MECHANISMS OF 4-HYDROXYTAMOXIFEN-INDUCED APOPTOSIS IN RHABDOMYOSARCOMA CELLS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT

Rhabdomyosarcoma (RMS) is a malignant soft-tissue sarcoma in children, accounting for about 40% of pediatric soft-tissue tumours. Five-year survival for metastatic RMS is only about 25%. Furthermore, there has been no significant improvement in RMS survival since 1975, pointing to a need for improved therapy.

Previous work in our lab has shown that 4-hydroxytamoxifen (4OHT) leads to increased apoptosis and decreased viability in RMS cells. Expanding on this work, the current project aims to elucidate the mechanisms behind 4OHT-induced apoptosis in RMS cells, focusing on the roles of estrogen receptors (ER) and MAP kinases (MAPK).

We found that: 1) 4OHT-induced apoptotic signaling was associated with increased MAPK phosphorylation, 2) Inhibition of MAPK protected cells against 4OHT, 3) Inhibition of ER also protected against 4OHT, and 4) ER inhibition blocked 4OHT-associated MAPK phosphorylation.
ACKNOWLEDGEMENTS

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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>4OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>AEBS</td>
<td>microsomal antiestrogen binding site</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>ARMS</td>
<td>alveolar rhabdomyosarcoma</td>
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<tr>
<td>ASK</td>
<td>apoptotic signaling kinase</td>
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<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
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<tr>
<td>E2</td>
<td>17-β-estradiol</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>ERα</td>
<td>estrogen receptor α isoform</td>
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<td>ERβ</td>
<td>estrogen receptor β isoform</td>
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<td>ERE</td>
<td>estrogen response element</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERMS</td>
<td>embryonal rhabdomyosarcoma</td>
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<tr>
<td>FOXO1A</td>
<td>forkhead box O1A</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>MAP2K</td>
<td>mitogen-activated protein kinase kinase</td>
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<td>MAP3K</td>
<td>mitogen-activated protein kinase kinase kinase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<td>MLK</td>
<td>mixed lineage kinase</td>
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<td>MMP-9</td>
<td>matrix metalloproteinase-9</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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<tr>
<td>MNAR</td>
<td>modulator of nongenomic activity of estrogen receptor</td>
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<td>MTP</td>
<td>mitochondrial transmembrane potential</td>
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<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PAX3</td>
<td>paired box gene 3</td>
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<tr>
<td>PAX7</td>
<td>paired box gene 7</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RMS</td>
<td>rhabdomyosarcoma</td>
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<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
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<tr>
<td>TAM</td>
<td>tamoxifen</td>
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<td>TGFβ</td>
<td>transforming growth factor β</td>
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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Rhabdomyosarcoma

1.1.1 Overview

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma [1], with an annual incidence of 4.5 per million in children ages 19 and under [2]. It accounts for 40% of all soft tissue sarcomas [2] and 10% of all solid tumours [3, 4] in children. RMS is thought to arise from immature mesenchymal cells committed to the skeletal muscle lineage [1]. However, primary RMS can present almost anywhere in the body, even in tissues where skeletal muscle is not normally found [5-7]. The most common sites of occurrence are the head and neck (including orbital and parameningeal regions), genitourinary tract, and extremities [1].

Although most cases of RMS are thought to occur sporadically, various familial syndromes have been linked to RMS. These include Li-Fraumeni Syndrome, Costello syndrome, neurofibromatosis, and Beckwith-Wiedemann Syndrome [2]. Li-Fraumeni Syndrome is an autosomal dominant disorder associated with mutations in p53, leading to elevated risk of many early-onset cancers, including sarcomas, acute leukemia, pre-menopausal breast carcinoma, brain tumours, and adrenocortical carcinoma [8]. Neurofibromatosis is characterized by mutations in neurofibromin 1 (NF1) or 2 (NF2), both of which are tumour suppressors [9]. The NF1 mutation results in a 20-fold increased risk of RMS relative to the general population [2]. Costello syndrome, caused by mutations in HRAS, is characterized by mental and physical developmental defects, as well as increased risk of various malignancies (including rhabdomyosarcoma) [10]. Beckwith-Wiedemann
Syndrome, caused by genetic errors at chromosome region 11p15, is associated with overgrowth, abdominal wall defects, and increased risk of tumours such as rhabdomyosarcoma [11].

RMS is diagnosed based on histological examination by light or electron microscope, as well as immunohistochemical detection of muscle-specific protein markers (including actin, myosin heavy chain, desmin, myoglobin, Z-band protein and MyoD) [12].

Treatment modalities include chemotherapy, surgery, and radiation. Unfortunately, most RMS tumours occur in the head and neck region or the genitourinary tract, and large surgical resections in these sensitive sites are often not feasible [13]. The most common chemotherapy regimen for RMS is the “VAC” combination, comprised of vincristine (an inhibitor of microtubule polymerization), actinomycin D (a DNA intercalator), and cyclophosphamide (a DNA alkylator) [14]. Ifosfamide (a DNA alkylator) and etoposide (a topoisomerase II inhibitor) are also frequently used, as part of the “VAI” (vincristine, actinomycin D, and ifosfomide) and “VIE” (vincristine, ifosfomide, and etoposide) combinations [14]. Doxorubicin (a DNA intercalator) is also commonly used, often in combination with vincristine and cyclophosphamide [15, 16].

With conventional multimodal therapy, patients with non-metastatic RMS have five-year failure-free survival of approximately 75% [14]. However, for metastatic RMS patients (which comprise 15% of cases) this figure drops to 25% [17]. Even more discouraging is a recent study that showed little or no improvement in overall RMS survival from 1976 to 2000 [2].
Figure 1: Comparison of survival for non-metastatic and metastatic RMS. Although non-metastatic RMS has a decent outlook with five-year survival around 75% (with minor differences depending on the chemotherapy regime used), metastatic RMS yields a five-year survival rate of only 25%.

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Most cases of RMS fall into one of two subtypes: embryonal and alveolar. They can be distinguished at the molecular, genetic, and histological levels, and are associated with different sites of occurrence and clinical outcomes. There are also several rarer subtypes. The definitions of these other variants are more vague and controversial, with many different classification systems in use [18].

Figure 2: Recent trends (1976-2000) in survival of RMS patients. There is a small increase in 4-year survival over the past few decades, but the improvement is not consistent (with large fluctuations). Furthermore, the difference in survival between 1976-1980 and 1996-2000 is not statistically significant. Reprinted with permission from Reference #2.
1.1.2 Embryonal rhabdomyosarcoma

Embryonal rhabdomyosarcoma (ERMS) accounts for about two thirds of RMS cases [2]. It occurs most commonly in children during the first few years of life [2, 19], and tends to arise in the head and neck region [20]. ERMS is associated with a more favourable prognosis compared to alveolar RMS, with 10-year survival around 75% [13].

At a molecular level, ERMS is characterized by a loss of heterozygosity at chromosome locus 11p15.5 [21, 22]. The exact implications of this LOH are unclear, but the region contains several putative tumour suppressors, including GOK [23, 24], WT2 [25], and p57kip2 [25].

Loss of imprinting (LOI) is also observed at 11p15.5 [12, 26]. Interestingly, the pro-proliferative insulin-like growth factor II (IGF-II) gene is maternally imprinted (paternally expressed), while the putative tumour suppressor p57kip2 is imprinted in the other direction [22]. Therefore, LOH (resulting from loss of the maternal allele and duplication of the paternal allele) leads to overexpression of IGF-II but loss of p57kip2 expression, both of which are changes that are thought to possibly contribute to tumourigenesis.

1.1.3 Alveolar rhabdomyosarcoma

The other major RMS subtype is alveolar rhabdomyosarcoma (ARMS), named for its histological appearance, which bears some resemblance to pulmonary alveoli. ARMS accounts for about a quarter of RMS cases, and is distributed evenly from ages 0 to 19 [2]. It occurs more commonly in the extremities [20], and is associated with poorer clinical outcomes compared to ERMS, including increased incidence of metastasis and higher mortality [20]. 10-year survival for ARMS is only 20% (compared to 75% for ERMS) [13].
ARMS is usually associated with one of two chromosomal translocations, each fusing together two transcription factors. The most common one, t(2;13)(q35;q14), fuses the DNA-binding domain of paired box 3 (PAX3) with the transactivation domain of forkhead box O1A (FKHR or FOXO1A) [12, 20, 27]. The resulting fusion protein is a potent transcription factor that can disrupt the regulation of PTEN [28], VEGF [29], and IGF-I [30]. Less frequently found is t(1;3)(q36;q14), which fuses the DNA-binding domain of PAX7 with the transactivation domain of FKHR [31]. ARMS with t(1;13) translocations appear to be clinically less aggressive than those with t(2;13). Translocation-negative ARMS are also reported and may confer an intermediate biological behavior between the more common subtypes.
Figure 3: Proportion of RMS subtypes. A survey of 848 patients aged 0-19, with RMS diagnosed between 1975 and 2005. Data taken from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) Program (Reference #2).
1.2 Estrogen Signaling in Rhabdomyosarcoma

1.2.1 Estrogen and its receptors

Estrogen is a steroid hormone (in fact, a group of three related steroid hormones: \(17\beta\)-estradiol, estrone, and estriol), in the same class of compounds as progesterone, testosterone, glucocorticoids, and mineralocorticoids. Steroid hormones share the characteristic four-ring carbon skeleton of cholesterol, the lipid precursor from which they are derived. They span an wide range of biological functions, from metabolism and electrolyte homeostasis, to functions in the reproductive, immune, and nervous systems. However, their basic mode of action is similar: the hormone diffuses into a target cell, binds to a specific intracellular receptor (a member of the steroid hormone superfamily of receptors), and together the hormone-receptor complex acts as a transcription factor [32].

Estrogen has wide-ranging functions, including roles in reproduction (including the buildup of the luteal surge during the menstrual cycle), development of breast tissue [33, 34] and other secondary sexual characteristics [35], maintenance and protection of the cardiovascular system in both men and women [36, 37], and preservation of bone integrity in post-menopausal women [34, 38-40].
Figure 4: Cholesterol and the steroid hormones. Steroid hormones share in common the characteristic four-ring structure of cholesterol, the precursor from which they are biosynthesized. The estrogens (17β-estradiol, estrone, and estriol) are the major female sex hormones. Progestogens (such as progesterone) are primarily involved in pregnancy. Androgens (such as testosterone) are the major male sex hormones. Glucocorticoids (such as cortisol) are involved in glucose metabolism. Mineral corticoids or mineralocorticoids (such as aldosterone) are involved in electrolyte resorption and balance.
Estrogen signaling begins with the synthesis of estrogen in endocrine cells. From there, they travel through the bloodstream to target cells, enter by simple or facilitated diffusion, and bind to estrogen receptors (ER) in the cytoplasm or nucleus. At this point, estrogen signaling branches into two paths: genomic and nongenomic signaling.

In genomic signaling, the estrogen-bound receptor dimerizes and acts as a transcription factor for the target cell. The dimer binds to specific recognition sites on DNA known as estrogen response elements (ERE) [41-44], and recruits transcriptional coactivators or corepressors upon binding [43, 45, 46].

The nongenomic mechanism is a more recent model in which the estrogen-bound ER feeds directly into other signaling pathways, including the ERK, p38, Protein Kinase C, and PI3K/Akt pathways [47-50]. For example, the estrogen-ER complex can interact with Src and Sos [51-53] to activate the ERK pathway. Nongenomic signaling tends to occur more rapidly, and can produce responses within seconds or minutes [43]. Although ERs reside predominantly in the cytoplasm and nucleus, a very small proportion is found attached to the plasma membrane, which may further contribute to nongenomic ER signaling.

Although the genomic and nongenomic pathways described above involve estrogen as the inducing ligand, it should be noted that many estrogen-like molecules (including synthetic compounds such as tamoxifen) could also bind to the ER and produce genomic and nongenomic responses. More details will be discussed in Chapter 1.3 (“Selective Estrogen Receptor Modulators”).

Estrogen receptors are modular proteins comprised of several functional elements, including sites for DNA-binding, ligand-binding, coactivator-binding, and dimerization [42, 54, 55]. There are also two transcriptional activation elements: activation function 1 (AF-1),
which is ligand-independent, and activation function 2 (AF-2), which is ligand-dependent [42, 56, 57]. Although AF-1 provides some transactivation function to ER that is not bound to ligand, full activity is only achieved by the synergistic combination of both AF-1 and AF-2 in a ligand-bound ER [42, 57].

There are two known ER isoforms in humans: estrogen receptors α (ERα or ESR1) and β (ERβ or ESR2), cloned in 1985 [58] and 1996 respectively [59, 60]. Both ERs share the basic functional elements described previously. Sequence similarities between ERα and ERβ [42, 60] in the DNA-binding, ligand-binding, and dimerization regions enable both receptors to recognize similar sites on DNA [42, 61], to bind most ligands with somewhat comparable affinities [62], and to heterodimerize with one another [42, 63]. The remaining regions are more distinct [42, 60], and as a whole ERα and ERβ often elicit different biological responses [64, 65].

More recently a membrane-bound estrogen receptor, structurally unrelated to ERα or ERβ, have garnered increasing attention. First cloned in 1996 [66], GPR30 is a G protein-coupled receptor (GPCR) shown to bind 17β-estradiol, tamoxifen, fulvestrant, and a number of other classical ER ligands [67]. It has been shown to activate several membrane-initiated signaling pathways, including ERK, cAMP, and PI3K [67]. However, much less is known about this newer estrogen receptor compared to the “classical” receptors ERα and ERβ.

1.2.2 Linking estrogen to rhabdomyosarcoma

Although there is a wealth of research on paracrine and autocrine signaling in RMS, including pathways involving IGF [22, 68-71], VEGF [72-74], and EGF [75-78], endocrine
signaling is a newer area of exploration. However, there are several clues that point to estrogen signaling as a potentially important player in the biology of RMS.

RMS incidence rate shows a bimodal distribution with respect to age of onset, peaking shortly after birth and during puberty [2]. It is intriguing to consider that both peaks correspond to periods of prolonged estrogen exposure.

Another clue comes from work on desmoid tumors, a rare non-metastatic but locally aggressive fibrous tumour. 80% of desmoid tumour patients are women, mostly in their childbearing years (ages 20 to 40) [79]. Therefore, it had been speculated that elevated hormone levels might be involved in desmoid tumourigenesis. Although the mechanisms are still unclear, many studies have shown that tamoxifen (a modulator of estrogen receptors) leads to reduced size and local invasiveness of desmoids [80-82]. Today, tamoxifen is a routine treatment option for desmoid tumors [83-85]. Given the success of tamoxifen in desmoid tumours, our lab had previously postulated that it might be worthwhile to explore the effects of this agent in related tumours of similar ontogeny, such as sarcomas [4].

There have been isolated case studies that suggest a possible connection between estrogen and RMS [86, 87]. More recently, experimental results from our group have pointed in the same direction [4]. It was shown that RMS primary tumours and cell lines express ERβ (though not ERα). In RMS cell lines, 17-β-estradiol (E2) stimulates cell proliferation, while 4-hydroxytamoxifen (an active metabolite of tamoxifen) inhibits cell proliferation and activates apoptotic signaling. Furthermore, removal of steroids from the culture medium sensitizes the cells to 4-hydroxytamoxifen, and supplementing E2 back into the medium partially reverses this effect.
Very little is understood about endocrine signaling in RMS, and given the possible links between estrogen and RMS, as well as the recent evidence pointing to the efficacy of 4-hydroxytamoxifen in RMS cell lines, it may be worthwhile to explore estrogen signaling as a potential therapeutic target in RMS. In this study, we explore the mechanisms behind 4-hydroxytamoxifen-induced apoptosis in RMS cell lines.

1.3 Selective Estrogen Receptor Modulators

1.3.1 Overview

Classically, most ER modulators can be considered agonists or antagonists, depending on their activity relative to that of 17-β-estradiol (E2). Agonists (such as diethylstilbestrol) mimic E2 by activating the ER in the same manner as E2. On the other hand, antagonists (such as fulvestrant) produce no biological response; they have affinity for the receptor, but no efficacy.

Over time, it became clear that this classic model is oversimplified. Many ER modulators were found to have mixed activity. For example, tamoxifen (TAM), initially classified as an antagonist or antiestrogen, actually exhibits both agonistic and antagonistic properties. It acts as an antagonist in the breast, but an agonist in the uterus and bone [88, 89]. These “mixed-activity” compounds are known as selective estrogen receptor modulators (SERM).

A key explanation for this mixed activity involves the differential expression of transcriptional coregulators in various tissues. Recall that in the genomic model of ER signaling, the dimerized complex binds to DNA and recruits coactivators or corepressors to regulate transcription. These coregulators are expressed at different levels and proportions in different tissues. SERMs may take advantage of these tissue-specific properties to produce
tissue-specific responses [90, 91]. For example, TAM acts as an antagonist in the breast by recruiting corepressors (preferentially over coactivators) to the DNA, thus blocking transcription of target genes. However, in the uterus (where TAM acts as an agonist), there is a higher concentration of the coactivator Src-1 [90]. The local abundance of coactivators effectively overwheels the corepressors, allowing TAM to function as an agonist in this environment.

Apart from this agonist-antagonist dichotomy, there is a further level of complexity in this system. Instead of blocking the E2 pathway (like an antagonist) or retracing the same pathway followed by E2 (like an agonist), SERMs can also redirect ERs into new genomic pathways [89, 92]. For example, TAM has been shown to induce expression of maspin (a tumor suppressor downregulated in many breast tumors) by binding onto ERα and following a genomic pathway [93]. E2 itself, however, does not regulate maspin expression. In this case, TAM is neither mimicking E2 nor opposing it, but rather, initiating an entirely new pathway of its own.

Furthermore, ERα and ERβ often produce different responses to the same ligand. For example, TAM is a partial agonist for ERα, but an antagonist (usually) for ERβ [54, 65, 94-97]. Therefore, differential expression of ERα and ERβ in different tissues adds further complexity to the mixed properties of SERMs.
Figure 5: Synthetic estrogen receptor modulators. Traditionally most estrogen receptor modulators were classified as either agonists (such as diethylstilbestrol) or antagonists (such as fulvestrant). However, many compounds were found to have mixed agonistic/antagonistic activity depending on the tissue. These are known as selective estrogen receptor modulators (SERM). Tamoxifen (and its metabolite 4-hydroxytamoxifen) is a first-generation SERM used primarily to treat and prevent breast cancer. Raloxifene, a second-generation SERM, is used to prevent breast cancer and osteoporosis. Lasofoxifene is a third-generation SERM used to prevent and treat osteoporosis.
Prior to the development of SERMs, endocrine therapy faced a long-standing dilemma: the benefits of estrogen supplementation (such as cardioprotection and preservation of bone mineral density) must be balanced against the drawbacks (such as increased risk for breast and uterine cancer). Therefore, a good ER modulator should work in opposing ways—namely, to mimic estrogen in the cardiac vasculature and bone, but not in the breast and uterus. The advent of SERMs opened up the possibility of new compounds that might offer such specific activity profiles.

Since the development of the first generation of SERMs like TAM, newer waves of agents have emerged. Second-generation SERMs (such as raloxifene, used for prevention and treatment of osteoporosis, and prevention of breast cancer) were designed to improve upon TAM by minimizing its associated risk of uterine cancer [98]. These first two generations of SERMs were mostly developed by modifications on a common triphenyl structure. The third generation (and beyond) of SERM development has expanded beyond this structural template, producing entirely different compounds with further refined tissue-specific activity profiles [98]. For example, lasofoxifene is a third-generation SERM with highly agonistic activity in the bone, but is antagonistic in both the breast and uterus, making it suitable for prevention and treatment of osteoporosis [99].

1.3.2 Tamoxifen

The story of tamoxifen began as an attempt to create a “morning-after” pill, following the discovery of several triphenyl compounds with contraceptive effects in laboratory animals [100, 101]. First synthesized in 1962, tamoxifen (initially known as ICI46474) was a failed
contraceptive (in fact, it was slightly profertile) recycled into one of the most widely used anti-cancer agents [101].

Today, apart from its use for the treatment and prevention of breast cancer [102], TAM is also used to treat a number of other malignancies, including desmoid [84], hepatocellular [103, 104], ovarian [105, 106], pancreatic [107, 108], and renal cell carcinomas [109, 110], as well as melanoma and glioma [111]. As a beneficial side effect, TAM helps prevent osteoporosis in post-menopausal women, evidenced by decreased incidence of osteoporotic fractures [112] and increased bone mineral density [113]. TAM may also provide some protection against coronary vascular disease by lowering cholesterol [114]. Negative side effects of TAM include elevated risk of uterine cancer, thrombosis, stroke, and cataracts [115, 116].

TAM has a prolonged half-life, which offers an advantage against late-stage tumours [102]. For example, TAM and raloxifene are structurally related, and equally effective in preventing breast cancer [117], yet TAM is superior to raloxifene in its ability to combat the tumour itself [102]. With a much shorter half-life, raloxifene is rapidly cleared from the body. Therefore, although raloxifene may have some efficacy in cancer prevention, it does not accumulate in sufficient concentrations to overcome late-stage breast tumours [102].

In the body, TAM is converted (mainly by liver enzymes) into several active metabolites, including 4-hydroxytamoxifen, desmethyltamoxifen, α-hydroxytamoxifen, and tamoxifen N-oxide [118, 119]. 4-hydroxytamoxifen is among the most commonly studied, and exhibits affinities up to 100 times stronger than TAM itself [119].
1.4 Mechanisms of Tamoxifen and 4-Hydroxytamoxifen

Tamoxifen (TAM) and 4-hydroxytamoxifen (4OHT) have been associated with a number of molecular targets. The following is an overview of some potential mechanisms proposed for TAM or 4OHT. These include direct binding targets (such as ERs, AEBS, and PKC), as well as downstream targets (such as MAPKs, TGF-β, and survivin). Some of these may be linked in the same pathway, whereas others belong to separate independent pathways.

1.4.1 Estrogen receptors

TAM was designed to modulate estrogen activity. Not surprisingly, estrogen receptors (ER) have been studied intensely in the search for the mechanisms of TAM. Evidence of ER involvement comes from many levels of evaluation, including structural studies, binding assays, molecular and functional data taken in vitro and in vivo, and clinical data.

TAM and 4OHT demonstrate competitive binding with estrogen [120-122]. Crystal structures of ER-4OHT complexes confirm a direct interaction [123, 124].

Many effects of TAM and 4OHT are ER-dependent. For example, mutations in ERα alter its response to 4OHT [125, 126]. On a more functional level, 4OHT-resistant ERα-negative HeLa cells become sensitized to 4OHT upon stable transfection of ERα [127]. In myoepithelial cells, TAM upregulates nitric oxide (which is growth-suppressive [128]) in an ERβ-dependent manner [129]. Breast cancer cells kill lymphocytes by expressing FasL, and TAM inhibits this FasL expression in an ER-dependent manner [130]. ERs have also been shown to recognize novel binding sites on DNA when activated by TAM or 4OHT. For example, binding of 4OHT to ER leads to transactivation of quinone reductase via
recognition of an electrophile response element (not the estrogen response element traditionally associated with ER signaling) [131].

Patients with ER-positive breast cancer respond better to tamoxifen compared to those with ER-negative tumours. Traditionally this was observed with respect to ERα status [132], but recently ERβ expression was also shown to be associated with more favourable prognosis [133].

Despite the great volume of evidence supporting a role for ERs, the downstream events are not as clear. Furthermore, it has long been observed that estrogen cannot fully protect against micromolar concentrations of TAM in vitro, suggesting the existence of non-ER targets [134]. Therefore, ERs are only the beginning of a subset of pathways.

The membrane-bound ER, GPR30, is also known to bind TAM and 4OHT, but little or no evidence exists to link it to apoptosis. In fact, quite the opposite, GPR30 has been implicated in TAM resistance [135], and in the progression of thyroid [136] and endometrial [137] cancers.

1.4.2 Caspases

Cell death following TAM or 4OHT treatment has been consistently linked to activation of caspases [4, 138]. For example, treatment of MDA-MB-231 and BT-20 breast cancer cells with TAM or 4OHT leads to increased activity of caspase-3, -8, and -9, followed by the appearance of apoptotic morphology [138]. Pretreatment with a caspase inhibitor blunted the apoptotic response. Other studies have shown cleavage of PARP (a caspase-3 substrate) following TAM or 4OHT treatment [139]. In N-nitroso-N-methylurea-induced rat mammary
tumours, TAM leads to elevated caspase activity and a 10-fold increase in tumour apoptotic index [140].

The evidence for caspase involvement is overwhelming, but the upstream events leading to caspases activation are not as well elucidated.

1.4.3 Microsomal antiestrogen binding site

The microsomal antiestrogen binding site (AEBS) is a membrane-associated complex with high affinity for SERMs bearing a cationic aminoethoxy side chain (including TAM and 4OHT), but no affinity for estrogen or non-cationic SERMs [141]. It is comprised of two enzymes (3β-hydroxysterol-δ8-δ7-isomerase and 3β-hydroxysterol-δ7-reductase) involved in cholesterol metabolism [142]. Binding of TAM to the AEBS inhibits its enzymatic functions, leading to accumulation of sterol substrates that, in turn, can induce cell cycle arrest and apoptosis [141, 143].

Although the mechanisms leading to apoptosis are unclear, accumulation of these sterols results in them becoming auto-oxidated into oxysterols that have been shown to depolarize the mitochondrial membrane [141]. Mitochondrial depolarization may trigger apoptotic release of cytochrome c.

While there is overwhelming evidence suggesting that TAM physically binds onto the AEBS, the downstream events leading to apoptosis, and the relative importance of this pathway to apoptosis, are less obvious.
1.4.4 Calcium signaling

TAM triggers a rapid increase of intracellular calcium [144]. Chelation of calcium blocks TAM-induced apoptosis in HepG2 human hepatoblastoma cells [145]. TAM-induced calcium also leads to generation of reactive oxygen species (ROS) in HepG2 cells, followed by apoptosis [146]. Inhibition of ROS protected against apoptosis, and chelation of calcium abrogated ROS generation. In MCF-7 and glioma cells, TAM leads to expansion of calcium waves and sensitized the cells to calcium ionophore-induced cell death [147]. More recently, Bollig et al. demonstrated an ER-independent mechanism for 4OHT, involving calcium release from the endoplasmic reticulum and upregulation of protein phosphatase 1α (PP1α) [148]. siRNA knockdown of PP1α inhibited calcium release and conferred protection against TAM.

TAM and 4OHT may also modulate calcium signaling by binding calmodulin, thus inhibiting calmodulin-dependent enzymes such as cAMP phosphodiesterase [149]. Finally, TAM has been shown to antagonize calcium-dependent induction of the mitochondrial permeability transition, a mechanism that is commonly implicated in chemical-induced apoptosis [150].

1.4.5 Mitochondria

Many drugs collapse the mitochondrial transmembrane potential (MTP) [151], triggering the release of cytochrome c into the cytoplasm [152]. Cytochrome c, in conjunction with apoptosis-inducing factor (AIF), initiates the intrinsic apoptotic pathway by activating caspase-9 [153, 154]. TAM and 4OHT may have a similar effect.
Incubation of TAM with isolated rat liver mitochondria leads to complete collapse of the MTP [155]. In human mammary epithelial cells, TAM treatment leads to MTP collapse and mitochondria condensation alongside caspase activation [156]. TAM-resistant cells obtained by repeated passage contained increased mitochondrial mass, and resisted MTP collapse [156].

TAM treatment in MCF-7 and MDA-MB-231 breast cancer cells under serum-free conditions led to rapid apoptosis within an hour, and is associated with MTP collapse and release of cytochrome c from the mitochondria [157]. Inhibition of mitochondrial permeability transition protected against TAM-induced apoptosis. Cell death was also associated with elevated reactive oxygen species (ROS). Inhibition of NADPH oxidase protected against apoptosis, but did not prevent cytochrome c release, suggesting that cytochrome c release precedes ROS production.

In a recent study, TAM led to mitochondrial depolarization, and translocation of cytochrome c and AIF from the mitochondria [141]. They also detected an increase in the ratio of Bax (pro-apoptotic) to Bcl-2 (anti-apoptotic) expression, shifting the balance towards apoptosis.

1.4.6 Transforming growth factor β

Transforming growth factor β (TGF-β) is a cytokine with seemingly contradictory roles. In primary cells and cell lines TGF-β is growth-inhibitory and pro-apoptotic, but in late-stage tumours it also stimulates angiogenesis and inhibits antitumour immune responses [158]. The net effect of TGF-β is unclear, but there seems to be a consistent association between TAM and TGF-β.
In some studies, TAM leads to increased TGF-β activity in breast cancer cells, and anti-TGF-β1 antibody inhibits TAM-induced apoptosis [159-161]. Moreover, TAM results in elevated secretion of TGF-β in TAM-sensitive cells, but not in TAM-resistant variants [158, 161]. In advanced metastatic breast cancer patients, those who respond to TAM tend to have elevated TGF-β2, whereas non-responsive patients show no changes in TGF-β2 expression [158, 161, 162].

However, there are also studies pointing in the opposite direction, showing a decrease in TGF-β following TAM treatment [163, 164]. This may be, in part, due to the timescale involved. For example, Perry et al. reported a slight decrease in TGF-β protein levels within 6 hours, followed by a significant increase after 12 hours [159].

1.4.7 Protein kinase C

It is well established that TAM and 4OHT can bind, translocate, and modulate protein kinase C (PKC) activity [165]. PKC inhibition has been heavily studied as an avenue for anti-manic therapy, and TAM has consistently shown to be a promising anti-manic agent [166, 167]. However, the relevance of PKC in the context of TAM-induced apoptosis is still being investigated.

TAM is often used to treat glioma. Given the extreme overexpression of PKC (orders of magnitude higher than normal) and the efficacy of PKC inhibitors in glioma cells, it is plausible that TAM works in part by targeting PKC [168].

However, there is contradictory evidence regarding whether TAM activates or inhibits PKC, depending on the system used, though more studies tend to point to TAM as an inhibitor [169-173]. For example, Ahn et al. proposed a mechanism in MCF-7 breast cancer
cells involving activation of PKC [169]. Gundimeda et al., on the other hand, showed that TAM and 4OHT are PKC inhibitory in MBA-MB-231 breast cancer cells [174].

This is further complicated by the fact that PKC modulators sometimes exhibit opposing effects: the same agent may be pro-apoptotic in one cell type, but anti-apoptotic in another [175].

1.4.8 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPK) are serine-threonine kinases involved in a wide range of cellular responses, including proliferation, differentiation, and apoptosis. They can be activated by a number of stimuli, including growth factors, radiation, cytokines, and oxidative stress, ultimately leading to phosphorylation of transcription factors, and subsequent transcriptional regulation. MAPK signaling cascades share in common the sequential phosphorylation of three central kinases: MAP3K (MAP kinase kinase kinase), MAP2K (MAP kinase kinase), and MAPK itself. By far the most commonly studied MAPKs are c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK). All three have been shown to have a possible role in TAM- and 4OHT-induced cell death.

When breast cancer cells are treated with TAM, JNK phosphorylation was observed alongside caspase cleavage and apoptosis [138]. A dominant-negative JNK1 mutant partially blocks TAM-induced apoptosis, suggesting a causal role for JNK1 in mediating TAM activity.

Early evidence for p38 involvement was shown in MCF-7 cells, where 4OHT activates p38 alongside apoptosis, and inhibition of p38 protects against 4OHT [127]. The same pattern was observed in HeLa cells stably transfected with ERα. One of the downstream
transcriptional targets of p38 is thought to be TGF-β. Both are activated in response to 4OHT, and inhibition of p38 also abolishes TGF-β activation [161, 176].

Studies have also shown an involvement for ERK. For example, treatment of MCF-7 breast cancer cells with TAM leads to rapid ERK activation, and inhibition of ERK using PD98059 protected against TAM-induced apoptosis [177]. Inhibition of epidermal growth factor receptor (EGFR) antagonizes both ERK activation and apoptosis, consistent with a nongenomic ER pathway. In another study, 4OHT leads to ERK activation and apoptosis in HeLa cells stably transfected with human ERα [92]. Inhibition of ERK using PD98059 protects against apoptosis, while activation of ERK using epidermal growth factor (EGF) enhances apoptosis.

### 1.4.9 Other proposed mechanisms

TAM inhibits glucosylceramide synthase, leading to accumulation of its substrate, ceramide [178, 179]. Ceramide, in turn, has been commonly linked to apoptosis in many systems [180, 181].

TAM also upregulates c-myc mRNA and protein, and downregulation of c-myc using an antisense oligonucleotide protects MDA-231 breast cancer cells against TAM (whereas a nonspecific oligomer had no effect) [182]. While this particular study shows strong evidence for c-myc involvement in TAM-induced apoptosis, in general c-myc has seemingly contradictory and multi-faceted roles. It is involved in both proliferation and apoptosis. Its effects are global; c-myc is believed to regulate 15% of all genes [183]. Despite its many roles in driving apoptosis [184], it is also a proto-oncogene, and found to be upregulated in many cancers [185, 186]. Overexpression of c-myc is also a basis of tumourigenesis in many
animal models [187, 188]. The downstream effects of TAM-induced c-myc will need to be established to complete this potential pathway.

TAM is a hydrophobic compound that can partition into biomembranes, which may have wide-ranging consequences, including disruption of membrane fluidity [111, 189]. The details are unclear, but conceivably, changes in membrane fluidity may trigger the release of membrane-anchored second messengers leading to downstream signaling events [111, 150]. TAM and 4OHT have also been shown to inhibit a number of potassium channels by partitioning into the membrane and disrupting interactions between the channels and phosphatidylinositol 4,5-bisphosphate (PIP2) [190, 191].

Insulin-like growth factors (IGF) are mitogens for breast cancer, which express the IGF-I receptor (IGF-IR) [134]. TAM has been shown to disrupt IGF signaling by inhibiting IGF-I-induced phosphorylation of IGF-IR, and by blocking downstream phosphatidylinositol 3-kinase (PI3K) signaling [134, 192]. TAM treatment in vivo is also associated with decreased serum IGF-I [134].

Survivin belongs to the inhibitor of apoptosis (IAP) family of proteins, and functions as a negative regulator of apoptosis. TAM treatment is associated with decreased survivin expression, thus promoting apoptosis [103, 104]. In human breast cancer cells, inhibition of survivin enhances TAM-induced apoptosis, and overexpression of survivin confers resistance against TAM [193].

Matrix metalloproteinase-9 (MMP-9) is involved in the degradation of extracellular matrix, and has been shown to have anti-angiogenic properties. Transduction of the MMP-9 gene leads to tumour regression in vivo [194]. Several studies have shown that TAM leads to
increased MMP-9 activity *in vitro* and *in vivo*, correlated with decreased tumour growth and angiogenesis [164, 194, 195].

Interestingly, due to the positive charge of TAM (imparted by an amino group), it is able to electrophoretically accumulate into the negatively charged mitochondrial matrix (40-fold more concentrated) [196]. There, it inhibits topoisomerases and downregulates the transcription of mitochondrial genes. Over time, it also leads to degradation of mitochondrial DNA. Although the authors of this study did not discuss their finding in the context of apoptosis, the idea of TAM eliciting global effects on mitochondrial genes is a novel and overlooked mechanism, and the implications are relatively unexplored.

Certain actions of TAM are, so far, not linked to apoptosis. For example, estrogen epoxidation is one of the reactions involved in breast tumourigenesis, and TAM competes with estrogen for epoxidation [197]. This was proposed as a mechanism for cancer prevention rather than cancer treatment. Estrogen-related receptors (ERR) are known to bind TAM and 4OHT, but little or no evidence exists to link them to apoptosis. In fact, ERRγ has instead been linked to TAM resistance [198].
1.5 Rationale and Hypothesis

Greater understanding of the mechanisms behind TAM- or 4OHT-induced apoptosis may help identify new therapeutic targets, or lead to improved combinatorial drug formulations for the treatment of RMS. In addition, we become better equipped to understand the mechanisms of TAM resistance once we understand the mechanisms of its anti-cancer effects.

Despite the wealth of research on the mechanisms of TAM and 4OHT, more work is needed to help connect these scattered leads, and many gaps are yet to be filled before the many pieces of data can be fit into a coherent network. Furthermore, the effect of 4OHT in RMS is a new area of research, and none of the mechanistic studies for TAM or 4OHT described in Chapter 1.4 have been applied to RMS.

In this study, we explore the relevance of ER, MAPK, and caspase signaling during 4OHT-induced cell death in RMS. We hypothesize that 4OHT-induced apoptotic signaling is mediated by a causal sequence from ERs to MAPKs to caspases.
CHAPTER 2: MATERIALS AND METHODS

Cell cultures

Cell lines (from Dr. A. Thomas Look of Dana-Farber Cancer Institute, Boston, MA) were derived from embryonal (RD) and alveolar (RH4, RH30) RMS tumours. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Wisent, Cat #: 319-005-CL) with 10% fetal bovine serum (FBS) (Wisent, Cat #: 080450). Drug treatments were carried out under serum-free conditions, using either DMEM or phenol red-free DMEM (prfDMEM) (Wisent, Cat #: 319-051-CL).

Drug treatments

RMS cells were plated at a density of $10^4$ cells/well in 96-well plates for MTT assay, or at $3\times10^5$ cells/well in 6-well plates for protein extraction. They were allowed to adhere and recover for at least 6 hr in DMEM + 10% FBS, then serum-starved overnight in DMEM or prfDMEM. After overnight serum-starvation, the cells were subject to the appropriate drug treatments (for details, see captions for Figures 6 to 14), and undergo either the MTT assay, or protein extraction for western blot.

The cell plating numbers for MTT assay ($10^4$ cells/well) and western blot ($3\times10^5$ cells/well) result in similar cell densities, as the area of each well in the 6-well plate is approximately 30 times that of a well in the 96-well plate.

Z-4-hydroxytamoxifen (4OHT) was purchased from Sigma (H7904-5MG). The MAPK inhibitors SP600125, SB203580, and PD98059 were purchased from Calbiochem, and reconstituted in dimethyl sulfoxide (DMSO). Fulvestrant (ICI182780) was purchased from
Tocris Bioscience, and reconstituted in ethanol. Vehicle controls ensured that concentrations of ethanol and/or DMSO in the medium remained constant within each set of treatments.

**MTT viability assays**

Cells were plated and treated in 96-well plates (as described above) in triplicates. Following the appropriate drug treatment, the cells were incubated in 100 µL/well of medium containing 10% TACS MTT Reagent (R&D Systems). After 3 hr, 100 µL of detergent solution (provided with the assay kit) was added to each well, and incubated for 4 hr to overnight at 37°C in the dark. Absorbance was measured at 570 and 650 nm, and the difference (A570 minus A650) was recorded as a raw measure of overall cell viability. These raw values were normalized to the vehicle average to yield percent cell viability. Data from each triplicate set were averaged, and the average values from 3 experiments (involving 3 independently passaged populations of cells) were used for statistical analysis. Results are shown as mean (from the 3 replicates × 3 experiments) +/- SEM (from the 3 experiments).

This assay measures the ability of the cells to metabolize the tetrazolium MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), as an indication of their combined metabolic activity. Therefore, the word “viability” in this context is used loosely to refer to the collective effects of proliferation, cell death, and changes in cellular metabolic rate.
**Western blots**

Total cellular protein was extracted using ice-cold EBC lysis buffer (120 mM NaCl, 50 mM Tris pH 8, 0.5% Nonidet P-40) containing Complete Mini Protease Inhibitor Tablets (Roche) (1 tablet per 7-10 mL) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche) (1 tablet per 7-10 mL). Lysates were run on a 12% SDS-PAGE gel under reducing conditions, transferred onto PVDF membrane (Millipore), blocked in Odyssey Blocking Buffer (LI-COR Biosciences), incubated overnight at 4°C with primary antibodies, and then for 30-45 min at room temperature with anti-rabbit (680 nm) or anti-mouse (800 nm) infrared dye-tagged secondary antibodies. Blots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences) at 700 and 800 nm (to detect anti-rabbit and anti-mouse antibodies, respectively). To sequentially detect different proteins on the same blot, the membrane was stripped with PVDF stripping buffer (LI-COR Biosciences), then re-blocked and re-probed as before.

Background-adjusted densitometric values were taken using the Odyssey Infrared Imaging System Application Software (LI-COR Biosciences). For the MAPK proteins (JNK, p38, and ERK), densitometric results are presented as a ratio of phosphorylated MAPK over total MAPK. For cleaved caspase-3 and cleaved PARP, results are normalized to vinculin (the loading control). JNK (and phospho-JNK) appear as 2 distinct bands at 46 and 54 kDa; densitometry was performed on each band separately. ERK (and phospho-ERK) appears as 2 distinct bands at 42 and 44 kDa; densitometry was performed on the collective intensity of both bands (which are too close to densitometrically differentiate with precision). Cleaved caspase-3 exists as 2 bands at 17 and 19 kDa, but densitometry was performed only on the 19 kDa band (the 17 kda band is weaker and does not show up consistently).
Primary mouse antibody for vinculin was purchased from Millipore. Primary rabbit antibodies for cleaved caspase-3, cleaved PARP, Thr202/Try204-phosphorylated ERK1/2, total ERK1/2, Thr183/Tyr185-phosphorylated JNK, total JNK, Thr180/Try182-phosphorylated p38, and total p38 were purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse infrared dye-tagged secondary antibodies were purchased from LI-COR Biosciences.

**Statistical analysis**

Statistical analyses were performed using two-tailed Student’s t-test, with unequal variance. A $p$ value of less than 0.05 was considered statistically significant. Data are presented as mean +/- SEM (standard error of the mean) from experiments involving 3 independently passaged populations of cells.
CHAPTER 3: RESULTS

The results are organized into four general sections. The first section establishes the effects of 4OHT on RMS cell lines (including caspase activation), consistent with a previous finding in our lab [4]. The second and third sections explore the mechanisms of 4OHT, focusing on the involvement of MAPKs and ERs. The final section addresses data that suggest a link between MAPKs and ERs.

3.1 Effects of 4OHT on RMS cell lines

A previous study from our lab [4], the first to explore the ER pathway in RMS, has shown that 4OHT leads to decreased cell viability and induces apoptotic signaling. The treatments were carried out in 10% serum, and lasted at least 24 hr. In my experiments, however, a shorter treatment time is preferred due to the extreme instability of the MAPK inhibitors. Serum-starvation sensitized the cells to 4OHT, allowing the treatment time to be reduced to 3 hr while retaining comparable levels of effect.

For most of my treatments, I used DMEM free of phenol red (a pH indicator that exhibits weak estrogenic activity). This was a precautionary measure in case phenol red interferes with the binding of 4OHT onto ERs, which would overcomplicate the mechanisms that we are attempting to explore.

3.1.1 4OHT leads to loss of cell viability

Before exploring the mechanisms behind 4OHT, the overall effect of 4OHT in the treatment regime was established: overnight serum-starvation followed by 3 hr 4OHT
treatment. Under these conditions, 10 µM 4OHT drastically reduced cell viability as detected by the MTT assay (Figure 6), although 5 µM had little or no effect. In subsequent mechanistic experiments, 10 µM 4OHT is used to establish the basal effect.

Figure 6: 4OHT leads to loss of viability in RMS cell lines. Cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight, and treated with 0, 5, or 10 µM 4OHT (and equal concentrations of vehicle) in serum-free medium. After 3 hr of treatment, cell viability was measured using MTT assay. Shown here are mean (+/− SEM) percent cell viability relative to the 0 µM treatment. * p < 0.05, *** p < 0.001, two-tailed unequal variance t-test.
3.1.2 4OHT induces apoptotic signaling

At the same treatment conditions (overnight serum-starvation followed by 3 hr 4OHT treatment), 4OHT also led to increased cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), both of which are markers of apoptosis (Figure 7). Consistent with the MTT results, 10 µM 4OHT had a significant effect, while 5 µM had little or no effect.

![Figure 7: 4OHT induces apoptotic signaling in RMS cell lines.](image)

Cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight in phenol red-free (prf) DMEM, and treated with prfDMEM containing 0, 5, or 10 µM 4OHT (and equal concentrations of vehicle). After 3 hr of treatment, whole-cell protein lysates were harvested for detection of cleaved caspase-3 (A) and cleaved PARP (B) via western blot. Background-adjusted densitometric values were taken (for cleaved caspase-3, only the 19 kDa band was measured by densitometry). Shown here are mean (+/- SEM) values, normalized to both the loading control vinculin and to the 0 µM values (defined as 1 relative unit). Note the logarithmic scale (y-axis).

* $p < 0.05$, *** $p < 0.001$, two-tailed unequal variance t-test.
3.2 Involvement of MAPK in the effects of 4OHT

Having established that 10 µM 4OHT leads to loss of cell viability, as well as increased apoptotic signaling, we next explored whether this may be linked to MAPK signaling.

3.2.1 4OHT-induced apoptotic signaling is associated with increased MAPK phosphorylation

At the same conditions in which apoptotic signaling is observed (Figure 7), an increase in the phosphorylated forms of JNK, p38, and ERK were detected, whereas total (phosphorylation + non-phosphorylated) levels of these proteins remained approximately constant (Figure 8).

3.2.2 MAPK inhibition protects against 4OHT-induced loss of viability

Having established a correlation between 4OHT-induced apoptotic signaling and MAPK activation, we next explored whether there is a causal link. To this end, we pretreated RD cells for 30 min with MAPK inhibitors, before initiating the 3 hr 10 µM 4OHT treatment (with sustained inhibitor treatment throughout this 3 hr period). In cells treated with 4OHT alone, an extensive loss of cell viability was detected, as expected (Figure 9). However, when a JNK inhibitor (SP600125) or ERK inhibitor (PD98059, a direct inhibitor of MEK1/2, which is responsible for ERK phosphorylation) was present, this effect of 4OHT was partially blunted (Figure 9).
3.2.3 MAPK inhibition blocks 4OHT-induced apoptotic signaling

Consistent with the functional data shown in Figure 9, inhibition of JNK or ERK under the same conditions also blunted the apoptotic signaling induced by 4OHT, suggesting a causal link between MAPK activation and apoptotic signaling. When treated with 4OHT alone, RD cells showed increased JNK phosphorylation and increased apoptotic markers (cleaved caspase-3 and cleaved PARP), but pre- and co-treatment with the JNK inhibitor SP600125 not only reduced phosphorylated JNK, but also reduced levels of the apoptotic markers (Figure 10). A similar effect is observed using the p38 inhibitor SB203580 and the ERK inhibitor PD98059 (Figure 10).
Figure 8: 4OHT leads to increased MAPK phosphorylation in RMS cell lines. Cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight in phenol red-free (prf) DMEM, and treated with prfDMEM containing 0, 5, or 10 μM 4OHT (and equal concentrations of vehicle). After 3 hr of treatment, whole-cell protein lysates were harvested for detection of the MAPK proteins JNK, p38, and ERK (and their phosphorylated forms). Shown are mean (+/− SEM) background-adjusted densitometric ratios between phosphorylated and total MAPKs, normalized to the 0 μM value (defined as 1 relative unit). JNK appears as two distinct bands (46 kDa and 54 kDa) due to alternative splicing. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed unequal variance t-test.
Figure 9: MAPK inhibition protects against 4OHT-induced loss of viability. RD cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight, pre-treated for 30 min with vehicle or a MAPK inhibitor (SP600125 or PD98059), and treated for 3 hr with the four basic permutations: no drug (vehicle), 4OHT alone, MAPK inhibitor alone, or a combination of 4OHT + inhibitor. Following treatment, cell viability was measured using MTT assay. Shown here are mean (+/- SEM from 3 experiments) percent cell viability relative to that of vehicle treatment (defined as 100%).
Figure 10: MAPK inhibition protects against 4OHT-induced apoptotic signaling. RD cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight, pretreated for 30 min with vehicle or MAPK inhibitor, and treated for 3 hr with the four basic permutations: vehicle, 4OHT, inhibitor, or 4OHT + inhibitor. Whole-cell protein lysates were harvested for western blot. Data shown here are background-adjusted mean (+/− SEM) densitometric values/ratios. For cleaved caspase-3 and cleaved PARP, values are normalized to both the loading control (vinculin) and the vehicle values (defined as 1 relative unit). For JNK, p38, and ERK, values shown are background-adjusted densitometric ratios between phosphorylated and total forms, normalized to vehicle ratios (defined as 1 relative unit). * p < 0.05, two-tailed unequal-variance t-test.
3.3 Involvement of estrogen receptors in the effects of 4OHT

Having established a likely causal role for MAPKs in activating apoptotic signaling following 4OHT treatment, we now move on to another candidate target: estrogen receptors. In this section, we explore whether estrogen receptors might also be involved in 4OHT-mediated effects.

3.3.1 ER inhibition protects against 4OHT-induced loss of viability

Fulvestrant is a pure antagonist of ERα and ERβ in humans. We decided to test whether inhibition of ERs using fulvestrant can block the effects of 4OHT. RMS cells were pretreated for 30 min with fulvestrant, before being subjected to the standard 3 hr 4OHT treatment (with continued fulvestrant co-treatment throughout this 3 hr period). When treated with 4OHT alone, RMS cells showed a drastic reduction in viability as expected, but pre- and co-treatment with fulvestrant partially abolished this effect (Figure 11). Note that treatment with fulvestrant had no effect on cell viability, which at first may be surprising given that fulvestrant is a potent ER inhibitor. However, recall that these experiments were done under serum-free (and hence, estrogen-free) conditions. In other words, there is no estrogen binding onto the ERs in the first place, so therefore ER inhibition by fulvestrant has no effect on the cells.

The cytotoxic effects of 4OHT can be easily observed under a light microscope. RMS cells rapidly lose their well-defined spindle shape and adopt a smaller rounded shape, and eventually lose their adherence to the culture plate. The striking protective effects of fulvestrant can also be observed under the microscope. Figure 12 shows images taken from a differential interference contrast (DIC) microscope, with four panels corresponding to each
of the four treatments (vehicle, 4OHT alone, fulvestrant alone, and 4OHT + fulvestrant) in RD cells. The vehicle panel shows the normal appearance of RD cells, a characteristic sharply defined spindle shape. In the 4OHT alone panel, the cells are shrunk into a small rounded shape, and have a darkened colour (indicative of floating cells). However, when treated with a combination of 4OHT and fulvestrant, the cells are largely reversed back to their normal appearance. No staining was done on these cells, so the specific biological conclusions from Figure 12 alone are limited. However, the images are meant to complement results from the MTT assay (Figure 11) and western blots (Figure 13), and to show the protective effect of fulvestrant against 4OHT on a raw appearance level.

![Figure 11: The ER inhibitor fulvestrant protects against 4OHT-induced loss of viability.](image)

Cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight in phenol red-free DMEM, pre-treated for 30 min with vehicle or fulvestrant, and treated for 3 hr with the four basic permutations: no drug (vehicle), 4OHT alone, fulvestrant alone, or a combination of 4OHT + fulvestrant. Cell viability was measured using MTT assay. Shown here are mean (+/- SEM) percent cell viability relative to that of vehicle treatments (defined as 100%).

* $p < 0.05$, ** $p < 0.01$, two-tailed unequal-variance t-test.
Figure 12: Visible changes in cell appearance associated with 4OHT-induced cell death can be partially blocked with the ER inhibitor fulvestrant. RD cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight in phenol red-free DMEM, pretreated for 30 min with vehicle or fulvestrant, and treated for 3 hr with the four basic permutations: no drug (vehicle), 4OHT alone, fulvestrant alone, or a combination of 4OHT + fulvestrant. Differential interference contrast (DIC) microscopy pictures were taken after the 3 hr treatment.

3.3.2 ER inhibition blocks 4OHT-induced apoptotic signaling

Consistent with the functional data shown in Figure 11, inhibition of ERs using fulvestrant also blunted the apoptotic signaling induced by 4OHT, suggesting that ERs may be involved in mediating apoptotic signaling. When treated with 4OHT alone, RMS cells showed increased apoptotic markers (cleaved caspase-3 and cleaved PARP) as expected, but pre- and co-treatment with fulvestrant almost completely abolished both apoptotic markers (Figure 13).
Figure 13: The ER inhibitor fulvestrant protects against 4OHT-induced apoptotic signaling. Cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight in phenol red-free (prf) DMEM, pretreated for 30 min with vehicle or fulvestrant, and treated for 3 hr with the four basic permutations: no drug (vehicle), 4OHT alone, fulvestrant alone, or a combination of 4OHT + fulvestrant. Whole-cell protein lysates were harvested for detection of cleaved caspase-3 (A) and cleaved PARP (B) via western blot. Shown here are mean (+/− SEM) background-adjusted densitometric values, normalized to both the vehicle values (defined as 1 relative unit) and to the loading control vinculin. * p < 0.05, ** p < 0.01, two-tailed unequal-variance t-test.
3.4 Linking the MAPKs with ERs

Having shown evidence that both ER and MAPK may be involved in the apoptotic signaling induced by 4OHT, I next asked whether these two targets are part of the same pathway, or represent independent mechanisms.

So far, I have shown that in RMS cells treated with 4OHT, there is an increase in JNK phosphorylation. However, when the cells were pre- and co-treated with fulvestrant, a reduced induction of JNK phosphorylation was observed (Figure 14). Fulvestrant is not a JNK inhibitor, yet it was somehow able to inhibit 4OHT-induced JNK phosphorylation. This suggests that 4OHT-induced JNK phosphorylation is dependent on ERs, thus inhibition of ERs by fulvestrant also led to downstream inhibition of JNK.

A similar effect was observed on ERK and p38, though to a smaller extent (data not shown).
Figure 14: The ER inhibitor fulvestrant blocks 4OHT-induced JNK phosphorylation but does not affect basal phospho-JNK levels. Cells were plated in DMEM + 10% FBS, recovered for 6 hours, serum-starved overnight in phenol red-free (prf) DMEM, pretreated for 30 min with vehicle or fulvestrant, and treated for 3 hr with the four basic permutations: no drug (vehicle), 4OHT alone, fulvestrant alone, or a combination of 4OHT + fulvestrant. Whole-cell protein lysates were harvested for detection of phosphorylated and total JNK via western blot. Shown here are mean (+/− SEM) background-adjusted densitometric ratios between phosphorylated and total JNK, normalized to vehicle ratios (defined as 1 unit). JNK appears as two distinct bands (46 kDa and 54 kDa) due to alternative slicing. Densitometric ratios are plotted individually for each JNK variant (46 kDa on the left, 54 kDa on the right).

** p < 0.01, two-tailed unequal-variance t-test.
CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Although a great wealth of research exists on the mechanisms of TAM and 4OHT, our understanding of their mechanisms consists of a mass of disconnected leads, with many gaps to be filled, and some contradictions to be clarified.

In this study, we explored the mechanisms of 4OHT in RMS, following a previous study [4] in our lab that demonstrated the anti-RMS effects of 4OHT. In particular, we looked at the involvement of ERs, MAPKs, and the apoptotic markers caspase-3 and PARP. All of these have been individually implicated in the mechanisms of TAM or 4OHT in various studies, though few studies show the connections between these targets in the context of 4OHT activity.

4.1 Involvement of caspases

We showed a strong induction of apoptotic signaling following 4OHT treatment, as indicated by the upregulation of cleaved caspase-3 and cleaved PARP. Caspases are cysteine proteases that initiate and execute apoptosis in many systems, generally divided into two classes: the initiators and effectors. Both forms exist in their inactive pro-enzyme forms in a non-apoptotic cell. Upon induction of apoptosis, initiator caspases are auto-activated by multimeric complexes (such as the apoptosome and DISC) [199]. The activated initiator caspases cleave downstream effector caspases, which in turn execute apoptosis by cleaving a wide range of cellular targets, leading to cell death. Caspase-3 is an effector caspase found in mammals, and one of its targets is poly(ADP-ribose) polymerase (PARP) [200].
Apoptosis can be initiated by two general pathways, either intrinsically triggered from signals within the cell (leading to release of apoptotic factors from the mitochondria and formation of the apoptosome), or extrinsically triggered from extracellular ligands binding onto death receptors on the plasma membrane (leading to formation of DISC) [199]. Although our experiments did not distinguish between the two (since caspases-3 is activated by both the intrinsic and extrinsic pathways), there is evidence that TAM alongside simultaneous activation of the extrinsic pathway leads to synergistic apoptotic effects, suggesting that TAM complements the extrinsic pathway, and therefore acts intrinsically [201]. This is consistent with many studies showing mitochondria-mediated apoptosis after TAM treatment [141, 155-157]. Therefore, we have reason to suspect that the apoptotic signaling we observed here is likely triggered via the intrinsic pathway mediated by the mitochondria. However, there is a recent report suggesting that estrogen itself could induce apoptosis in estrogen-sensitive cells via both the intrinsic and extrinsic pathways [202]. Further experiments will need to be done to confirm whether the 4OHT-induced pathway is intrinsic or extrinsic (or both), possibly by monitoring the translocation of apoptotic mitochondrial factors such as cytochrome c and AIF, or by observing the sensitivity of RMS cells to 4OHT while blocking the extrinsic pathway. It will also be informative to determine whether extrinsically activated apoptosis can combine synergistically with 4OHT in our experimental system.

In addition to apoptosis, there may be other modes of cell death not accounted for in our assays and experimental conditions. Apoptosis itself is classified as Type I cell death [203]. Type II cell death involves autophagy, characterized by the formation of vacuoles that digest the host cell from the inside [203, 204]. Type III cell death, necrosis, loosely refers to forms
of seemingly “uncontrolled” cell death leading to chaotic release of inflammatory cellular contents [203, 204]. Oncosis is a form of cell death resulting from the collapse of membrane ion gradients, leading to cell and organelle swelling as well as membrane permeation, with eventual release of inflammatory cellular contents [204].

### 4.2 Involvement of MAPKs

In addition to apoptotic signaling, we observed a simultaneous activation of MAPKs, especially JNK. To distinguish whether the MAPKs are acting to mediate apoptotic signaling, or whether they are part of a protective response to cell death, we used inhibitors to block MAPK phosphorylation. This led to partial protection against 4OHT, suggesting that MAPKs are involved in mediating apoptotic signaling.

Both JNK and p38 have been commonly associated with apoptosis, including TAM- or 4OHT-induced apoptosis [127, 138]. While ERK is traditionally associated with proliferation, many studies link ERK to apoptosis in a wide variety of systems [205-207]. More work will be needed to confirm our results, using alternative methods of MAPK inhibition such as siRNA knockdown or dominant-negative constructs. Once MAPK involvement is confirmed, we may then further explore their downstream targets induced by 4OHT. So far, our preliminary data (not shown) demonstrates an increase in ATF2 phosphorylation, but no effect on c-Jun phosphorylation, both of which are classical JNK targets. Conversely, we may trace the pathway in the other direction using inhibitors of upstream targets along the MAPK pathways. Immediately upstream of the MAPKs are the MAP2Ks, including MKK4/7 (which targets JNK), MKK3/6 (which targets p38), and MEK1/2 (which targets ERK). Further upstream, at the MAP3K level, there are a much greater number of potential
targets, with multiple members from many kinase families, including the mixed lineage
kinases (MLK) and apoptotic signaling kinases (ASK). A screening approach may be needed
to trace the various targets involved at this level of the pathway.

4.3 Involvement of ERs

Finally, we have shown that ER inhibition using fulvestrant partially blocks both 4OHT-
induced apoptotic signaling, as well as 4OHT-induced phosphorylation of JNK (and to a
lesser extent, p38 and ERK). Therefore, we propose that ERs act upstream of the MAPKs,
which in turn lead to apoptotic signaling. A previous study in RMS showed that 17β-estradiol
(E2) was also able to partially block the effects of 4OHT, consistent with the idea that ERs
may be involved [4].

One immediate argument against this interpretation of our data is the possibility that
fulvestrant might be nonspecifically inhibiting JNK phosphorylation. However, this is
unlikely as fulvestrant is completely unrelated to any kinase inhibitors, and to our knowledge
there are no reports of fulvestrant inhibiting JNK phosphorylation. Furthermore, closer
examination of our data also argues against this possibility. As shown in Figure 14,
fulvestrant only affected 4OHT-induced phospho-JNK levels, and had no effect on basal
levels of phospho-JNK, in contrast to the true JNK inhibitor SP600125 (Figure 10), which
reduced both basal and 4OHT-induced phospho-JNK. This helps to rule out the possibility
that fulvestrant might be nonspecifically targeting JNK.

Alternative methods of inhibition (including siRNA knockout, dominant-negative
constructs, or other inhibitors) should be used to confirm this conclusion. Conversely, it
would also be informative to overexpress the ERs and observe whether this sensitizes the cells to 4OHT.

Although we did not attempt to distinguish between ERα and ERβ in our experiments (fulvestrant inhibited both), a previous study in our lab by Greenberg et al. [4] has shown that RMS primary tumours and cell lines express ERβ but not ERα, at both the mRNA and protein levels. This suggests that ERβ is likely the isoform that is mediating MAPK and caspase activation in our experiments, with little or no contribution from ERα. To confirm this idea, isoform-specific inhibition of ERβ will need to be achieved, possibly using siRNA to downregulate ERβ mRNA, or ERβ-specific small-molecule inhibitors. We have attempted siRNA knockdown of ERβ, but with only mild effects at the mRNA level. A stable inducible shRNA system may be needed to achieve more robust and durable knockdown.

According to Greenberg et al., the various RMS cell lines (RD, RH4, and RH30) express different levels of ERβ. We observed no correlation between ERβ expression and 4OHT sensitivity. However, to confidently assess the role of ERβ without confounding factors from other differences in the cell lines, we would need to vary ERβ expression across an otherwise isogenic set of cell lines. This can be achieved by stable shRNA knockdown and overexpression of ERβ to generate variant RMS cell lines with altered ERβ expression.

**Mode of Action at the ER**

Upon binding of 4OHT to the ER, one can postulate two general modes of action. First, 4OHT may occupy the ER, blocking it from endogenous ER ligands, leading to downstream consequences of this blockade. In the second scenario, 4OHT not only binds to the ER, but also continues on with a genomic or nongenomic pathway of its own. There is overwhelming
evidence for both possibilities, and both are likely to occur under different systems and conditions [92, 93, 131, 134]. However, in our experimental system we can eliminate the first possibility, due to two reasons.

First, all our experiments were carried out in serum-free conditions. Therefore, we have removed estrogens, as well as other components of the serum that might bind onto the ERs. In most of our experiments, we also removed phenol red (an artificial component of the medium that weakly mimics estrogen and can bind onto the ERs). Since we have removed all the ER ligands as much as possible, it is unlikely that 4OHT is merely acting to blockade the ER (because there are no ligands left to block in the first place).

Second, in our experiments using fulvestrant, a potent inhibitor of the ERs, no effect was observed on cell viability or on apoptotic signaling. In other words, simple blockade of the ERs cannot account for the effects observed following 4OHT treatment.

Therefore, 4OHT is most likely binding onto the ER and initiating a pathway of its own that leads to JNK activation and apoptotic signaling. 17β-estradiol alone cannot recapitulate the effects of 4OHT under the same conditions (data not shown), supporting our notion that 4OHT may be distorting the normal estrogen pathway to achieve the observed anti-RMS effects. The timescale of our treatments (3 hr) do not allow us to further narrow down the possibility of genomic versus nongenomic signaling, as both events can occur within this relatively large time window. To make this distinction, treatment times on the scale of minutes may be needed, as nongenomic effects tend to occur much more rapidly.
Linking ERs with MAPKs

MAPK activation has traditionally been considered as part of an ER-independent mechanism of TAM and 4OHT [138, 208, 209]. However, our study provides evidence for ER-dependent MAPK activation. Despite the prevailing view that TAM- or 4OHT-induced MAPK activation is ER-independent, there are still some studies that support our conclusions.

Zhang et al. provided strong evidence that causally linked p38 to ERα as part of a mechanism for 4OHT [127]. ERα-negative HeLa cells are 4OHT-resistant and do not induce p38 activation after 4OHT treatment. However, stable transfection of ERα not only sensitizes the cells to 4OHT, but also leads to 4OH-induced p38 activation. When these ERα-transfected HeLa cells are selected for resistance to 4OHT, p38 activation vanishes as well.

Prifti et al. demonstrated ER-dependent activation of JNK by E2 and synthetic estrogens [210]. T47D breast cancer cells treated with estrogen leads to increased JNK phosphorylation, and pretreatment with fulvestrant completely abolishes JNK phosphorylation. As a control, they showed that EGF-induced JNK phosphorylation (which is ER-independent) is not affected by fulvestrant.

Various groups have also established a nongenomic ER pathway leading to MAPK activation [211-213]. For example, Migliaccio et al. showed that E2 leads to activation of ERK and upregulation of GTP-ras (another member of the ERK pathway) in MCF-7 cells [212]. This E2-induced ERK pathway can be inhibited using fulvestrant. ERα-negative Cos-7 cells do not activate ERK in response to E2, but transfection of human ERα leads to E2-responsive ERK activation. Cheskis et al. described a novel scaffold protein they called modulator of nongenomic activity of estrogen receptor (MNAR), which is required for the interaction between ERα and Src (a tyrosine kinase that can lead into the ERK pathway by
activating Raf), thus providing a mechanism for ER signaling to feed into the ERK pathway [213].

Wong et al. originally identified MNAR by incubating a GST-tagged ERβ with MCF-7 whole-cell extracts in the presence or absence of E2 [214]. E2-dependent protein binding partners were pulled down, separated by SDS-PAGE, silver-stained, and identified by mass spectrometry. If the effects of 4OHT in RMS cells are mediated through a nongenomic pathway, we might be able to use a similar approach to find 4OHT-dependent protein binding partners that might shed light on the molecular events following binding of 4OHT to the ER.
Figure 15: Model of 4OHT activity in RMS cells. 4OHT-induced cell death in RMS cells involves binding of 4OHT to the ER, phosphorylation of MAPKs, and activation of apoptotic markers caspase-3 and PARP. These appear to be linked in a causal sequence, from ER to MAPK to apoptotic signaling. Furthermore, blockage of the ER alone is not sufficient to induce cell death. Therefore, the antiestrogen model does not fit our observations, leading us to propose a “pseudo-estrogen” model, in which 4OHT acts upon the ER to distort the pathway away from normal estrogen signaling, presumably leading to activation of MAPK and apoptotic signaling. It is conceivable for 4OHT to hijack the genomic pathway (leading to transactivation of target genes by the 4OHT-bound ER) or the nongenomic pathway (feeding into cytoplasmic signaling pathways, eventually also leading to transcriptional effects). Many blanks are yet to be filled in this still-simplistic model, and many intermediates are yet to be identified (candidates include MNAR and Src) to form a more complete picture.
4.4 Concluding remarks

Our study aims to define the mechanisms of 4OHT-induced cell death in RMS cell lines, focusing on three groups of targets: ERs, MAPKs, and caspases. Furthermore, we show that these targets may be causally linked in a sequence from ERs to MAPKs to caspases.

The immediate next steps are to confirm these conclusions using alternative inhibition methods. Once we are confident that our basic pathway is valid, we can then begin to expand it from each of the targets. From the caspases, we may explore whether the upstream apoptotic triggers are intrinsic or extrinsic. From the MAPKs, we can monitor the phosphorylation status of upstream and downstream targets. From the ERs, we may attempt to narrow down the downstream events to a genomic or nongenomic pathway, and to pull down 4OHT-dependent protein interaction partners.

Furthermore, 4OHT can be tested on normal cells to identify effects and mechanisms that select against RMS cells (or tumour cells in general). New selective mechanisms might be identified by comparing the changes in expression profile (in response to 4OHT treatment) between normal versus RMS cells. Finally, primary RMS cells and murine xenografts may offer more realistic systems to model the effects and mechanisms of 4OHT that are relevant in a clinical setting.

A greater understanding of the pathways behind 4OHT-induced cell death may lead to new therapeutic targets. Furthermore, it would allow us to design combinatorial treatments to enhance the effects of TAM or 4OHT: drugs that employ a complementary mechanism to 4OHT may yield synergistic effects. Finally, greater understanding of the mechanisms behind 4OHT or TAM will better equip us to understand the mechanisms of resistance to these drugs.
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