then, that there is a substantial amount of melatonin that is synthesized other than in the pineal gland, where it is likely to occupy paracine functions.

1.7.3 Melatonin's receptor-mediated canonical activity

In humans, melatonin exerts its effect by binding to two GCPRs, termed melatonin receptor type 1 and 2 (MT1 and MT2) (Nikolaev *et al.*, 2021). These receptors can be found in peripheral tissues such as the ovaries, skin, pancreas, placenta, and many more (Slominski *et al.*, 2012). The canonical role of melatonin in sleep and regulation of the circadian rhythm, however, is largely mediated by the presence of these receptors in the brain (Oishi *et al.*, 2021). From there, the hormone can induce deep physiological changes such as reduced neuronal excitability and blood pressure (Grossman, 2013; Oliveira-Abreu *et al.*, 2019).

The MT1 and MT2 receptors are GCPRs and thus follow the well-characterized signalling dynamics (Reviewed in Emet *et al.* (2016)). Briefly, upon ligand binding, these receptors can homo- or heterodimerize, although the MT2 homodimer occurs to a lesser extent (Ayoub *et al.*, 2004). Both receptors can then interact with G_i or $G_{q/11}$ proteins to induce different signal transduction mechanisms (Jockers *et al.*, 2008). G_i proteins are well-known inhibitors of certain adenylyl cyclase (AC) isoforms (AC1, AC5, and AC6) highly expressed in neuronal cells (Hanoune & Defer, 2001; Sadana & Dessauer, 2009). AC inhibition leads to a reduction in cAMP levels, which prevents dissociation of the regulatory subunits from the catalytic subunit of PKA (Sassone-Corsi, 2012). As such, PKA is kept in an inactive state, and cannot phosphorylate CREB, thereby preventing its activation and nuclear localization (Guo *et al.*, 2017; Wang *et al.*, 2018b). CREB acts as a transcription factor for many genes related to the promotion and stabilization of wakefulness, as the deletion of CREB decreases time spent awake in mice (Graves *et al.*, 2003). Thus, binding of melatonin to the MT1/MT2 receptors leads to decreased cAMP levels and, ultimately, repression of the transcriptional activity of CREB.

1.7.4 Melatonin as an antioxidant in healthy cells

In healthy cells, melatonin is characterized as a powerful antioxidant, a property first linked to its ability to act as an ROS scavenger. Indeed, the hormone has been found to react with H_2O_2 to form a product that can then be reduced by CAT (Tan *et al.*, 2000). The ROS quenching behaviour of melatonin has also been described for $^{\bullet}OH$, $^1O_2$, and many others (Galano *et al.*, 2018; Matuszak *et al.*, 2003; Purushothaman *et al.*, 2020).

The antioxidant potential of melatonin is also believed to be driven by its ability to upregulate the activity of antioxidant enzymes. Indeed, *in vivo* mice studies have shown that melatonin (as well as closely related molecules 5-methoxytryptamine and, to a lesser degree, 5-methoxytryptophol) increased the activity of antioxidant enzymes SOD and glutathione reductase in regions such as the liver and the kidneys (Liu & Ng, 2000). Similarly, Emamgholipour *et al.* (2016) have reported that melatonin may increase the expression and activity CAT in blood cells.

Finally, melatonin has also been found to downregulate the activity of the two major endogenous ROS generators: NOX and XO (from section 1.6). Interestingly, melatonin is believed to prevent NOX assembly as *in vivo* rat studies have demonstrated that pinealectomy (removal of the pineal gland, the major source of melatonin) increases NOX activity while administration of melatonin decreased transcription of $p22^{phox}$, an essential protein for NOX activity (Simões *et al.*, 2016). This inverse relationship between melatonin and NOX was then corroborated by further *in vivo* and *in vitro* studies (Tain *et al.*, 2013; Zhou *et al.*, 2008). Similarly, using murine models once again, Richter *et al.* (2009) suggest that melatonin reduces the expression of placental XO. Thus, melatonin is also believed to exhibit antioxidant potential via the inhibition of pro-oxidant enzymes.

1.7.5 Melatonin as a pro-oxidant in cancer cells

Melatonin appears to have somewhat of a paradoxical behaviour in that whereas it acts as an antioxidant in healthy cells, it induces ROS production in cancers (Reviewed by Zhang and Zhang (2014)). To that effect, Radogna *et al.* (2009) have confirmed melatonin's pro-oxidant capabilities in myeloid leukaemia cells while also demonstrating it to be independent of its receptor binding. Instead, they showed that the inhibition of the melatonin-calmodulin (CaM) interaction appears to dampen the hormone's pro-oxidant capabilities.

The involvement of CaM in inducing ROS can be solved by the work of Jenkins *et al.* (2001), who proposed that binding of CaM to calcium-independent phospholipase A2 (iPLA2)

results in an inactive conformation of the latter protein. Active iPLA2 serves to hydrolyze phospholipids which can result in the release of arachidonic acid (AA) (Reviewed by Burke and Dennis (2009)). AA can then be picked up by 5-Lipoxygenase (5-LOX) to produce a variety of eicosanoids with diverse physiological effects (Reviewed by Funk (2001)). In the process, 5-LOX participates in the production of ROS (Catalano *et al.*, 2005). Then, since iPLA2 and 5-LOX have been shown to be upregulated in multiple cancer types, it is logical that the inactivation of CaM induces more ROS production in cancer cells than in healthy cells (Jiang *et al.*, 2002; Soumaoro *et al.*, 2006; Zhou *et al.*, 2015). Thus, by interacting with CaM, melatonin prevents the CaM/iPLA2 interaction and enables the tumoral-enhanced activity of 5-LOX, and of ROS production.

1.7.6 Melatonin and DNA

Melatonin's role as an antioxidant (section 1.7.4) inherently protects DNA from oxidative damage, but its *genoprotective* role does not end there. Treatment with melatonin has been shown to upregulate multiple proteins involved in DNA repair pathways, thus leading to an enhance DNA repair ability (Liu *et al.*, 2013). This is believed to be mediated by the MT1 and MT2 receptors, as knockdowns of either of the receptors have been shown to tamper with p38 and p53 phosphorylation, and inhibit the hormone's protective role on DNA (Santoro *et al.*, 2013b).

Santoro *et al.* (2012) have proposed that melatonin protects DNA via the phosphorylation of p53 at Ser15 (an activating phosphorylation event, from section 1.3.1), an event heavily dependent on the kinase activity of p38. These findings corroborate previous studies that had suggested a role for p38 in p53 activation (Sanchez-Prieto *et al.*, 2000; She *et al.*, 2000). Meanwhile, Roy *et al.* (2018) suggest alternate p38-dependent genoprotective pathways in cells where p53 activity is compromised. Thus, p38 appears to have a central role in melatonin-induced DNA protection. Yet, studies on the molecular mechanisms of p38 activation by melatonin are largely lacking, but as mentioned earlier, some MT1/MT2 receptors isoforms can interact with $G_{q/11}$ proteins (from section 1.7.3). Yamauchi *et al.* (1997) have demonstrated that these $G_{q/11}$ proteins may stimulate p38 activation, albeit in receptors other than MT1 and MT2. Nonetheless, these findings may begin to explain the role of melatonin in genomic protective, although this proposed molecular mechanism remains to be explicitly demonstrated.

1.7.7 Melatonin and the cytoskeleton

The diverse role of melatonin continues to be apparent in its ability to regulate cytoskeletal components, as reviewed by Benítez-King (2006). To that effect, melatonin appears to induce the polymerization of microtubules once again by interacting with CaM rather than by receptor binding (discussed in section 1.7.5). Indeed, CaM (activated by the presence of Ca^{2+}) inhibited the tubulin polymerization by 40% while this inhibition was reverted by the addition of melatonin, and CaM antagonists. Yet, at higher doses, and in the absence of CaM, melatonin was also able to induce depolymerization (Huerto-Delgadillo *et al.*, 1994).

Melatonin also appears to have an involvement in the organization of actin filaments. In melanoma cells, the hormone induced altered distribution of the filaments, leading to increased cell surface (Alvarez-Artime *et al.*, 2020). Similarly, Benítez-King (2006) demonstrated the hormone's ability to induce the thickening and differential localization of these filaments. Melatonin appears to affect the structure of the actin cytoskeleton by regulating the expression of genes involved in their organization (Tan *et al.*, 2015).

2 HYPOTHESIS AND RESEARCH OBJECTIVES

2.1 Problematic

The incidence of choriocarcinomas is rare, and current treatment options make for a good prognosis (Capobianco *et al.*, 2021). However, multiple reports have begun to highlight the emergence of resistance to traditional treatments (Goldfarb *et al.*, 2020; Wong *et al.*, 2022). Resistance to chemotherapeutic agents is well documented in other types of cancer such as breast cancer and chronic myelogenous leukemia and may explain the increase in their occurrence (Hu *et al.*, 2021; Raguz & Yagüe, 2008; Schneider *et al.*, 2014). Thus, finding novel, effective treatment against choriocarcinomas before traditional ones begin to fail consistently is key to preventing yet another cancer-related epidemic.

Previous work from our research team has shown that melatonin upregulates cell survival mechanisms in response to stress induced by hypoxia/reoxygenation (H/R) in healthy trophoblasts, while downregulating these same mechanisms in tumoral trophoblasts (Sagrillo-Fagundes *et al.*, 2019). Furthermore, our team has also outlined melatonin's ability to increase ROS production in tumoral trophoblasts (Kharrat, 2020). Although these findings are promising, a clear link between melatonin's ability to induce stress in tumoral cells and its use as an adjuvant to chemotherapeutic agents has yet to be shown.

2.2 Hypothesis

Taking into consideration information from literature, as well as previous findings from our research team, the hypothesis of this study is as follows:

"Melatonin increases ROS production in BeWo cells when combined with various chemotherapeutic agents, which will in turn induce an increase in tumoral cell death."

2.3 Specific objectives

The specific objectives of this study are to determine if BeWo human choriocarcinoma cells are responsive to melatonin in the following ways:

1. **Melatonin increases ROS production in combination with various chemotherapeutic agents.**

2. **Melatonin induces an increase in tumour cell death in combination with various chemotherapeutic agents.**

3. **Melatonin continues to exhibit antineoplastic potential in a model resembling more that of a solid tumour.**

3 MATERIALS AND METHODS

3.1 Cell culture

BeWo cells (clone CCL-98) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS; Multicell, Woonsocket, RI). The cells were grown in an incubator (ThermoFisher, Waltham, MA) at 37°C and 5% CO_2. Cells were split at a 1:10 ratio once 80% confluence was achieved, up to passage 30.

Viable cells counts were performed by mixing cells at a 1:1 ratio with a 0.4% Trypan blue solution (Sigma, St. Louis, MO) and counted using the TC10 Automated Cell Counter (BioRad, Hercules, CA). Only cultures exhibiting over 90% viability were plated for experimental analysis. Unless stated otherwise, cells were seeded at 3.0 x 10^5 cells/well for 6-well plates, 1.0 x 10^5 cells/well for 12-well plates, and 8.0 x 10^3 cells/well for 96-well plates 24h prior to any treatment. All plates were purchased from Corning Inc. (Corning, NY)

3.2 Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Cells were seeded on clear, flat-bottom 96-well plates as previously mentioned, and treated for 24h, 48h, and 96h with decreasing concentrations of 5-FU, ETO, MTX, OXA and PTX, all purchased from Cedarlane Labs (Burlington, ON). Fresh growth media and chemotherapeutic agents were supplied every 24h. An MTT (Sigma, St. Louis, MO) solution in phosphate buffer saline (PBS; Sigma, St. Louis, MO) was added to each well at a final concentration of 0.5 mg/mL. The cells were incubated for 2h at 37°C and 5% CO_2. The resulting crystals were dissolved in 2% (V/V) glycine (glycine (ThermoFisher, Waltham, MA), dH_2O, pH 11) and absorbance at λ = 570 nm was measured using the SpectraMax M5 spectrophotometer (Molecular Devices, San Jose, CA). Viability curves were derived from the absorbance readings by normalizing between 0% (no cells; media containing 0.5% DMSO) and 100% viability (untreated cells; media containing 0.5% DMSO).

3.3 Intracellular reactive oxygen species production measurements

Cells were seeded in black, flat-bottom, 96-well plates as mentioned and treated with 75 μM 5-FU, 1 μM ETO, 80 μM MTX, 1 μM OXA or 1 nM PTX with and without melatonin (1 μM or 1 mM; Sigma, St. Louis, MO). Cells were treated for 24h, 48h, or 72h, with fresh chemotherapeutic agents being supplied every 24h. The CM-H2DCFDA (ThermoFisher, Waltham, MA) probe in PBS was added to each well at a final concentration of 10 μM. Cells were then incubated for 40 mins at 37°C and 5% CO_2. Fluorescence was measured using the SpectraMax M5 fluorimeter with λ_{ex} = 485 nm and λ_{em} = 535 nm. Positive control was 100 μM (3.4 x 10^{-4} %) H_2O_2, added to the cells for 20 mins.

3.4 Flow cytometry

Cells were grown and plated as previously described and treated with 75 μM 5-FU, 1 μM ETO, 80 μM MTX, 1 μM OXA or 1 nM PTX with and without 1 mM melatonin for 48h. Flow cytometry analysis was performed using Invitrogen's eBioscience Annexin V apoptosis detection kit (Invitrogen, Waltham, MA), as per manufacturer protocol. Briefly, cells were harvested, and centrifuged at 12,000 rpm for 5 mins. Cells were then washed with PBS and resuspended in binding buffer (part of apoptosis detection kit provided by Invitrogen). Cells were stained for 10 mins with Annexin V (diluted 1:40; part of apoptosis detection kit provided by Invitrogen), followed by 1 min with 0.95 μg/mL propidium iodide (PI; part of apoptosis detection kit provided by Invitrogen). Positive controls were obtained by incubating cells with 1 μM staurosporine (Cell Signalling Technologies, Danvers, MA) for 1h. Positive controls and no treatment controls were used to set quadrant boundaries as per Supplemental Figure 7.2. Data was obtained using the CellQuest Pro (BD Biosciences, Franklin Lakes, NJ) software for the Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Data was subsequently analyzed using the FlowJo v.10 software (BD Biosciences, Franklin Lakes, NJ).

3.5 DNA extraction and gel electrophoresis

Cells were plated on 6-well plates as previously described. Total cell count was obtained as before, without trypan blue exclusion. 10^6 cells were harvested, washed in PBS and pelleted at 3000 rpm (4°C, 5 mins). Cells were incubated in lysis buffer (10 mM Tris-HCl pH 7.4, 0.5% SDS,

10 mM EDTA, 0.5 mg/mL proteinase K), overnight in a 37°C water bath. An RNAse solution was added and incubated 30 mins in 50°C water bath.

Extracted DNA was ran on 1% and 2% agarose (BioRad, Hercules, CA) gels in Tris-Borate-EDTA buffer (TBE; 65 mM Tris, 22.5 mM boric acid, 1.25 mM EDTA) containing SybrSafe DNA Gel stain (ThermoFisher, Waltham, MA). Samples were run at 60V for 15 mins, then 100V for 150 mins. Images were obtained using the Gel Doc™ EZ imager (BioRad, Hercules, CA) running the ImageLab software v.4.0.1 (BioRad, Hercules, CA).

3.6 Immunocytochemistry (ICC) and image acquisition

Coverslips (12CIR-1.5; ThermoFisher, Waltham, MA) were washed with 0.1 M HCl and 70% EtOH, and placed in 24-well plates. Cells were seeded at 10,000 cells per well and allowed to recover for 24h, and treated with 1 nM with or without 1 mM melatonin for 48h.

Cells were washed with PBS Ca^{2+}/Mg^{2+} (Gibco, Waltham, MA) and fixed with 4% PFA (Acros Organics, Waltham, MA) for 20 mins. PFA autofluorescence was neutralized by incubating cells at RT with a 50 mM NH_4Cl solution and permeabilization was done with 0.3% Triton X-100 (Sigma, St. Louis, MO), for 10 mins each. Blocking was performed with 7% normal donkey serum (NDS; Sigma, St. Louis, MO) at RT for 1h. Primary antibodies (α-tubulin DM1A mouse mAb (Cell Signalling Technologies, Danvers, MA) and β-actin 13E5 rabbit mAb (Cell Signalling Technologies, Danvers, MA)) were diluted 1:1000 in 7% NDS, and allowed to bind in a humidified chamber for 2h at RT. Cells were then washed with PBS containing 0.1% Tween-20 (Sigma, St. Louis, MO). Secondary antibodies (anti-mouse IgG Fab2 Alexa-488 (Cell Signalling Technologies, Danvers, MA) and anti-rabbit IgG Fab2 Alexa-594 (Cell Signalling Technologies, Danvers, MA)) were diluted 1:1000 in 7% NDS and allowed to bind for 1h at RT. Nuclei were stained with 0.5 µg/mL 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Waltham, MA) for 15 mins. Coverslips were mounted on slides (Fisher Scientific,Waltham, MA) with ProLong Gold Antifade Mountant (ThermoFisher, Waltham, MA).

Images were obtained using the Zeiss LSM780 confocal microscope (Zeiss, Oberkochen, Ostalbkreis) under 40X magnification using immersion oil. Fluorescence was monitored using the X-Cite 120 LED illuminator system (XCELITAS Technologies, Waltham, MA) with $\lambda_{ex}/\lambda_{em}$ = 358 nm/ 461 nm (DAPI), $\lambda_{ex}/\lambda_{em}$ = 488 nm/496 nm (anti-mouse IgG Fab2 Alexa-488) and $\lambda_{ex}/\lambda_{em}$ = 590

nm/ 618 nm (anti-rabbit IgG Fab2 Alexa-594). 5 fields of view per slide were inspected, as per figure 3.1.

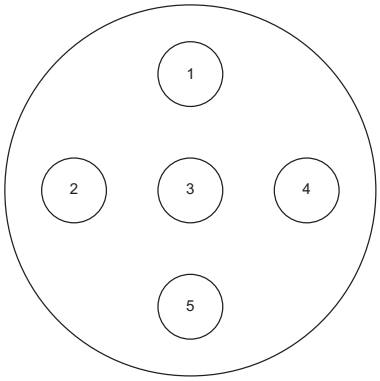


Figure 3.1: Diagram Indicating the Fields of View Observed for Immunocytochemistry

The microscope's field of view was placed in five distinct places (circles numbered 1 through 5) on the coverslip for analysis. This was done to prevent bias and overlapping of fields of view.

3.7 xCELLigence real-Time viability assay

Cells were plated on special 96-well PET E-plates (Agilent, Santa Clara, CA), custom for the xCELLigence Real-time viability apparatus (Agilent, Santa Clara, CA) running on the RTCA software v1.0.0.1304 (Agilent, Santa Clara, CA). Cells were allowed to grow until cell index (CI) reached at least 6.0 before treating them with 75 µM 5-FU, 1 µM ETO, 80 µM MTX, 1 µM OXA or 1 nM PTX with and without melatonin (1 mM). Treatments were performed over the course of at least 4 days, supplying the cells with fresh media containing chemotherapeutic agent and melatonin every 24h.

3.8 Statistical analysis

All data was represented as mean ± SD with n=3 to 6. All biological replicates were performed on different BeWo cell passages. Effects of melatonin are expressed relative to each drug alone and was set to 100%. Statistical significance ($p \leq 0.05$) was analyzed using student t-test with Holm-

Šídák p-value correction for multiple t-tests. All analyzes were performed using GraphPad Prism v9.4.0 (GraphPad Software, San Diego, CA).

4 RESULTS

4.1 Consistent BeWo cell death is observed after 48h in response to chemotherapeutic agents

We began our analysis by determining suitable *in vitro* concentrations and timeframes for the various chemotherapeutic agents by means of MTT assays. Since all agents but one (OXA was dissolved in water) were dissolved in dimethyl sulfoxide (DMSO), the toxicity of this solvent was empirically determined, and was found to be negligible to BeWo cells at contents below 0.5% (Supplemental Figure 7.1). Thus, based on this limitation and the solubility of the antineoplastics in DMSO, tested concentrations ranged from 400 pM to 400 μM for 5-FU, 21.38 pM to 106 μM for ETO, 100 pM to 500 μM for MTX, 5 pM to 25 μM for OXA, and 5.85 pM to 29.25 μM for PTX.

Following 24h treatments, 5-FU, OXA and PTX unequivocally failed to induce a 50% decrease in BeWo cell viability (Fig. 1, top row). Respectively, these drugs reduced cell viability to 81.71% ± 6.03%, 68.00% ± 7.64%, and 79.58% ± 6.20% at their highest concentration. MTX appears to induce close to 50% BeWo cell death at 5.0 μM, albeit with high variability (SD = 17.40%), and accompanied with a resurgence in cell viability at higher concentrations. ETO reduced BeWo cell viability down to 33.15% ± 3.11% at a concentration of 106.2 μM, with 21.37 μM inducing 50% cell death (absolute IC_{50} = 21.37 μM at 24h).

At 48h, maximum tested concentrations (stated above) of all drugs brought BeWo cell viability well below 50% (Fig. 1, middle row). At this timeframe, 5-FU could reduce viability down to 29.72% ± 1.59%, ETO to 2.03% ± 0.80%, MTX to 19.99% ± 6.02%, OXA to 8.52% ± 8.11% and PTX to 12.37% ± 1.87%. Absolute IC_{50} values were found to be 72.65 μM, 1.126 μM, 78.14 μM, 1.046 μM, and 0.8488 nM for these same drugs respectively, as shown in Table 4.1.

To further characterize these agents, their cytotoxicity was also assessed after 96h of exposure (Fig. 1, bottom row). 5-FU, ETO, MTX and OXA showed little to no cytotoxicity at low concentrations, with viabilities as follows: 93.92% ± 5.87% at 0.8 nM, 84.84% ± 16.16% at 2.13 pM, 92.06% ± 5.35 % at 100 pM, and 100.23% ± 13.98% at 5 pM, respectively. Absolute IC_{50} values at 96h for these four drugs were found to be 7.558 μM, 2.008 μM, 0.906 mM, and 0.19 μM, again respectively. PTX was found to be highly toxic, allowing only 20.99% ± 11.93% cell viability at 5.85 pM (lowest concentration), and near complete cell death (0.08% ± 0.73%) at 5.85 μM. Thus, an absolute IC_{50} for PTX at 96h could not be calculated.

4.2 1 mM, but not 1 μM, melatonin induces a transient increase in ROS production in bewo cells when paired with chemotherapeutic agents

Next, we sought to investigate whether melatonin affected ROS production when combined with the chemotherapeutic agents described earlier. To that effect, BeWo cells were treated with the 48h IC_{50} concentration of each drug, with and without melatonin (1 μM and 1 mM) and ROS production was assessed relative to the drugs alone. To measure the progression in ROS production, measurements were taken at 24h, 48h, and 72h post-exposure.

In the first 24h, addition of 1 μM melatonin had no significant ($p > 0.05$) nor consistent effect on relative ROS production for all (fig. 2, top left). In some cases, relative ROS production was consistently close to 100% (defined as the level of ROS production for each drug individually): 97.51% ± 5.63% for 5-FU, and 102.37% ± 15.41% for OXA. Similar results were also obtained for ETO, MTX and PTX, but with a much higher variability: 101.80% ± 47.48%, 99.67% ± 48.11%, and 147.50% ± 49.69%, respectively. The effects of the addition of 1 mM melatonin also appeared to induce a highly variable increase in ROS production: 116.66% ± 83.57% for 5-FU, 119.09% ± 16.85% for ETO, 136.43% ± 81.30% for MTX, 189.15% ± 103.67% for OXA, and 117.51% ± 39.10% for PTX. No statistically significant change in ROS production was found for any of the drugs at 24h, with neither 1 μM nor 1 mM melatonin ($p > 0.05$).

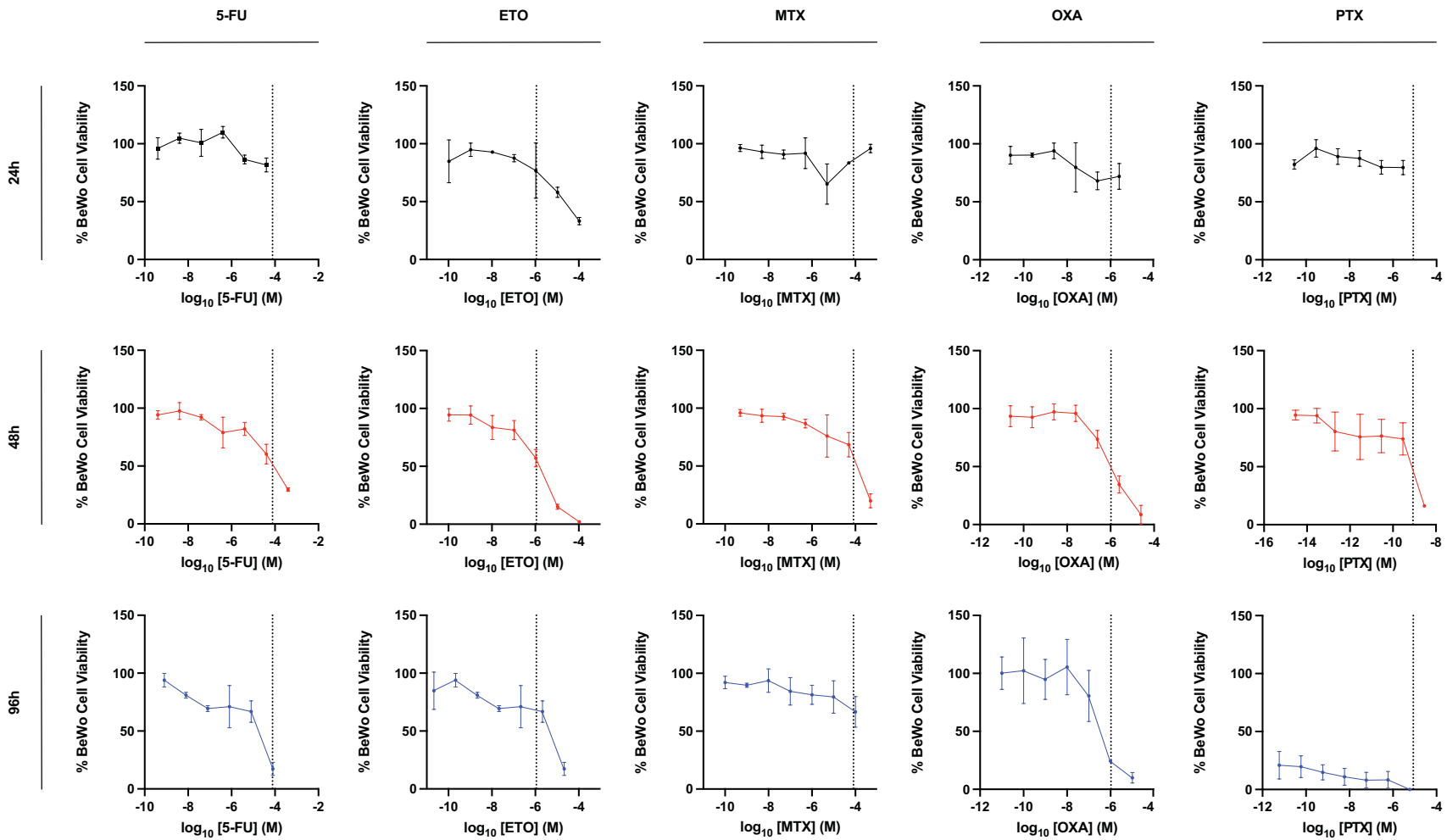


Figure 4.1: Viability Curves of BeWo Cells in Response to Various Antineoplastics

BeWo cells were subjected to varying concentrations of 5-FU, ETO, MTX, OXA, and PTX and cell viability was assessed using the MTT assay for exposure times of 24 hours (top row, black), 48 hours (middle row, red), and 96 hours (bottom row, blue). Viability was normalized to the control (media containing 1% DMSO) without antineoplasticand fresh media (± antineoplastic) was supplied every 24 hours. Vertical dashed line represents the IC50 concentration at 48 hours, values subsequently used in the obtention of other data. Abbreviations: 5-FU, 5-fluorouracil; ETO, etoposide; MTX, methotrexate; OXA, oxaliplatin; PTX, paclitaxel. (N=3)

Table 4.1: Summary of the IC50 Values for BeWo Cells in Response to Various Antineoplastics

	Absolute IC50 Value (μM)		
	24h	48h	96h
5-FU	N/A	72.65	7.558
ETO	21.37	1.126	2.008
MTX	5.0	78.14	906
OXA	N/A	1.046	0.19
PTX	N/A	8.488×10^{-4}	N/A

The values presented here were extracted from the viability curves shown in figure 4.1. IC50 values were determined by means of an MTT viability assay for exposure times of 24 hours, 48 hours, and 96 hours, with fresh media (± antineoplastic) being supplied every 24 hours. Unless otherwise indicated, all values are expressed in micromolar (μM). The not applicable (N/A) denotation was given to all cases in which the IC50 could not be calculated. Abbreviations: 5-FU, 5-fluorouracil; ETO, etoposide; MTX, methotrexate; OXA, oxaliplatin; PTX, paclitaxel.

At 48h, as shown in figure 4.2 (top, right), ROS production in response to 1 μM melatonin varied with respect to the drug, from having little to no effect (87.98% ± 17.35% for 5-FU, 97.58% ± 7.17% for OXA, and 94.75% ± 30.98% for PTX), to inducing increased, but variable ROS production (146.81% ± 41.38% for ETO, and 168.44% ± 57.84% for MTX). Whereas no statistically significant increase in ROS production was observed in response to 1 μM melatonin ($p > 0.05$), the addition of 1 mM melatonin induced a significant increase for all drugs. Indeed, relative ROS production levels were found to be 127.63% ± 11.29 for 5-FU ($p \leq 0.05$), 306.00% ± 65.65% for ETO ($p \leq 0.05$), 197.87% ± 31.72% for MTX ($p \leq 0.05$), 254.93% ± 10.54% for OXA ($p \leq 0.0001$), and 183.80% ± 5.33% for PTX ($p \leq 0.0001$).

At 72h, only OXA and PTX combined with 1 mM melatonin continued to display a statistically significant increase in ROS production, valued at 168.12% ± 22.68% ($p \leq 0.05$) and 248.31% ± 22.28% ($p \leq 0.01$). When paired with 1 mM melatonin, ROS production in the 5-FU, ETO and MTX-treated BeWo cells was of 97.17% ± 9.28%, 174.15% ± 55.20%, and 182.43 ± 143.68%, respectively, all non-significant ($p > 0.05$). Relative ROS production was found to be

130.24% ± 23.99% for 5-FU, 91.88% ± 25.30% for ETO, 134.99% ± 50.78% for MTX, 126.01% ± 18.80% for OXA, and 92.06% ± 8.37% for PTX when paired with 1 μM melatonin, none of which were significantly different to the drug alone ($p > 0.05$).

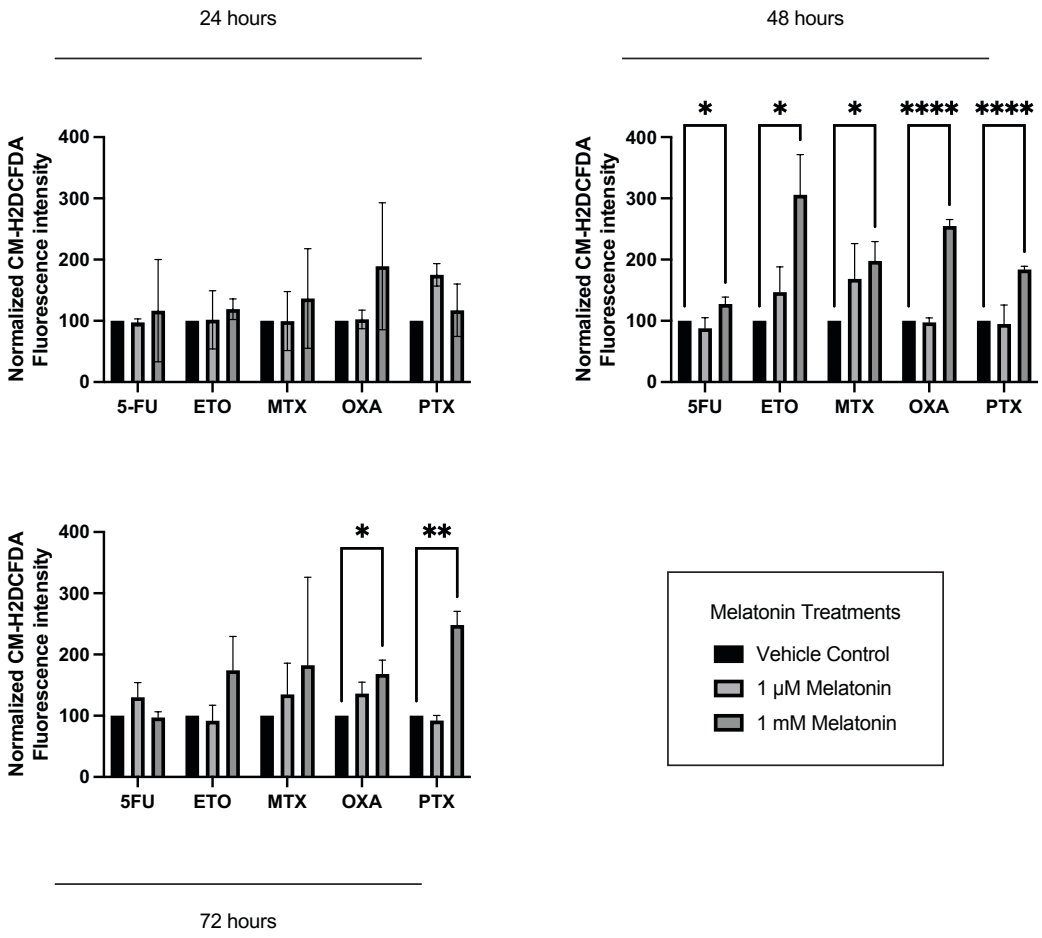


Figure 4.2: Melatonin (1 mM) Induces a Significant, but Transient Overproduction of ROS in BeWo cells when Combined with Various Antineoplastics

To determine the effect of melatonin on ROS production, a total ROS assay was performed on BeWo cell cultures by means of the CM-H2DCFDA probe. Melatonin (1 μM or 1 mM) was added in the culture media and cells were allowed to growth for 24 hours (top left), 48 hours (top right), and 72 hours (bottom left), supplying fresh media (± antineoplastic; ± melatonin) every 24 hours. ROS production was normalized to that of cells responding to the corresponding antineoplastic alone. Vehicle control was 1% DMSO. Positive control was 100 μM H_2O_2 added to control cells for 20 mins. Statistical significance is expressed as: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Abbreviations: 5-FU, 5-fluorouracil; ETO, etoposide; MTX, methotrexate; OXA, oxaliplatin; PTX, paclitaxel. (N=3-7)

4.3 Increased ROS production induced by 1 mM melatonin is concurrent with reduced cell viability when co-administered with 5-FU, ETO and OXA

Then, we sought to determine whether the increased ROS production in response to 1 mM melatonin induced cell death. To that effect, flow cytometry analysis was performed, using PI and Annexin 5/Fluorescein isothiocyanate (A5/FITC) as markers of cell death. As 1 μM did not appear to induce ROS overproduction, this concentration was not further investigated. The gating method is shown in Supplemental Figure 7.2. Representative flow cytometry data obtained is shown in Figure 12A.

In Figure 4.3B, the percentage of viable cells ($A5^-/PI^-$) in response to each drug combined with 1 mM melatonin was represented relative to the drug alone. Treatment with 1 mM melatonin induced a significant decrease in viable cells for the 5-FU, ETO, and OXA groups. Respectively, viability dropped from 100% (drug alone) to 68.54% ± 14.44% ($p \leq 0.05$), 40.17% ± 10.85% ($p \leq 0.0001$), and 72.24% ± 17.76% ($p \leq 0.01$). Slight decreases in average viability counts for MTX and PTX (78.66% ± 28.52% and 78.08% ± 19.31%, respectively) were also observed but were found to be statistically non-significant ($p > 0.05$).

Next, the normalized total proportions of apoptotic (both early and late) cells in response to 1 mM melatonin with various antineoplastics are shown in Figure 4.3C. We base our analysis on the consensus that A5/FITC staining allows for analysis of the various stages of apoptosis but, as is discussed later (in section 5.7), we challenge this consensus. Where a significant decrease in viable cells was observed, an increase in total apoptotic cells was concurrent: 147.86% ± 3.73% ($p \leq 0.0001$) for 5-FU, 123.12% ± 14.82% ($p \leq 0.05$) for ETO and 147.03% ± 35.19% ($p \leq 0.05$) for OXA. Non-significant increases were observed for MTX (126.72% ± 26.03%; $p > 0.05$) and PTX (162.41% ± 47.00%; $p > 0.05$).

The ratios of apoptotic cells were analyzed more in depth, and normalized proportions were separated into 2 groups: early and late apoptotic BeWo cells. In response to 1 mM melatonin, the fractions of early apoptotic cells ($A5^+/PI^-$) were found to be variable and did not differ significantly in response to 1 mM melatonin ($p > 0.05$; Figure 4.3D). In most cases, treatment with melatonin induced a (non-significant) increase in mean early apoptotic cell counts (116.85% ± 22.64 for 5-FU, 105.00% ± 22.74% for MTX, 117.45% ± 36.78% for OXA, and 141.43% ± 39.44% for PTX; $p > 0.05$ in all cases). In the case of ETO-treated cells, we observed a slight, non-significant, decrease in $A5^+/PI^-$ cells (114.46% ± 83.25%) in response to 1 mM melatonin.

On the other hand, from Figure 4.3E, we observe that the addition of 1 mM melatonin induced a significant increase in late apoptotic BeWo cells when co-cultured with 5-FU (193.28% ± 17.82%; $p \leq 0.001$), ETO (148.55% ± 35.54%; $p \leq 0.05$) and OXA (184.28% ± 54.60%; $p \leq 0.05$). The proportions of late apoptotic BeWo cells treated with MTX or PTX and 1 mM melatonin increased slightly, but non-significantly ($p > 0.05$), to 151.97% ± 48.67% and 195.00% ± 64.85%, respectively.

4.4 Melatonin inconsistently protects BeWo cells from antineoplastic-induced DNA damage

Having verified that melatonin does induce a decrease in BeWo cell viability when combined with most chemotherapeutic agents, we sought to understand if its cytotoxicity is linked to the cytotoxic mechanism of the agents. 5-FU, ETO, MTX and OXA, through different mechanisms, eventually induce DNA damage in cancer cells. Thus, to determine the effect of melatonin on DNA fragmentation, the integrity of the genomic content of 10^6 cells treated with these four antineoplastics for 48h was assessed via 2% agarose gel electrophoresis.

On the agarose gels, the presence of DNA fragmentation was represented as a smear. From figure 4.4, we observe that the addition of all antineoplastics tested here induced DNA fragmentation. Concomitant addition of 1 mM melatonin appears to induce inconsistent results: in some cases, stronger fluorescence is observed in the high molecular weight range while, in other cases, a weaker fluorescence is apparent (relative to treatment with each antineoplastic alone). However, we report that, for the most part, BeWo cells treated with 5-FU or OXA, and melatonin, displayed higher fluorescence in the high molecular weight range accompanied by lower fluorescence in the low molecular weight area. The opposite was apparent in BeWo cells subjected to ETO/melatonin. In the MTX group, addition of 1 mM melatonin left the pattern of fluorescence essentially unchanged.

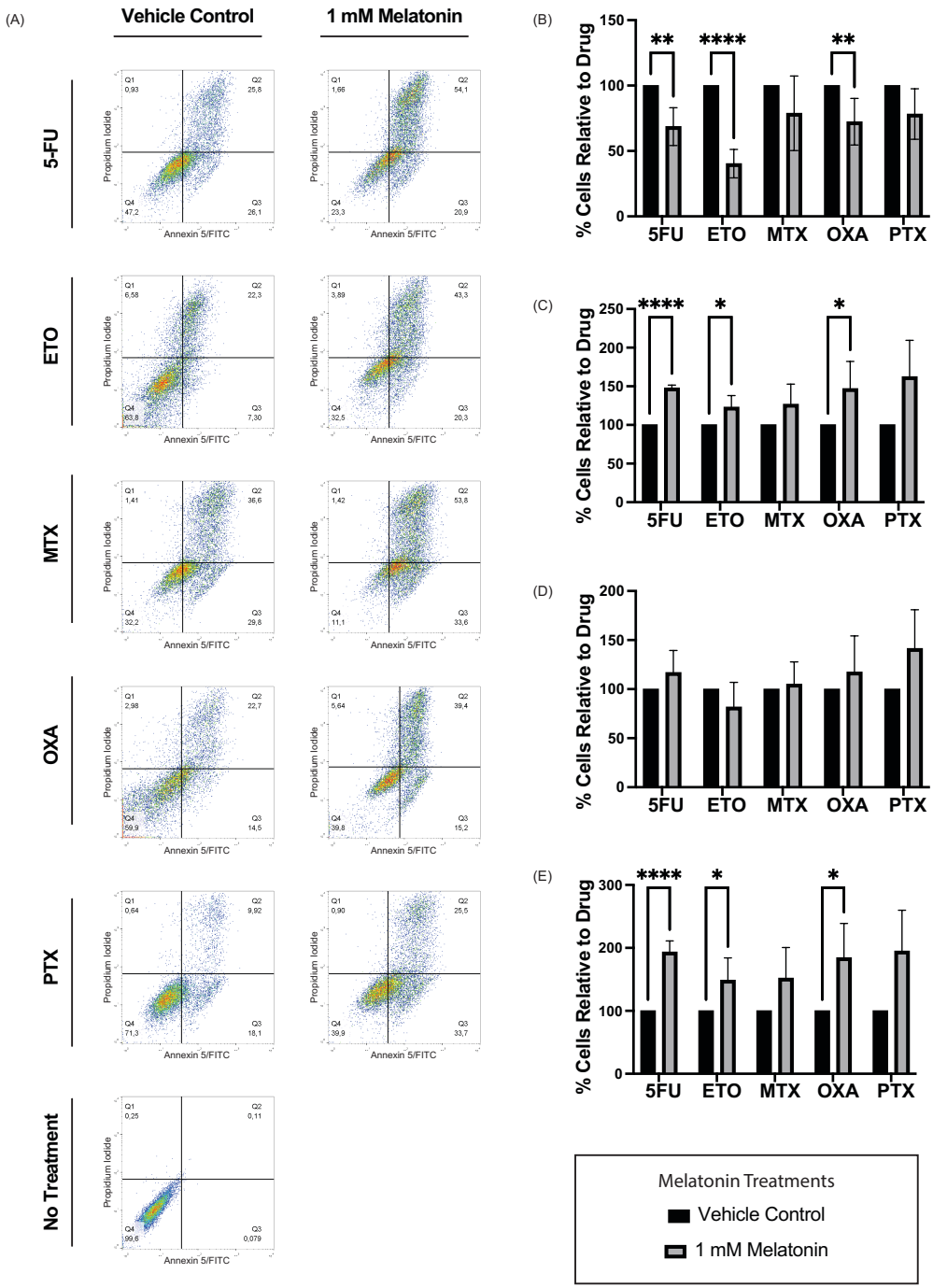


Figure 4.3: Melatonin (1 mM) Enhances Antineoplastic-Derived Apoptosis of BeWo Cells

BeWo cells were treated with various antineoplastics with and without melatonin, and viability was assessed using flow cytometry according to staining intensities of the Annexin 5 and PI probes. (A) Representative flow cytometry results obtained for the antineoplastics tested. (B) Compiled proportions of viable cells. (C) Compiled proportions of total cells in all stages of apoptosis. (D) Compiled proportions of cells in early phase of apoptosis. (E) Compiled proportions of cells in late phase of apoptosis. (A-E) Vehicle control was 1% DMSO. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001. Abbreviations: 5-FU, 5-fluorouracil; ETO, etoposide; MTX, methotrexate; OXA, oxaliplatin; PTX, paclitaxel. (N=3-6)

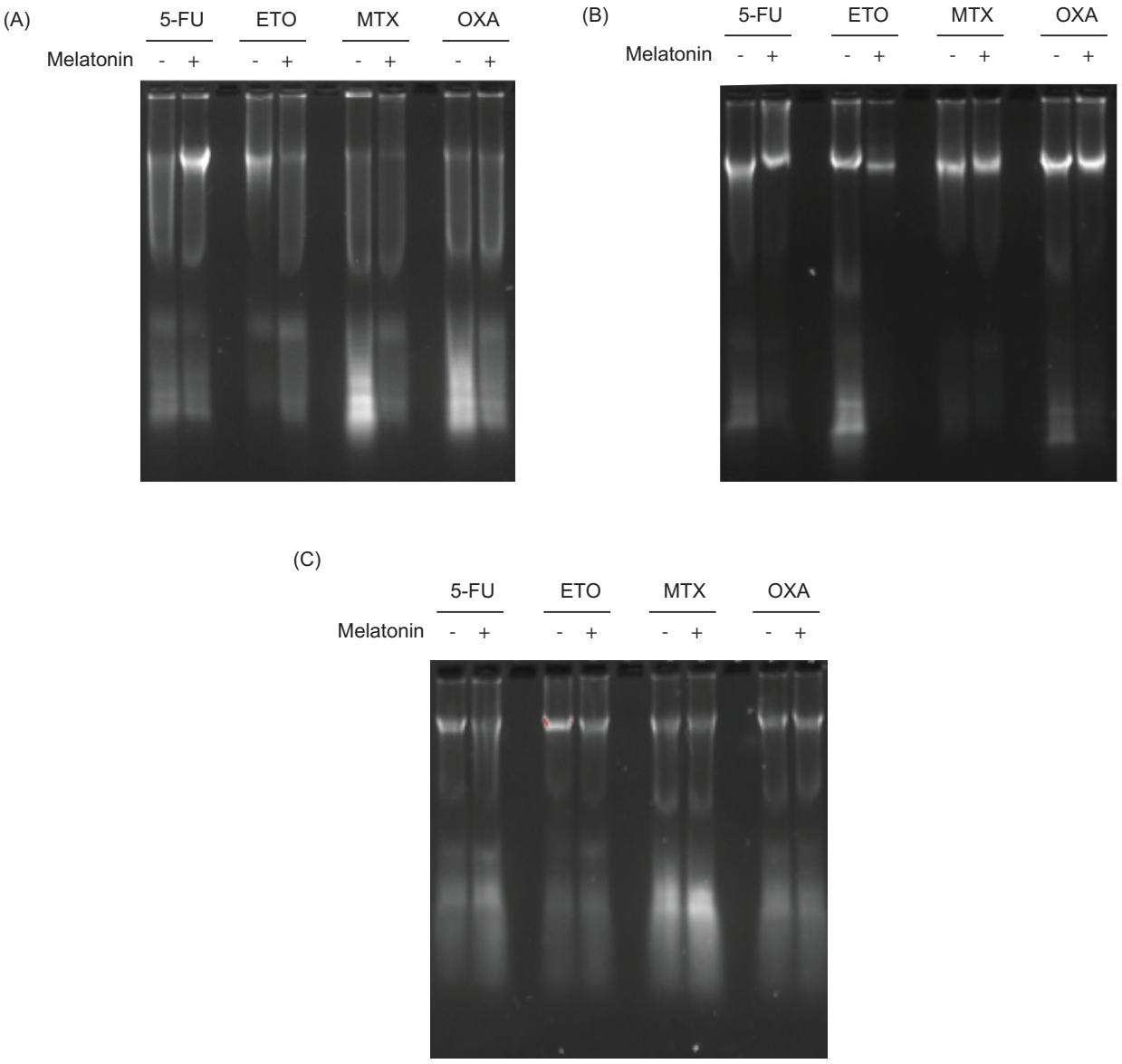


Figure 4.4: Melatonin (1 mM) Unreliably Hinders Antineoplastic-Driven DNA Fragmentation in BeWo Cells

BeWo cells were subjected to a concurrent administration of 5-FU, ETO, MTX and OXA, and 1 mM melatonin for 48h. The DNA of 10^6 cells for each treatment group was extracted, and its integrity was assessed via 2% agarose gel electrophoresis. Overall, melatonin appeared to prevent or revert the genotoxicity of the antineoplastics, but results were highly variable. (A) First biological replicate (N1) indicates that melatonin has genoprotective effects in cells treated with 5-FU, and OXA. (B) Second biological replicate (N2) shows that melatonin prevents DNA damage in 5-FU, ETO and OXA-treated BeWo cells. (C) Third biological replicate (N3) suggests that melatonin did not protect BeWo cells against DNA damage when coupled to any antineoplastic. (A-C) Melatonin did not appear to significantly alter the occurrence of DNA lesions when coupled to MTX. (N=3)

4.5 Melatonin (1 mM) reverts the effects of PTX-driven alterations in mitotic spindle occurrences and phenotypes in BeWo Cells

Instead of inducing DNA fragmentation, PTX's cytotoxic mechanism is through the stabilization of microtubules and subsequent induction of mitotic arrest. To address the combinatorial effect of 1 mM melatonin with PTX in BeWo cells, the DNA, microtubules and F-actin filaments were stained using the ICC technique described in the Materials and Methods (section 3.6).

Representative cytoskeletal structures are shown in Figure 4.5A, where the no treatment control (Figure 4.5A, top row), and treatment with PTX alone (Figure 4.5A, middle row) or with 1 mM melatonin (Figure 4.5A, bottom row) are depicted. A qualitative analysis failed to show any visible alteration in the staining pattern of F-actin. Thus, the positioning of DNA and microtubules were used to identify cells in mitosis, where mitotic spindles could be clearly visualized.

We began by assessing the effect of PTX on the proportions of cells in metaphase arrest and found that BeWo cells treated with this antineoplastic exhibited a significant decrease of such, down from 100% (normalized value from in the no-treatment control) to 56.53% $\pm$ 15.22% ($p \leq 0.05$). Then, in cells treated with PTX and 1 mM melatonin, the proportion of cells in mitotic arrest returned to its initial state: 98.16% $\pm$ 47.60% (Figure 4.6A). Understanding that PTX may not only tamper with spindle formation per se, but may also tamper with how they are formed, spindle phenotypes (bipolar (Figure 4.5B) vs multipolar (Figure 4.5C)) were also assessed. Figure 4.6B summarizes the proportion of BeWo cells exhibiting bipolar or multipolar spindles, normalized to the number of mitotic spindles observed. This analysis revealed that, in the control group, 97.67% $\pm$ 5.77% and 3.33% $\pm$ 5.77% of mitotic cells exhibited bipolar spindle phenotypes. These were found to be significantly different from those of the PTX-treated group, where bipolar and multipolar spindles were observed in 69.44% $\pm$ 4.81% ($p \leq 0.01$) and 30.56% $\pm$ 4.81% ($p \leq 0.01$) of mitotic cells, respectively. Addition of 1 mM melatonin to PTX-treated cells essentially restored phenotypical proportions to those of the control: 97.62% $\pm$ 11.85% (bipolar) and 9.79% $\pm$ 2.29% (multipolar). Mitotic spindle phenotypes were also normalized to the total number of cells (Supplemental Figure 7.3). There, the proportion of bipolar spindles followed the same trend (decreasing in response to PTX alone), although no significance in the proportion of multipolar spindles was observed.

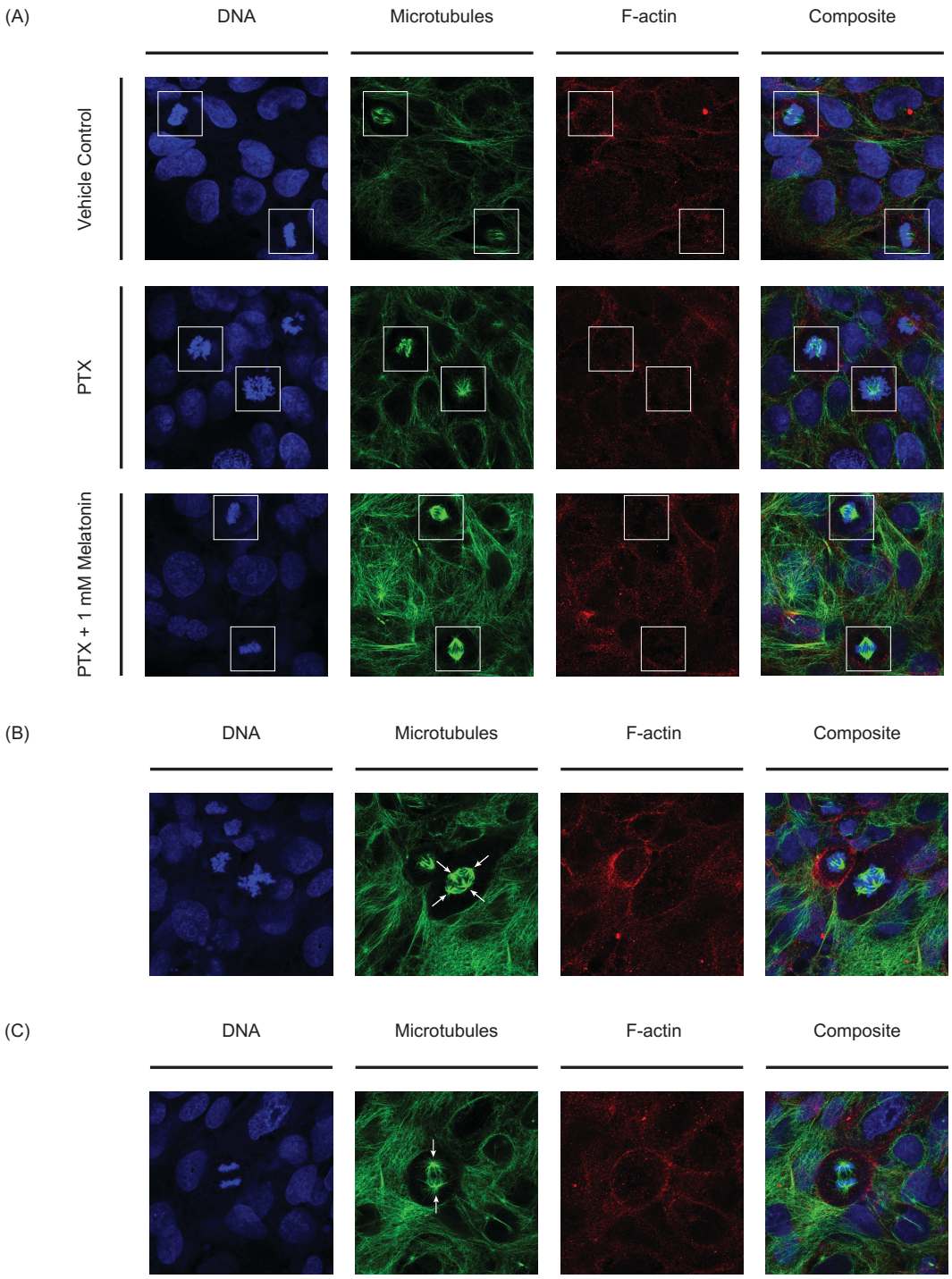


Figure 4.5: Melatonin (1 mM) Restores Normal Mitotic Spindle Proportions and Phenotypes in BeWo Cells

(A) Representative images showing mitotic spindle phenotypes in untreated (top row), PTX-treated (middle row) and PTX/melatonin-treated BeWo cells (bottom row). White boxes outline suspected spindles. Vehicle control was 1% DMSO. (B) A mitotic spindle exhibiting a multipolar phenotype. (C) A mitotic spindle exhibiting a bipolar phenotype. (B-C) White arrows indicate the origin of the spindle poles. (N=3)

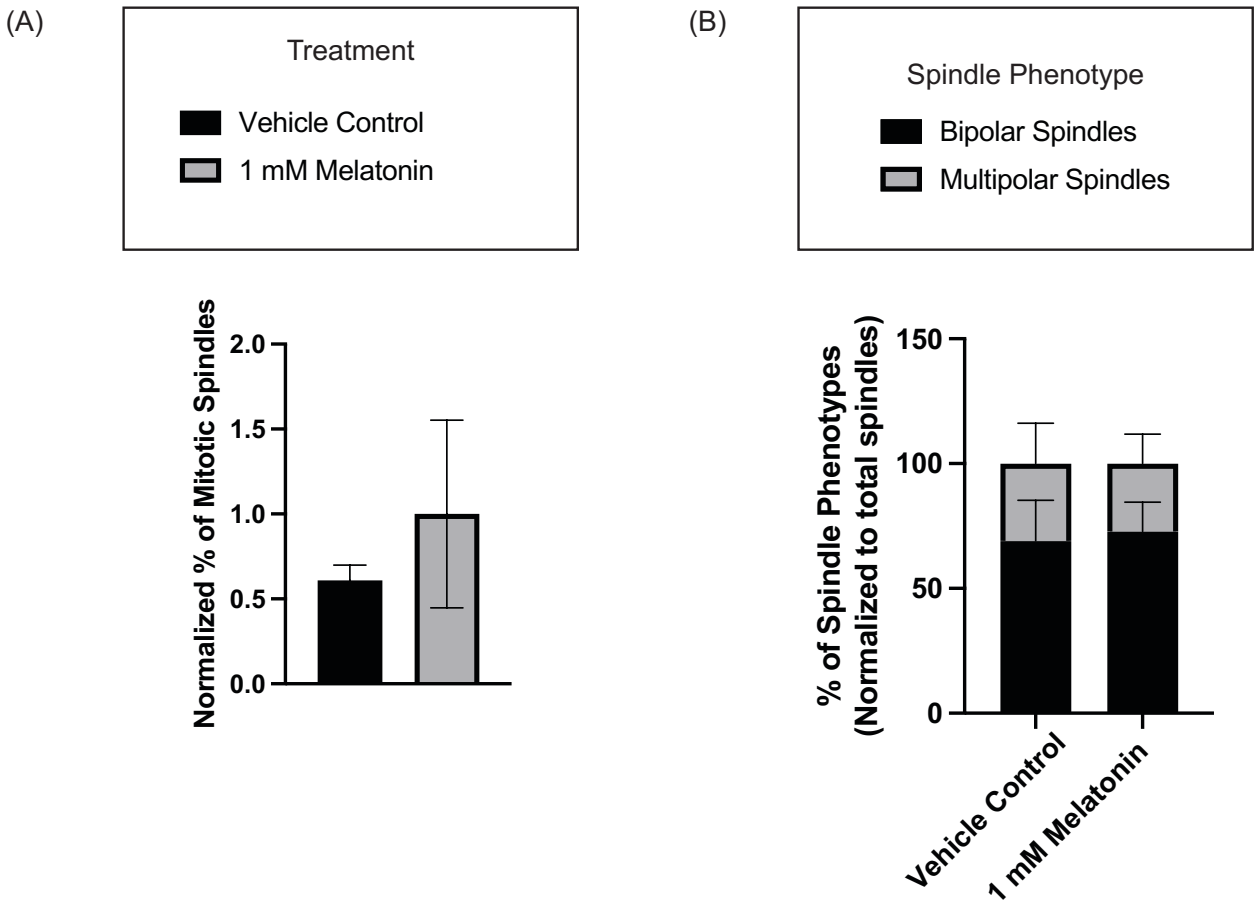


Figure 4.6: Melatonin (1 mM) Reverts the Effect of PTX on the Proportions and Phenotypes of Mitotic Spindles in BeWo Cells

(A) The total number of mitotic spindles in response to treatment with PTX ± 1 mM melatonin, normalized to the total number of cells in all fields of view. Vehicle control was 1% DMSO. (B) Proportions of bipolar and multipolar spindle phenotypes in BeWo cells, normalized to the total number of spindles observed. Abbreviations: NT: no treatment; PTX, paclitaxel. (N=3)

4.6 Melatonin continues to enhance the cytotoxic effects of 5-FU and ETO in a multilayered BeWo culture model

Finally, we sought to understand whether 1 mM melatonin can induce cell death in a model that resemble more that of a solid tumor. To that effect, cells were plated on 96-well PET E-plates (Agilent, Santa Clara, CA) for the XCELLigence apparatus. Cells were allowed to grow for about 4 days, until a CI value of 8.0 was obtained – as opposed to the 24h growth period allowed in previous experiments. Cells were then treated with the same concentrations of 5-FU, ETO, MTX,

OXA and PTX with and without 1 mM melatonin over the course of 90h, supplied with a fresh dose of antineoplastic every 24h.

In the case of 5-FU (Figure 4.7A), we observed a consistent decrease in normalized cell index (nCI) from the initial exposure (t = 0h) to the final time point of the experiment (t = 90h). Although the decreasing trend in nCI in response to 1 mM melatonin was observed from the start of the experiment, a statistically significant decrease in nCI was only obtained at 70 hours post exposure and thereafter: t = 70h ($p \leq 0.05$), t = 80h ($p \leq 0.05$), and t = 90h ($p \leq 0.01$).

Figure 4.7B shows nCI of BeWo cells in response to 1.126 µM ETO with and without 1 mM melatonin. Here, we again observed a decrease in nCI value in response to the dose of melatonin, but the decrease only began to emerge after 70h of exposure. At t= 10h and t= 20h, melatonin did not appear to have any effect on nCI as indicated by nCI close to 1.0 and the low degree of variability in the average nCI values (nCI_{avg}): nCI_{avg} was 0.95 ± 0.06 at t= 10h and 0.93 ± 0.06 at t= 20h. nCI_{avg} continued to revolved around 1.0 between 30 hours and 50 hours post-exposure for the melatonin-treated group, but with high degree of variability: 1.02 ± 0.32 for t = 30h, 1.00 ± 0.34 for t= 40h, 0.92 ± 0.43 for t= 50h. A non-significant decrease in nCI was observed at t= 60h but was still associated with a high degree of variability, where nCI_{avg} was 0.81 ± 0.37 (Supplemental Table 7.1). The variability decreased thereafter, and a statistically significant reduction in nCI was observed starting at t= 80h as nCI_{avg} was found to be 0.65 ± 0.24 for t= 70h, 0.51 ± 0.20 ($p \leq 0.05$) for t= 80h, and 0.27 ± 0.24 ($p \leq 0.01$) for t= 90h.

In the case of MTX (Figure 4.7C), the addition of 1 mM melatonin induced a first significant decrease in cell viability 10h post-treatment (t= 10h), which was transient as statistical significance was lost at t=20h. A second, transient, decrease in cell viability in response to co-treatment with MTX and melatonin was observed at t=50h, and was maintained until t=70h (Supplemental Table 7.1). All other timeframes failed to exhibit a significant alteration in BeWo cell viability. No significant alteration in cell viability was found throughout in the OXA/melatonin-treated (Figure 4.7D) and PTX/melatonin-treated (Figure 4.7E) groups. Of note, a large variability was observed in the data in the latter treatment group.

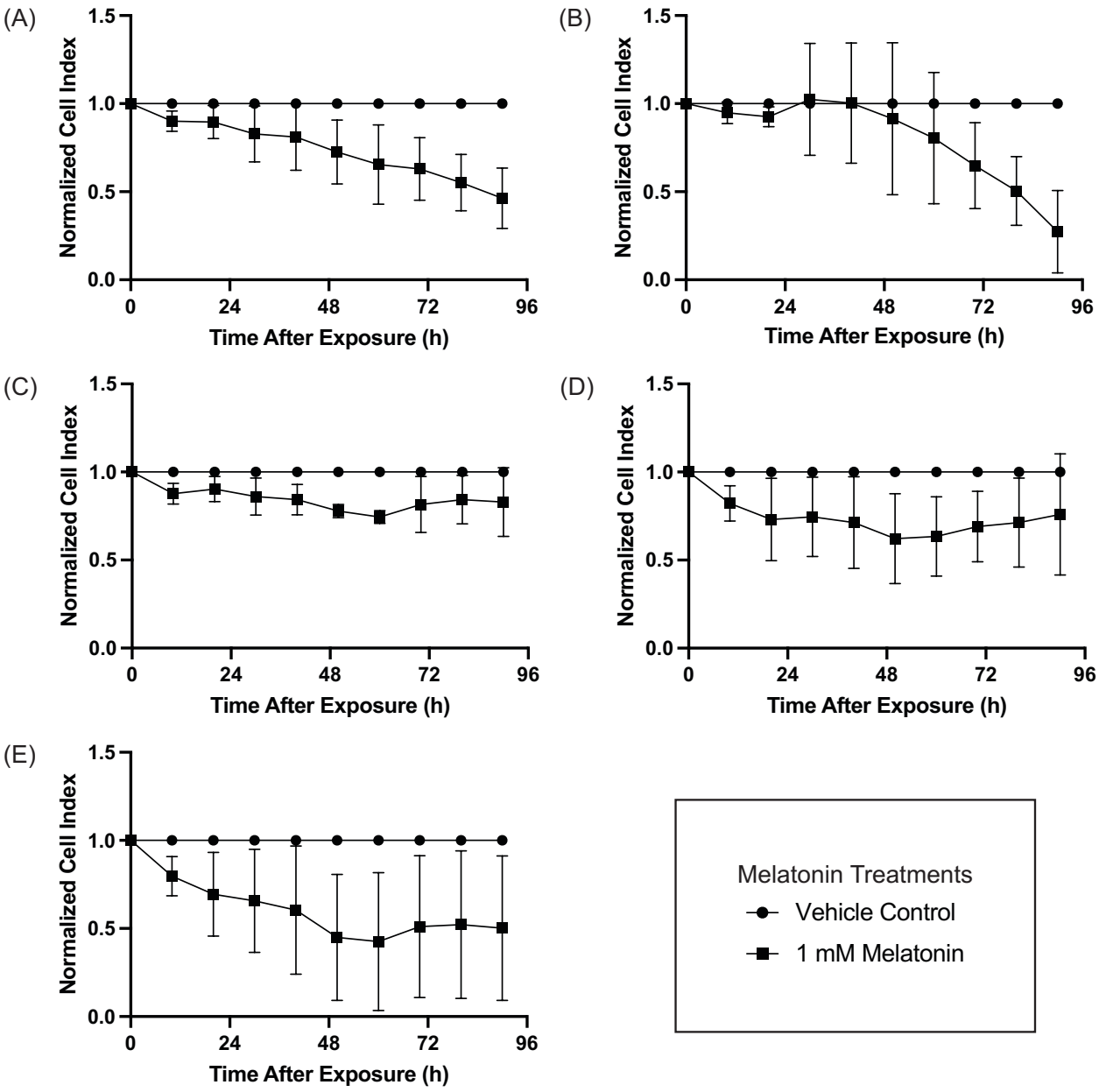


Figure 4.7: Melatonin (1 mM) Continues to Exhibit Antineoplastic Potential when Combined with Certain Antineoplastics in a Multi-layered BeWo Culture Model

Cells were grown for approximately 5 days, until a cell index value of 8.0 was reached. Cell index values were normalized to the time of addition of each of the chemotherapeutic agents (t = 0). Cell index was measured with and without the addition of 1 mM melatonin in presence of (A) 72.65 µM 5-FU, (B) 1.126 µM ETO, (C) 78.14 µM MTX, (D) 1.046 µM OXA and (E) 0.8488 nM PTX. Vehicle control was 1% DMSO. (N=4)

5 DISCUSSION

5.1 Determining optimal doses and exposure times of the antineoplastics in BeWo cells

The first step in our study was to determine the optimal time and concentration of each of the antineoplastics in BeWo cells specifically. We sought to obtain the concentrations that would reduce cell viability to 50% (*i.e.,* IC50) such that any increase or decrease in viability could be easily monitored thereafter. To that effect, we performed MTT assays with increasing concentrations of the chemotherapeutic agent at different timeframes. It is of note that the antineoplastics were dissolved in DMSO, a chemical solvent known to have the potential to disrupt cellular membranes (de Ménorval *et al.*, 2012). As such, to be confident that our assays were truly monitoring the effect of the chemotherapeutic agent rather than the solvent, the DMSO content was kept at 0.5%, well below the 1.25% which preliminary analyses showed to be non-toxic to BeWo cells (Supplemental Figure 7.1). Thus, based on this constraint and on the dissolvability of the agents in DMSO, higher concentrations than the ones used here could not be achieved.

We first tested the effects of each antineoplastic on cell viability with a 24h exposure time (Figure 4.1, top row). Under such conditions, only treatment with ETO yielded a consistent decrease in cell viability that reached 50%. For 5-FU and PTX, we noted that we could not reach the 50% cell viability mark, instead reaching viabilities of 81.71% ± 6.03% and 79.58% ± 6.20% at the maximal dose, respectively. Still within a 24h timeframe, treatment with 90 nM OXA began to hint towards a decrease in cell viability, but a plateau revolving ~ 75% viability was observed thereafter. The appearance of plateaus for the 5-FU, PTX, and OXA-treated groups indicate that the doses to which the cells were subjected were simply not enough to induce significant cytotoxicity in BeWo cells specifically. BeWo cells treated with MTX first exhibited a decrease in viability (achieving 50% viability at 5.0 µM) but increased at higher doses. Of course, such an observation could be attributed to the variability of results, especially considering the lack of statistically significant change in cell viability at any concentration (analysis not shown). The decrease in viability at 5.0 µM MTX was nonetheless quite pronounced and lead us to questioning the veracity of this claim. Interestingly, Hussa and Pattillo (1972) reported that MTX does not induce net cellular death in BeWo cells at similar doses as tested here while Friedman and Skehan (1979) explicitly refer to the cell line as being derived from an MTX-resistant tumor. Another group reported

that MTX induces BeWo cell differentiation into STBs rather than inducing cell death (Taylor *et al.*, 1991). Thus, previous studies suggest that MTX is ineffective in inducing cytotoxicity in BeWo cells, in line with our results. All in all, the lack of consistently observable effect of all antineoplastics (less ETO) imply that a 24h treatment regimen is not suitable. Furthermore, administration of a single dose of antineoplastics *in vitro* is unrepresentative of clinical therapies, in which patients are often given multiple rounds.

Therefore, we next turned to the other extreme and subjected BeWo cells to long treatments: 96h regimens (4 doses, once every 24h). At this timeframe, in most cases we were able to obtain an IC50 value: 7.59 µM for 5-FU, 2.01 µM for ETO, 90.62 µM for MTX and 192.5 nM for OXA. On the other hand, the cytotoxic effect of PTX was so potent that the highest viability was of 20.99% $\pm$ 11.93% for the smallest dose (5.85 pM). Further diluting the PTX solution to decrease the dose while satisfying the constraint of the DMSO content was an option, but the drawbacks of a 96h assay outweighed said option. Indeed, not only is such a long assay at increased risk of contamination, the controls, which were untreated, were often overconfluent, which could influence cell viability and many other parameters to skew results. Of note, MTX was found to be the least effective of the drugs tested (highest IC50), consistent with the studies reported earlier wanting the drug to be ineffective in BeWo cells. All other antineoplastics followed the expected trend: higher cytotoxicity upon repeated exposure.

To circumvent the limitations of 96h treatments, we attempted to determine the IC50 values at an intermediary timeframe: 48h (2 doses, every 24h). Under these conditions, all antineoplastics exhibited a 50% decrease in cell viability at 72.65 µM for 5-FU, 1.126 µM for ETO, 78.14 µM for MTX, 1.046 for OXA and 0.8488 nM for PTX (From Figure 4.1 and Table 4.1). At this timeframe, the sources of error attributed to 96h-long assay were largely eliminated while mimicking, to some degree, administration of chemotherapeutic agents in a clinical setting. Thus, we chose to perform all subsequent experiments with exposure times of 48h, with the concentrations of antineoplastics listed in above, and in Table 4.1. With these observations came another consideration: why would MTX induce considerable cell death at 48h, but not at 24h or 96h? We propose that while a 24h exposure to MTX begins to trigger intracellular mechanisms of cytotoxicity (described in section 1.4.2.2), these mechanisms only become observable in a portion of cells, later (somewhere between 24h and 48h). Then, prolonged exposure to this relatively inefficient antineoplastic would allow the cells to adapt and trigger resistance mechanisms (briefly discussed in section 1.5.2).

5.2 Co-treatment of 1 mM melatonin, but not 1 µM, with all antineoplastics induces a transient ROS overproduction in BeWo Cells at 48h

At least some chemotherapeutic agents are known to induce ROS production and, in this manner, may induce cell death (Yang *et al.*, 2018). Indeed, ROS can trigger cell death by many means, such as inducing lipid peroxidation and DNA damage. Previously, studies have shown that melatonin alone can also act as a pro-oxidant in a variety of cancer cell lines, in a dose-dependent manner (Estaras *et al.*, 2019; Laothong *et al.*, 2015). As a result, we sought to determine if the co-administration of melatonin and antineoplastics could induce the overproduction of ROS in BeWo cells, beyond that of what the antineoplastic alone can. If so, this would provide a first hint as to a synergistic effect of melatonin with antineoplastics. Unpublished data from this same lab indicated that *in vitro* administration of 1 mM melatonin alone suffices to induce oxidative stress (Kharrat, 2020). Then, understanding that the combination of the hormone with antineoplastics may potentiate this effect, we tested both 1 mM melatonin as well as a lower dose: 1 µM. The progression of ROS induction was monitored at 24h, 48h and 72h.

At 24h, we found that neither 1 µM nor 1 mM melatonin was able to significantly induce the overproduction of ROS. The average production of ROS in response to 1 µM melatonin/antineoplastic resembled that of the antineoplastic alone, and the results exhibited low variability. As such, we attributed the lack of statistically significant ROS overproduction to imply that this dose does not suffice to induce oxidative stress at 24h. Conversely, the average ROS levels in response to the co-administration of 1 mM melatonin and antineoplastic was higher than with treatment with the antineoplastic alone but exhibited high degrees of variability. We thus stipulated that perhaps this dose of melatonin began to induce ROS production, but that 24h did not allow enough time to produce a consistently observable effect.

At 48h, the co-administration of 1 mM, but not 1 µM melatonin, induced an increase in ROS production relative to all antineoplastics. The lack of visible overproduction of ROS in response to 1 µM melatonin continues to imply that the dose does not suffice to induce oxidative stress. As previously mentioned, the induction of ROS in BeWo cells in response to 1 mM melatonin alone was previously suggested by unpublished data from this same lab (Kharrat, 2020). Thus, it is unsurprising that the same trend is observed when the same

dose is co-administered with ROS-inducing antineoplastics. Florido *et al.* (2022) obtained similar results in that melatonin-induced ROS production began to be apparent at 48h, albeit when combined with a nicotinic stimulant rather than antineoplastics, and in different cell lines. Melatonin, however, has a quick distribution and a short half-life, suggesting that the hormone is fast-acting (Tordjman *et al.*, 2017). This is supported by various pharmacokinetic studies, in which its T_{max} (time to achieve maximal concentration *in vivo*) was found to range between minutes to a few hours depending on the administration route (Reviewed by Zetner *et al.* (2016)). As such, we sought to determine why the ROS-producing activity of 1 mM melatonin would take considerably longer, only observed at 48h and not earlier. Melatonin's pro-oxidant nature stems not from the binding to the MT1/MT2 receptors, but rather from its ability to bind CaM (Radogna *et al.*, 2009). Later, Liu *et al.* (2019) summarized the kinetic parameters of melatonin binding to various proteins, and outline that the binding of the hormone to CaM is much weaker (Kd > 2 mM) than to other proteins (Kd ranging between the nM and the µM range), in turn weaker than to its receptor (Kd = 0.1 nM). Since Lanoix *et al.* (2006a) have discovered the presence of MT1/MT2 receptors on BeWo cells, we propose that the initial dose of melatonin bound primarily to these receptors and partially to other proteins. Then, the second dose provided later would saturate melatonin's binding sites on other proteins, enabling the less-likely interaction with CaM. Consequently, this proposition (herein referred to as the binding site saturation theory) brought about the following interrogation: is the melatonin provided in the initial dose still present at intracellular levels? Gooneratne *et al.* (2012) reviewed the pharmacokinetics of melatonin, setting the half-life in the range of minutes to about an hour in biological systems while Daya *et al.* (2001) have shown that the stability of melatonin in aqueous solutions at 37ºC only decreases after 3 days. Since the hormone elimination capabilities of *in vitro* systems likely lie between that of complete biological systems and those of aqueous solutions, the exogenous hormone is likely to remain stably bound to various proteins from anywhere between a few minutes to 72 hours, rendering our proposition possible. Furthermore, Lanoix *et al.* (2008) have shown that BeWo cells demonstrate AANAT and HIOMT (important enzymes in the melatonin biosynthetic pathway) enzymatic activity similar to that of the pineal gland at night. Endogenous production of melatonin would then further enhance the likelihood of the hormone binding to CaM and support our theory. Yet, the lack of concrete evidence as to the retention of melatonin in *in vitro* cultured BeWo cells and its stability when bound to various proteins make this proposition speculative and would require further analysis.

Aft 96h post-exposure, the pro-oxidant effect of melatonin appears to dissipate when co-administered with 5-FU, ETO and MTX. This appears to support our earlier proposition, as the initial dose of melatonin (72h prior) is likely degrading, freeing binding sites to which newly added melatonin (endogenously produced or exogenous) can then bind without inducing ROS production. Yet, the overproduction of ROS retains statistical significance in cells treated with 1 mM melatonin and OXA. We noted that the average ROS production in the OXA/melatonin-treated group was lower at 72h than at 48h and thus may still support our earlier proposition. Melatonin may still bind CaM to induce ROS production but is less *likely* to do so when other binding sites are present, explaining the decrease in average ROS production.

The same observation is seen with PTX as with OXA. Of course, it is possible that the sustained ROS overproduction is the result of a continuous CaM/melatonin binding: a lower Kd simply means the interaction is weaker, not non-existent. Then, we could attribute the apparent increase in relative ROS production (from 183.80% ± 5.33% at 48h to 248.31% ± 22.28% at 96h, as seen in Figure 4.2) to sample variation. However, the involvement of CaM in both melatonin-mediated ROS production and in microtubule dynamics warrants a more in-depth explanation. Indeed, studies have outlined that CaM may interact with many microtubule-associated proteins (MAPs) (Bouvier *et al.*, 2003; Padilla *et al.*, 1990). Then, depending on the presence or absence of MAPs, CaM can inhibit or promote microtubule polymerization, respectively, and may potentiate or antagonize the effects of PTX (Lee & Wolff, 1982; Schiff *et al.*, 1979). Recently, data has suggested that exposure to PTX can lead to altered gene expression in cancer cells to attempt to induce resistance, as early as day 1 post-exposure (Koussounadis *et al.*, 2014; Sun *et al.*, 2014). It is possible, then, that PTX-treated BeWo cells quickly adapt by repressing CaM's activity or expression. Repression of the iPLA2/5-LOX system would then be relieved to induce ROS production. Stanislaus *et al.* (2012) demonstrate that, depending on the dose of PTX, CaM knockdown may substantiate the antineoplastic's cytotoxic effects, thus outlining a dependency between the CaM system and PTX, a notion that may support our theory.

All in all, then, we observed that whereas 1 μM melatonin does not induce ROS production when combined with antineoplastics, a 1 mM dose begins to have an effect at 48h, which tends to dissipate at later times. We speculate that these observations rely on the binding kinetics of melatonin to various ligands and requires a minimal dose in which

binding sites on these ligands must first be saturated, before binding to CaM, and subsequent induction of ROS is possible.

5.3 Co-treatment of 1 mM melatonin with 5-FU, ETO and OXA results in a reduction in BeWo cell viability

We have demonstrated that 1 mM melatonin, but not 1 μM, induces a significant and consistent overproduction of ROS at 48h post-exposure. Yet, depending on the levels of ROS produced, they may induce death or confer a survival advantage to cells. Thus, whereas melatonin's ability to induce ROS overproduction hints at the possibility that the hormone may induce cell death, making such a determination requires further analysis. To that effect, we performed a flow cytometry analysis using A5/FITC and PI staining (Figure 4.3). Considering that 1 μM melatonin did not influence ROS production, we chose instead to focus on the administration of 1 mM of the hormone, at 48h of exposure (the timeframe from which the IC50 was derived).

Using this technique, we determined that 1 mM melatonin, when combined with 5-FU, ETO or OXA significantly reduced the proportion of viable cells. Then, although the average proportions of viable cells decreased with the co-administration of the hormone with MTX and PTX, this drop in cell viability was found to not be statistically significant (Figure 4.3A). As expected, the inverse trend was observed in terms of the proportions of apoptotic cells (Figure 4.3B). The flow cytometry technique used here is believed to enable the differentiation between cells in early and late apoptosis. From Figure 4.3C, we observed that none of the treatments considerably induced cells into early apoptosis, instead driving them into late apoptosis (Figure 4.3D). Apoptosis appears to be, at least in part, the mechanism through which 5-FU, ETO and OXA induce cell death (Lamberti *et al.*, 2012; Ma *et al.*, 2021; Yoo *et al.*, 2012). Meanwhile, melatonin alone has been shown to induce this same type of cell death in BeWo cells specifically (Lanoix *et al.*, 2012; Sagrillo-Fagundes *et al.*, 2019). Other studies have demonstrated that the hormone induces the downregulation of Bax and MDM2, while upregulating Bcl-2 and have thus provided a causal link between treatment with melatonin and the induction of apoptosis (Mohseni *et al.*, 2012; Proietti *et al.*, 2014). Thus, it is likely that the combinatorial effect of the antineoplastic and melatonin provides a *double-apoptotic signal* which accelerates its execution. Conversely, ROS are known inducers of several signalling pathways, including apoptosis (Reviewed by Redza-Dutordoir

and Averill-Bates (2016)). We considered that perhaps melatonin was also inducing apoptotic pathways via the previously demonstrated generation of ROS. Such a mechanism would, however, fail to explain the lack of observable increase in cell death in the MTX and PTX-treated cells. Thus, although the ROS-mediated induction of apoptosis may be occurring, such a mechanism appears to be secondary.

MTX is also known to induce apoptosis in cancer cells (AlBasher *et al.*, 2019). As such, we would expect the double-apoptotic signal theory we have previously mentioned to hold true. However, no significant decrease in BeWo cell viability was observed (Figure 4.3A). We were tempted to attribute this observation to the hormone's well-characterized ability to induce DNA repair, but such an explanation failed to explain why the co-administration of melatonin with 5-FU, ETO, and OXA did induce a reduction in viability. A first clue as to a potential explanation was provided by the dose-response curves shown in Figure 4.1. Logically, we would expect the same dose of MTX to induce higher cytotoxicity upon 96h of exposure than at 48h. Graphically, however, we observe that an MTX exposure in the range of ~ 100 µM suffices to induce a 50% reduction in BeWo cell viability at both time frames. Thus, there appears to be some inconsistency in MTX's cytotoxic effect. As previously discussed, Taylor *et al.* (1991) demonstrated that MTX, under similar conditions than those presented here (lower micromolar range of exposure and for 48h), can induce trophoblast differentiation rather than cell death in BeWo cells. Furthermore, work from this same lab indicated that resistance to this antineoplastic can be quickly induced (data not shown), consolidating the knowledge that BeWo cells are inherently resistant to MTX (Friedman & Skehan, 1979). Therefore, we propose that (1) the bivalent effect of MTX on BeWo viability and (2) their predisposition to MTX resistance is concurrently being observed, skewing our observations. Of course, MTX's apparent inefficacy in killing BeWo cells may in itself explain our observations but would not explain why melatonin would not induce *further* cell death.

Understanding why melatonin would not further induce cell death when coupled to PTX could be explained by the involvement of CaM. Indeed, PTX induces the stabilization of microtubules to prevent the essential dynamicity of these structures throughout formation of the mitotic spindle (and prior), thereby inducing mitotic arrest (Kampan *et al.*, 2015; Schiff & Horwitz, 1980). Thus, treatment with PTX results in net polymerization of microtubules (Orr *et al.*, 2003; Parness & Horwitz, 1981). On the other hand, while Huerto-Delgadillo *et al.* (1994) demonstrated that melatonin, similarly to PTX, can promote microtubule

polymerization, they also show that higher concentrations (micromolar range), it may also induce depolymerization in the absence of CaM by binding to the tubulin polymer. Then, PTX may induce differential gene expression, particularly via the activation of c-Jun N-terminal kinases (JNK) (Lee *et al.*, 1998). However, a direct involvement of PTX in expression of CaM has yet to be established. Instead, CaM-dependent systems have been shown to enhance JNK-mediated transcription (Enslen *et al.*, 1996). As such, although the possibility that PTX inhibits CaM expression cannot de disproved, it becomes unlikely. Thus, we extend our binding site saturation theory (outlined in section 5.2) to explain our observations. We propose that 1 mM melatonin largely suffices to also saturate its binding sites on CaM present in the cellular environment, essentially mimicking the absence of CaM. The unbound melatonin fraction could then perhaps induce depolymerizing and directly counter the stabilizing effect of the antineoplastic, resulting in a reversal to *normal* dynamicity.

Deriving possible explanations from our results requires that we apply due criticism and skepticism. Based on our assessment of cell viability via MTT assay (sections 4.1 and 5.1), we were expecting to observe ~50% viability with the flow cytometry analysis in the cells treated with the antineoplastic alone. This was not the case. Instead, we obtained viabilities ranging from 30% to 70% (representative data shown in Figure 4.3A), which led us to question our results. The MTT assay (described in section 3.2) relies on the reduction of MTT by mitochondrial enzymes, and the resulting formation of formazan can be assessed using colorimetric methods. Thus, the assay ultimately monitors for mitochondrial activity as an indirect indication of viability as proper mitochondrial function is critical to cells, and its dysfunction is involved in many forms of cell death (Crompton, 1999; Karch & Molkentin, 2015). However, it has been demonstrated that cytosolic enzymes such as GST may also reduce MTT (York *et al.*, 1998). GST is involved in ROS homeostasis and can be upregulated in response to these species (Tew & Townsend, 2012). Thus, while antineoplastics seek to induce toxicity by raising ROS levels, cells may respond by upregulating GST, which in turn leads to increased production of formazan. Assay measurements would then likely overestimate the proportions of viable cells. Conversely, Takahashi *et al.* (2002) describe how certain substrates in the culture media may reduce the rate of MTT reduction, without correlating to a decrease in cell viability. Thus, while the MTT assay *approximates* cell viability, many factors may skew the results, explaining the disparity between this method and that of flow cytometry (Ghasemi *et al.*, 2021). The flow cytometry

technique used here relies on the specific binding of Annexin 5 and PI to phosphatidylserine (PS) and DNA, respectively, and offers a more robust alternative to measurements of cell viability (Crowley *et al.*, 2016; Rosenbaum *et al.*, 2011). Thus, we are confident that the viability, as measured by flow cytometry, is reliable and accurate.

5.4 Melatonin (1 mM) inconsistently inhibits antineoplastic-driven DNA fragmentation in BeWo cells, depending on the agent

Having verified that melatonin may induce cancer cell death when co-administered with some antineoplastics, we next sought to determine if this apparent cytotoxic predisposition is dependent on the mechanism of action of each antineoplastic. 5-FU, ETO, MTX and OXA all induce, in various ways, DNA damage (from section 1.4.2.1, 1.4.4.1, 1.4.2.2, 1.4.1.1, respectively). Thus, the integrity of the DNA was assessed using DNA gel electrophoresis, during which we ran 2% agarose gels.

We begin by discussing the results that occurred in most cases across replicates, understanding that these should be analyzed cautiously given their variability. We first observed that treated with every antineoplastics tested here (5-FU, ETO, MTX and OXA) resulted in a smear in their respective lane. This demonstrated that DNA fragments of many sizes were present within treated BeWo cells, indicative of genetic fragmentation. This was expected as all these agents are known inducers of DNA damage as part of their cytotoxic mechanism. For the most part, co-administration of 5-FU with 1 mM melatonin in BeWo cells resulted in a band in the high molecular weight range exhibiting higher fluorescence, coupled with a weaker signal in the low molecular weight range. The same trend was observed in cells treated with OXA and melatonin. From the works of Dong *et al.* (2020), the *genoprotective* role of the hormone appears to be receptor-dependent, while Lanoix *et al.* (2006b) had already demonstrated the expression of these receptors in BeWo cells specifically. Thus, our observation that melatonin appears to reduce the degree of DNA fragmentation is not unexpected. Next, we consider the MTX-treated group. There, the addition of 1 mM melatonin did not appear to affect DNA fragmentation. We attribute this to this cell line's inherit resistance against MTX. Indeed, BeWo cells have been shown to have decreased MTX import and higher DHFR levels, two events associated to resistance to the antineoplastic (Kaiho *et al.*, 1983). Thus, MTX likely is limited in its ability to induce DNA fragmentation. In the absence of DNA damage, melatonin is essentially left without any DNA

to repair, or at least to a degree not detectable by our method. On the hand, addition of melatonin with ETO appeared to induce more DNA fragmentation than treatment with the antineoplastic alone. Although ETO inhibits the re-ligation activity of topoII (which would presumably induce DSBs, from section 1.4.4.1), the antineoplastic predominantly induces SSBs, only inducing DSBs in ~3% of cases (Muslimović *et al.*, 2009). Although studies explicitly quantifying the proportions of SSBs and DSBs induced by 5-FU and OXA lack, both agents are generally implied to induce both SSBs and DSBs (Chiu *et al.*, 2009; Faivre *et al.*, 2003; Kunz *et al.*, 2009; Van der Wilt *et al.*, 1992; Van Triest *et al.*, 2000). As such, the incidence of SSBs seems to be driving the unresponsiveness to melatonin in ETO-treated BeWo cells. This would explain the variability of the results: melatonin would have failed to induce DNA repair in replicates where 5-FU or OXA-induced SSBs predominated and would have been successful in replicates where these same agents induced more DSBs. The hormone, however, appears to have an involvement in the induction of the DDR in response to both types of lesions (Reviewed by Majidinia *et al.* (2017)). Thus, it remains unclear why melatonin failed to induce DNA repair in ETO-treated cells. Our results become even more perplexing when considering that Chen *et al.* (2018a) demonstrated that the hormone does indeed decrease the DNA repair capabilities, but via the inhibition of Double Strand Break Repair Protein RAD51 (RAD51), itself involved in DSBs repair. Thus, we fail to provide a clear reasoning for our observations based on current, published knowledge of the topic.

Thus, we, of course, questioned the validity of the results given the inconsistencies in the fluorescence patterns observed across replicates. We noted that the inconsistency was not specific to a single drug, nor was it specific to a given replicate. Thus, this provided a first hint as to the fact that the irregularity of the *genoprotective* role of melatonin was indeed reflective of its true efficacy in biological systems. Melatonin is a known inducer of many molecules of the DDR (Reviewed by Majidinia *et al.* (2017)). Similarly, Khan *et al.* (2015) demonstrated that melatonin, in breast cancer cells, can reduce the expression ATM, and that this effect in turn inhibits the expression of pro-apoptotic proteins. Yet, ATM is also heavily involved in the induction of the DDR (from section 1.3.1). Thus, it is plausible that the administration of melatonin may inhibit ATM expression, thereby inducing either (1) inhibition of apoptosis, or (2) inhibition of the DDR (which would ultimately induce apoptosis), a paradoxical effect perhaps dependent on the cellular environment. The same paradoxical properties of the hormone are also observed in its ability to induce (or prevent) oxidative

stress (from sections 1.7.4 and 1.7.5) and cell death (from Sagrillo-Fagundes *et al.* (2019)). Studies on the requirements for melatonin to induce one effect or the opposing one largely lack. As such, it is difficult to ascertain whether the intracellular environment of BeWo cells treated with the antineoplastics discussed here reliably satisfy the requirements of melatonin's ability to induce DNA repair. Furthermore, it has been shown that melatonin can induce the phosphorylation of H2AX (an event required for the induction of the DDR in response to certain lesions), an event that is, at least in part, dependent on CaM expression (Santoro *et al.*, 2013a; Smallwood *et al.*, 2009). While melatonin can interact with CaM (from section 1.7.7), whether this interaction promotes or inhibits H2AX phosphorylation has not yet been investigated. Thus, a lot remains to be elucidated as to the hormone's involvement in various pathways, making our seemingly inconsistent results still potentially representative of its true efficacy.

5.5 Melatonin (1 mM) reverts PTX-driven alterations of mitotic spindle proportions and phenotypes in BeWo cells

Unlike the other antineoplastics tested in this study, PTX's cytotoxic effects are mediated by its ability to stabilize microtubules and induce mitotic arrest and apoptosis (Kampan *et al.*, 2015; Schiff & Horwitz, 1980). Thus, to assess the combinatorial effect of 1 mM melatonin with PTX, the intracellular components (DNA, microtubules, and F-actin) of treated cells we stained using immunocytochemistry.

During mitosis, actin filaments are involved in cell rounding and cortical stiffening (Kunda & Baum, 2009). Indeed, using live-cell imaging, Mitsushima *et al.* (2010) show that the actin filaments of mitotic cells primarily localize to the cortical region, while a subset clusters and revolves around the cells. Then, the spherical shape of mitotic cells is believed to be essential to proper mitotic spindle dynamics, suggesting cross-talk between the actin and the actin cytoskeletons (Rizzelli *et al.*, 2020). The dependence of the microtubule organization on actin filaments thus outlines the relevance of assessing the later structure in this study. From Figure 4.5A, we observe that the actin filaments localize to the cortical surface spherically (as expected), independently of subjected treatment (± antineoplastic ± melatonin). In line with our results, PTX reportedly does not induce any observable effect on the organisation of F-actin (Tsai *et al.*, 1998). Meanwhile, the effect of melatonin on actin is largely attributed to its ability to restore *normal* dynamics in dysfunctional cells (Tan *et al.*,

2015). Thus, since PTX has no observable effect on actin filaments, we propose that although the regulatory effects of melatonin on said structure may still be at play, the effect of the hormone may simply not be distinguishable as it further promotes *normal* organization, in cells already exhibiting *normality.*

Next, we assessed the effect of PTX ± melatonin on the microtubule components of the cytoskeleton. Qualitative analysis revealed that BeWo cells tended to aggregate in clusters of varying sizes, a property also revealed by Benaitreau *et al.* (2010). Considering that the ICC technique executed here (section 3.6) did not provide markers for cell membranes, the delimitations of each individual cell could not have been determined accurately. As such, rather of performing a fluorescence-based quantitative analysis, we instead chose to assess the proportions of cells in metaphase. To that effect, the number of mitotic spindles (relative to the total number of nuclei) was determined (Figure 4.6A). Although the presence of a spindle does not directly imply that cells are arrested in mitosis, the assumption here is that the total number of cells in metaphase, at any given timepoint, is proportional to the number of cells *truly* arrested in metaphase. We found that the proportions of cells in which mitotic spindles could be identified significantly decreased in response to PTX (56.53% ± 15.22%; $p \leq 0.01$) relative to the non-treated group (set to 100%) and was restored in the PTX/melatonin-treated group (98.16% ± 47.60%; $p > 0.05$). The decrease in mitotic spindle counts could imply that the PTX-treated cells do not progress into metaphase, instead arrested in earlier phases of mitosis. This is corroborated by an earlier study by Choi and Yoo (2012) who indicate that PTX induces G2/M arrest and thus prior to the formation of the spindles. Why then, would melatonin restore the proportions of mitotic spindles? Zhai *et al.* (1996) demonstrate that the physiological changes during the G2/M transitions involve an abrupt decrease, then increase, in the levels of polymerized microtubules. As such, the transition is itself dependent on the microtubules' depolymerization capabilities, which are hindered by PTX, and restored by melatonin (from our earlier proposition in section 5.3), explaining our findings.

Yet, some cells treated with PTX may still progress into the M phase, in which case cells can be arrested in metaphase, but the concomitant formation of multipolar spindles with improper chromosome alignment (Choi & Yoo, 2012; Yvon *et al.*, 1999). Thus, we also verified the phenotype of the mitotic spindles (bipolar vs multipolar) in response to each treatment, normalized to the number of total spindles observed (Figure 4.6B). Of note, the occurrence of monopolar spindles was essentially ignored as making the distinction between

truly monopolar spindles and spindles oriented along the vertical axis (one visible pole masking the other one behind) would have been somewhat arbitrary. We found that the proportions of cells exhibiting a multipolar phenotype was significantly increased in response to PTX alone (30.56% ± 4.81%; $p \leq 0.01$) relative to the no treatment control (9.79% ± 2.29%). These proportions were, once again, restored by the co-administration of PTX with 1 mM melatonin. PTX is believed to induce spindle multipolarity by lowering the concentration of tubulin required to induce polymer formation (denoted as the critical concentration) (De Brabander *et al.*, 1981; Hornick *et al.*, 2008). Indeed, a net polymerization relies on greater microtubule rescue (elongation) than catastrophe (shortening) (Reviewed by Gardner *et al.* (2013)). PTX stabilizes microtubules, thereby preventing their shortening, resulting in net polymerization, even at lower tubulin concentrations. Once again, for the same reasons as proposed in section 5.3, we would expect melatonin counter the effect of PTX and restore proper spindle phenotypes, as is observed here. Although a clear link between melatonin and microtubule depolymerization has yet to be provided, Huerto-Delgadillo *et al.* (1994) imply this observation to be dependent on the interaction (or lack thereof) of the hormone with CaM.

5.6 Melatonin (1 mM) continues to exhibit potential as an adjuvant to 5-FU and ETO in a solid tumor model of BeWo cells

We have so far provided evidence that 1 mM has potential as an adjuvant to some antineoplastics, particularly when co-administered with 5-FU, ETO and OXA. Earlier models employed in this study allowed for 24h of recovery before dosing the cells with antineoplastics with and without melatonin (see materials and methods, section 3.1), ensuring a monolayer of BeWo cells was formed. This model, however, largely ignores important components of a solid tumour, where nutrient and drug accessibility within the core of the tumor become important considerations. To address this limitation, we used the XCELLigence apparatus, which measures the conductivity of electricity across wells and integrates the data into a measure of viability: a greater number of viable adherent cells will induce more electrical resistance (as stated in the manufacturer's protocol). To that effect, cells were allowed to grow to over confluence (for 3-4 days) and viability was assessed in real time. In such a way, cells were more likely to form multilayered cultures and a core in which both antineoplastic and melatonin have presumably limited accessibility.

Using this model, we observed that co-administration of 5-FU and ETO with 1 mM melatonin induced a significant decrease in cell viability with respect to the antineoplastic alone, starting at 70h and 80h, respectively, and from which the cells did not recover thereafter (Figure 4.7A and B, and Supplemental Table 7.1). These results suggest that melatonin may perhaps be used as an adjuvant in the treatment of *in vivo* placental choriocarcinomas, although further studies are required to make such a determination. In line with our results, the use of melatonin as an adjuvant to 5-FU and ETO has previously been shown to inhibit tumor progression and even allow to overcome their resistance in various tumors (Ao *et al.*, 2020; Chen *et al.*, 2018a; Liu *et al.*, 2021; Sakatani *et al.*, 2019). However, to our knowledge, this study provides novel insight as to the use of melatonin in combination with antineoplastics in placental choriocarcinomas specifically.

On the other hand, co-administration of 1 mM melatonin with MTX (Figure 4.7C) resulted in an initial, significant (from Supplemental Table 7.1), decrease in cell viability at 20h, followed by a period of recovery in which cell viability was not significantly lower than with treatment with the antineoplastic alone. Another significant, but transient decrease in cell viability was then observed at 50h and 60h post-treatment. These results are directly in line with the apparent inconsistency in the cytotoxicity of MTX, as observed in the dose-response curve (Figure 4.1) and further consolidate our earlier proposal wanting that several mechanisms, other than those inducing cytotoxicity, are concurrently being observed. Again, we refer to the study conducted by Taylor *et al.* (1991) who characterized MTX's ability to induce CTB fusion, which could explain the XCELLigence measurements as both (1) BeWo cell viability (as expected) and (2) the size of the resulting syncytium are likely to alter the electrical conductivity measured by the apparatus. Perhaps BeWo exposure to MTX induces apparent (inefficient) cytotoxic mechanisms at 20h, followed by the induction of fusional pathways at 50h and 60h, where a statistically significant decreases in cell viability are observed (Figure 4.7C and Supplemental Table 7.1). Alternatively, the cells may have developed some degree of resistance which could also explain the transient decrease in cell viability observed. Resistance to MTX is not uncommon in clinical settings (Georgiou *et al.*, 2022; Jun *et al.*, 2020).

In OXA (Figure 4.7D) and PTX-treated (Figure 4.7E) BeWo cells, no significant change in cell viability was observed for any timeframe. Previous analysis shown in section 5.3 suggested that melatonin synergizes with OXA's cytotoxic effects on BeWo cells cultured in a monolayer. Thus, in the multilayered model presented in here (data shown in Figure

4.7), it appears that the distribution of the drug within the core of the mass is a limiting factor to its toxicity. Yet, OXA is a relatively small and uncharged compound that readily crosses the cell membrane via passive diffusion and its transport can be further enhanced by many transporters (*e.g.* CTR1 and Organic Cation Transporters 1/2/3 (OCT1/2/3)) (Reviewed by Perego and Robert (2016)). While BeWo cells express some of these transporters, others (OCT1/2/3) have little to no expression in these cells (Klomp *et al.*, 2002; Sata *et al.*, 2005; Vachalova *et al.*, 2022; Zeng *et al.*, 2019). Yet, OCT3 has been shown to have a major involvement in the toxicity of OXA (Gu *et al.*, 2019). As such, the absence of some transporters may be limiting the drug's distribution, especially in this model where accessibility is limited. On the other hand, a lack of significant reduction in BeWo cell viability in the PTX/melatonin-treated group (relative to PTX alone) was to be expected considering the earlier lack of statistically significant change resulting from the flow cytometry analysis (Figure 4.3), and the opposing effect of the hormone on microtubule assembly, discussed in sections 5.3 and 5.5.

The observation that the co-administration of 1 mM melatonin with some antineoplastics, but not all, induces a significant decrease in cell viability suggests that the intratumoral penetration of melatonin is not the limiting factor of its cytotoxic tendency. Instead, since the ability of melatonin to induce cell death in this pseudo-solid tumor model is highly dependent on the antineoplastic to which it is combined, it appears as though the diffusion properties of the drugs themselves drive the cytotoxicity.

5.7 Study limitations

We have begun to characterize a potential role for melatonin as an adjuvant to antineoplastics in the treatment of human placental choriocarcinomas. *In vitro* assays using both monolayered and multilayered BeWo cells have shown that 1 mM melatonin exhibits potential as an adjuvant to certain antineoplastics, a potential that appears to be independent on the cytotoxic mechanism of the agents. Nonetheless, we understand the present study has its limitations, making the conclusions that can be drawn here far from sufficient to claim that the hormone *can* be used in a clinical setting, and simply suggesting that further studies are warranted.

Diversity of models. A major limitation we have identified is the use of a single model (*i.e.,* BeWo cells) for choriocarcinomas. First, as was previously mentioned, MTX is

largely inefficient against BeWo cells, but widespread and efficient in the treatment of placental choriocarcinomas *in vivo* (section 1.2.4.3) (Friedman & Skehan, 1979; Lurain & Elfstrand, 1995). Thus, BeWo cells may only be representative of a subset of this cancer type. Then, this cell line has long been considered a model for EVTs, which represents but a single cell type that may be found in this type of tumor. Common tumoral VT models include JEG-3 and JAr cells, which have been suggested to express at least one of the melatonin receptors, thus enabling the possibility of observing similar results (Lanoix *et al.*, 2006b; Shiu *et al.*, 1999). Yet, differences in delayed cell cycle progression between JEG-3 and JAr cells induced by the hormone indicates that the mere presence of these receptors does not suffice to guarantee its clinical efficacy (Shiu *et al.*, 2000). Furthermore, morphological and behavioural differences between these cell types, likely due to the altered expression of a variety of proteins (discussed in section 1.2.3.3 and reviewed by Hannan *et al.* (2010)), further highlight the need for considering other cell types, a need not addressed here.

Furthermore, the effect of the co-administration of melatonin with antineoplastics ought to be verified in healthy trophoblasts. Optimally, the use of melatonin as an adjuvant to chemotherapy would reduce the dose of antineoplastics required and limit the side effects associated with their *off-target* cytotoxicity (briefly discussed in section 1.5.1). *In vitro* and *in vivo* studies where melatonin was co-administered with a variety of antineoplastics have shown the hormone has cardioprotective, hepatoprotective and neuroprotective properties, amongst others (Reviewed by Ma *et al.* (2020)). More specific to the context of choriocarcinomas, the hormone alone has been shown to be cytoprotective to healthy trophoblasts, exhibiting antioxidant and anti-apoptotic properties (Lanoix *et al.*, 2013). However, studies on melatonin's protective role in healthy trophoblasts, when co-administered with chemotherapeutic agents, are largely lacking and such an analysis was not provided here.

Diversity of antineoplastics. In this study, we have chosen to address the effect of the co-administration of melatonin with various antineoplastics. In doing so, we focussed on the diversity of the cytotoxic mechanisms of these agents rather than on how common their use is in the treatment of placental choriocarcinomas in a clinical setting. Thus, whereas analyses regarding MTX (the standard treatment, as of now) were performed, none were performed using ActD, another commonly used antineoplastic in both single and multi-agent chemotherapy. Similarly, the combinatorial effect of melatonin with cyclophosphamide and

vincristine was not investigated, although they make up the EMA/CO regimen, by far the most employed treatment option in more severe cases of the cancer under scrutiny here. Therefore, although the current study addressed the potential use of melatonin as an adjuvant to antineoplastics against placental choriocarcinomas *in general,* the focus was not on currently employed treatment options, nor was an exhaustive analysis of all types of chemotherapeutic agents performed.

Technical limitations. Next, we interpreted our results flow cytometry results based on the current consensus that Annexin 5/PI staining indicates the proportions of apoptotic cells. Yet, understanding the reasoning behind the technique outlines that obtention of positive results may not only be driven by apoptotic cells. Indeed, while PI binds to DNA, it is impermeable to cell membranes and thus the fluorescence it produces is only indicative of the integrity of said membrane (Marsh, 2007). Conversely, Annexin 5, also membrane-impermeable, binds to PS found in the outer leaflet of the cell membrane (Reviewed by Demchenko (2013)). Thus, the technique intrinsically allows for the detection of (1) loss of membrane integrity, and (2) flipping of PS, events that are largely associated with apoptosis (Martin *et al.*, 1995). Yet, loss of membrane integrity is observed is also observed in ferroptosis, necrosis, pyroptosis and necroptosis, to name but a few (Pedrera *et al.*, 2021; Zhang *et al.*, 2018). Conversely, recent studies have shown that PS exposure on the outer portion of the cell membrane is also observed in these alternate forms of cell death (Brouckaert *et al.*, 2004; de Vasconcelos *et al.*, 2019; Gong *et al.*, 2017; Klöditz & Fadeel, 2019; Krysko *et al.*, 2004; Shlomovitz *et al.*, 2019). Thus, whereas we were somewhat skeptical of the specific *mechanism* of cell death this technique implied was at stake, we remained confident that it was at least indicative of cell viability.

Finally, we understood that the solid tumor model provided by the XCELLigence apparatus does not address many of the important conditions found in the tumor microenvironment. A major element of said environment is the presence (or lack thereof) of components of the immune system. PTX has been demonstrated to enhance the production of some cytokines (IL-1β and TNF-α) (Allen *et al.*, 1993). Thus, it is evident that the immune system plays a major role in tumour progression, a role overlooked here.

5.8 Future directions

Given the limitations discussed in section 5.7, we reiterate that the data presented in this study simply sets the stage for further studies. As such, subsequent analyses should begin by consolidating the results we have shown here, *in vitro.* In doing so, we plan on performing largely the same analyses to ensure that melatonin continues to exhibit potential as an adjuvant to chemotherapy in other cancerous cell lines, where altered gene expression may impact factors implicated in cancer cell viability: metabolic activity, invasiveness, and so on. Then, we plan on explicitly demonstrating the cytoprotective effects of melatonin on healthy trophoblasts, when combined with antineoplastics, hopefully showing that the side effects implicated in chemotherapeutic treatment may be dampened.

We will also seek to determine the optimal time of melatonin treatment relative to the administration of the antineoplastics. Indeed, in this study, we have focussed on the co-administration of melatonin with chemotherapeutic agents but have failed to address the potential beneficial effects of *pre*- and *post*-treatment with the hormone.

Having verified that melatonin may have synergistic properties with antineoplastic, it would be of primary importance to elucidate the pathway through which the hormone exhibits this effect such that it can be exploited in clinical settings. CaM appears to be heavily involved in both ROS production and cytoskeletal reorganization (Reviewed by Benítez-King (2006) and Zhang and Zhang (2014)). Considering that current knowledge of the signalling pathways regulated by CaM are largely in line with the results presented here (ROS production described in section 5.2 and microtubule organization described in section 5.3), the protein provides a first clue in elucidating melatonin's cytotoxic mechanism. Thus, although informative, the current study opens the door for further characterization of melatonin as an adjuvant to antineoplastics in the treatment of human placental choriocarcinomas.

6 CONCLUSION

Human placental choriocarcinoma is a rare, but highly aggressive type of tumour. As of now, these tumours are treated quite effectively with single or multi-agent chemotherapy. However, resistance to these agents has begun to emerge, thus outlining a need for novel treatment options. First using monolayered BeWo cell cultures, we have demonstrated that, *in vitro*, 1 mM melatonin stimulates the production of ROS in BeWo cells when co-administered with 5-FU, ETO, MTX, OXA and PTX. This increase in ROS production was concurrent with a decrease in cell viability in cells treated with the former three antineoplastics (but not the others), where it was found to correlate with a significantly enhanced progression through apoptosis. Thus, we proposed that whereas the chemotherapeutic agent provides a first pro-apoptotic signal, the hormone provides a second, further committing these cells to this mode of cell death. Since melatonin has pleiotropic and seemingly paradoxical effects in various cell types, we also assessed whether the apparent cytotoxic potential of the hormone is dependent on the cytotoxic mechanism of the antineoplastic to which it is coupled. We found that, in cases where the antineoplastics primarily aim to induce genotoxic lesions, melatonin (unreliably) induced the repair of said lesions, while also countering the microtubule-stabilizing activity of PTX. The involvement of the hormone in maintaining DNA and microtubule dynamics integrity did not correlate with its synergistic abilities (or lack thereof) with the chemotherapeutic agents studied here. This suggested that melatonin acted independently of the the agents' cytotoxic mechanisms. Given these data, we proposed that melatonin's ability to interact with CaM drives its synergistic role in the inhibition of cancer progression, given the latter protein's involvement in ROS production and microtubule dynamics. Finally, we employed a multi-layered culture model to mimic, to some degree, solid tumours, in which drug accessibility often limits its efficacy. We found that melatonin retained its synergistic potential when coupled to 5-FU and ETO, but not the other drugs, and thus proposed that the distribution of the agents themselves, not melatonin, limits the hormone's efficacy in inducing cancer cell death. Here, we provided a first hint as to the fact that, via its interaction with CaM, melatonin may be used as Adjuvant to *certain* chemotherapeutic agents in the treatment of human placental choriocarcinomas.

7 SUPPLEMENTAL FIGURES AND TABLES

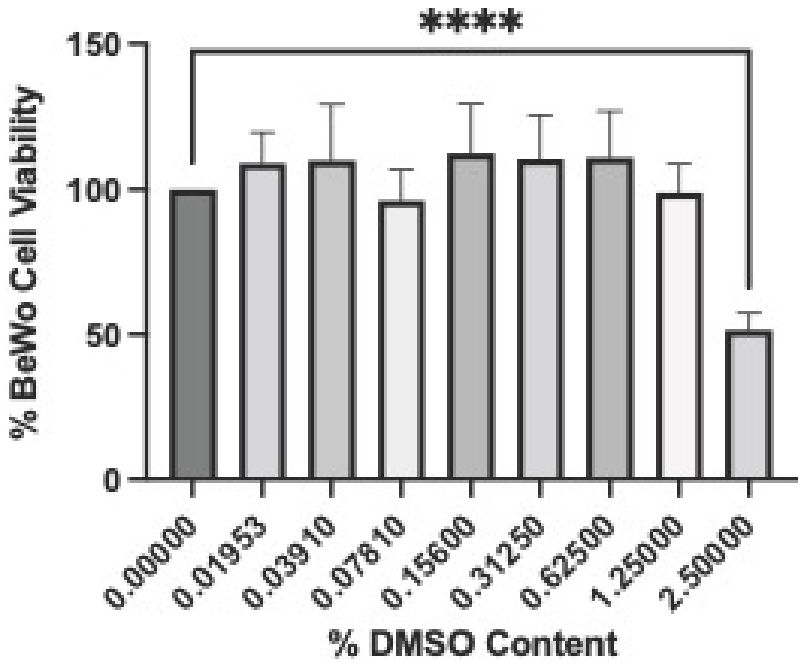


Figure 7.1: Effects of DMSO Content on BeWo Cell Viability

BeWo cells were grown in DMEM/F-12 media containing 10% FBS with DMSO contents ranging from 0.0% to 2.5%. Cell viability was measured using an MTT assay as previously described. BeWo cell viability was only found to be significantly reduced for a DMSO content of 2.5%.

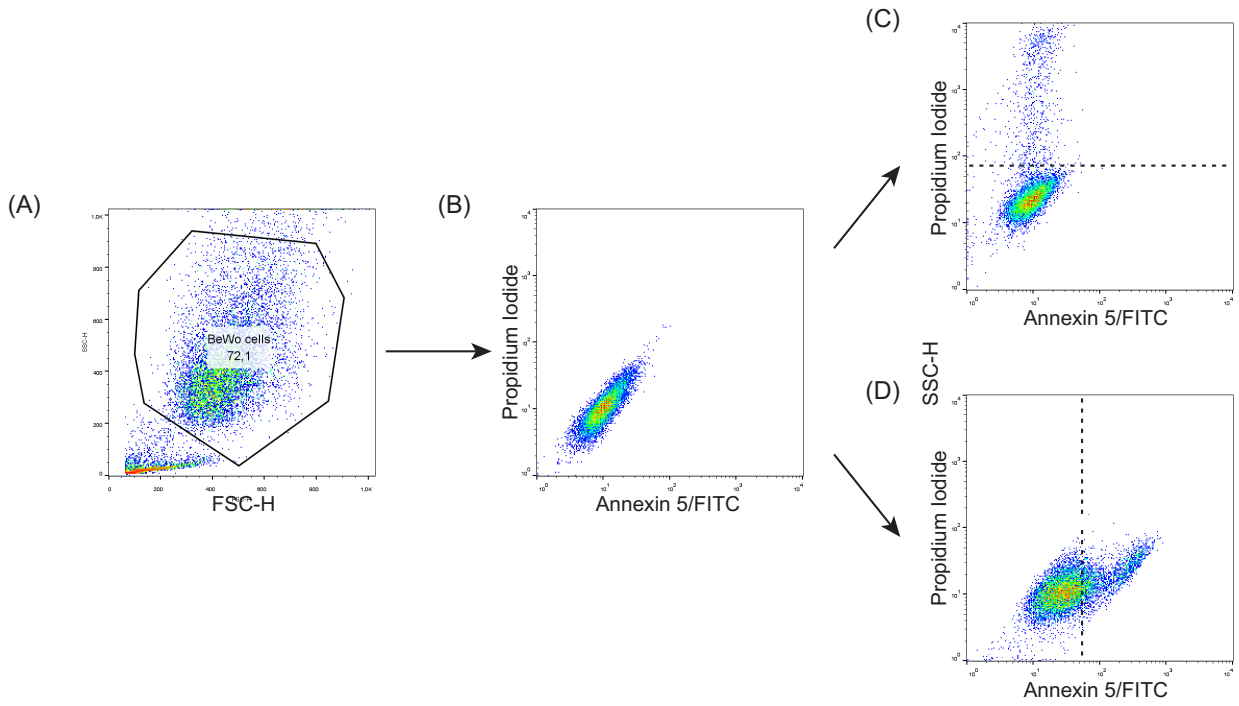


Figure 7.2: Flowchart of Gating and Controls Implemented in the Analysis of BeWo Cell Viability via Annexin-5/PI Fluorescence-Associated Cell Sorting

(A) Gating was performed first based on the side scatter (SSH) vs forward scatter (FSH) dot plot to remove constituents with aberrant granularity and size. (B) The fluorescence in the Annexin 5 and PI channels for the negative control (No treatment; NT) was then analyzed to set as the baseline value. (C-D) A positive control (Stauropsorine) was used to identify the increase in both Annexin-5 (C) and PI (D) caused by apoptosis and set the threshold between negative and positive signals. Dashed lines represent the thresholds.

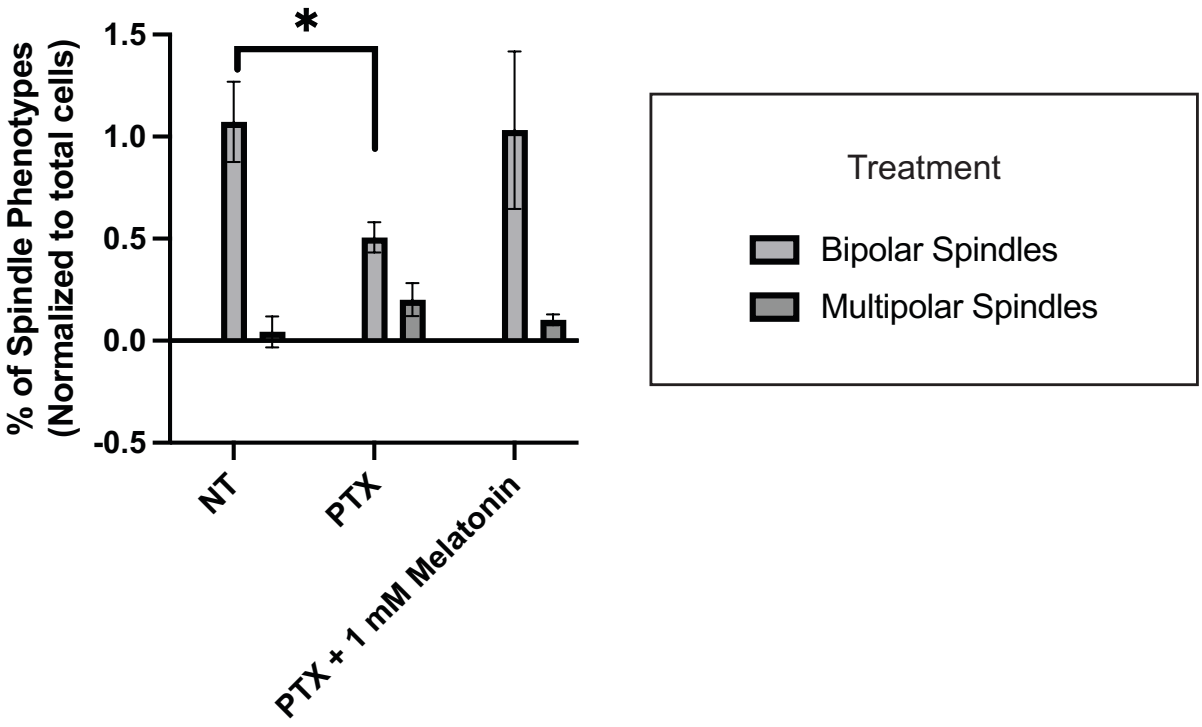


Figure 7.3: Melatonin (1 mM) Decreases the Proportions of Bipolar Spindles in BeWo Cells

BeWo cells were subjected to treatment with PTX ± 1 mM melatonin for 48h, and the proportions of bipolar and multipolar spindles were obtained via the ICC method described in section 3.6. The proportions shown here are relative to the total number of cells (exhibiting mitotic spindles, or not) counted per slide. A significant decrease in the proportions of bipolar spindles was observed in response to PTX, and normal spindle phenotypes were restored with the concurrent addition of 1 mM melatonin. No statistically significant change in multipolar spindle proportions were found, presumably due to their low occurrence overall.

Table 7.1: Melatonin (1 mM) Contributes to Increased Cell Death When Combined to Some Antineoplastics in a Multi-layered Solid Tumour Model.

	5-FU			Etoposide			MTX			Oxaliplatin			Paclitaxel		
Exposure Time	nCI_{avg}	SD	p-value	nCI_{avg}	SD	p-value	nCI_{avg}	SD	p-value	nCI_{avg}	SD	p-value	nCI_{avg}	SD	p-value
0	1.0000	0.0000	N/A	1.0000	0.0000	N/A	1.0000	0.0000	N/A	1.0000	0.0000	N/A	1.0000	0.0000	N/A
10	0.9007	0.0577	0.0802	0.9486	0.0614	0.5435	0.8768	0.0587	0.0391	0.8214	0.1003	0.1024	0.7971	0.1113	0.0928
20	0.895	0.0946	0.2515	0.9259	0.0559	0.207	0.9033	0.0719	0.1673	0.7307	0.2336	0.3129	0.6948	0.2383	0.2955
30	0.8279	0.1593	0.2515	1.0244	0.3179	0.9863	0.8602	0.1049	0.1673	0.7457	0.225	0.3129	0.6564	0.2935	0.3070
40	0.8111	0.1895	0.2515	1.0027	0.3412	0.988	0.8428	0.0869	0.0648	0.7136	0.2603	0.3129	0.6049	0.3644	0.3070
50	0.7252	0.1822	0.2515	0.9149	0.4322	0.9844	0.7781	0.0371	0.0039	0.6225	0.2556	0.3129	0.4498	0.3580	0.3070
60	0.6546	0.2248	0.2515	0.8048	0.3729	0.8836	0.7446	0.0363	0.0023	0.6346	0.2253	0.2933	0.4261	0.3920	0.3070
70	0.6291	0.1779	0.0404	0.6485	0.2442	0.1808	0.8152	0.1587	0.1673	0.6903	0.1999	0.1572	0.5106	0.4025	0.3070
80	0.5517	0.1597	0.0109	0.5047	0.1948	0.0179	0.8431	0.1381	0.1673	0.7135	0.2523	0.3129	0.5222	0.4193	0.3070
90	0.4629	0.1709	0.0068	0.2726	0.2349	0.0073	0.8299	0.196	0.1673	0.7593	0.3433	0.3129	0.5018	0.4100	0.3070

BeWo cells were grown until a CI value of at least 8.0 was obtained, as measured by the xCELLigence real-time viability apparatus. CI values for cells grown with the antineoplastic alone were normalized to 1.0 for all timeframes. Normalized CI values for BeWo cells treated with each antineoplastic in combination with 1 mM melatonin are shown. Statistical significance of cell indexes relative to treatment with the antineoplastic are shown in yellow ($p \leq 0.05$) and blue ($p \leq 0.01$). Abbreviations: 5-FU: 5-fluorouracil; MTX: methotrexate; nCI_{avg}: averaged normalized cell index; SD: standard deviation.

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