Hrk mediates 2-methoxyestradiol-induced mitochondrial apoptotic signaling in prostate cancer cells


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Title

Hrk mediates 2-methoxyestradiol-induced mitochondrial apoptotic signaling in prostate cancer cells

Inik Chang, Shahana Majid, Sharanjot Saini, Mohd S Zaman, Soichiro Yamamura, Takeshi Chiyomaru, Varahram Shahryari, Shinichiro Fukuhara, Guoren Deng, Rajvir Dahiya and Yuichiro Tanaka

Department of Urology, San Francisco Veterans Affairs Medical Center and University of California at San Francisco, San Francisco, California, USA

Running Title

Hrk is a novel target of 2-ME in prostate cancer.

Keywords

Apoptosis / Hrk / 2-methoxyestradiol / prostate cancer

Corresponding Author:

Yuichiro Tanaka, Department of Urology, San Francisco Veterans Affairs Medical Center and University of California San Francisco, 4150 Clement Street, San Francisco, CA 94121. Phone: 415-750-2031; Fax: 415-750-6639; E-mail: yuichiro.tanaka@ucsf.edu
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Abstract

Prostate cancer is one of the most prevalent cancers in males and ranks the second most common cause of cancer related deaths. 2-methoxyestradiol (2-ME), an endogenous estrogen metabolite, is a promising anticancer agent for various types of cancers. Although 2-ME has been shown to activate Jun N-terminal kinase (JNK) and mitochondrial dependent apoptotic signaling pathways, the underlying mechanisms including downstream effectors remain unclear. Here, we report that the human Bcl-2 homology 3 (BH3)-only protein harakiri (Hrk) is a critical effector of 2-ME-induced-JNK/mitochondria dependent apoptosis in prostate cancer cells. Hrk mRNA and protein are preferentially up-regulated by 2-ME, and Hrk induction is dependent on JNK activation of c-Jun. Hrk knockdown prevents 2-ME-mediated apoptosis by attenuating the decrease in mitochondrial membrane potential, subsequent cytochrome c (cyt c) release and caspase activation. Involvement of the pro-apoptotic protein Bak in this process suggested the possible interaction between Hrk and Bak. Thus, Hrk activation by 2-ME or its over-expression displaced Bak from the complex with anti-apoptotic protein Bcl-xL, while deletion of the Hrk BH3 domain abolished its interaction with Bcl-xL, reducing the pro-apoptotic function of Hrk. Finally, Hrk is also involved in the 2-ME-mediated reduction of X-linked inhibitor of apoptosis (XIAP) through Bak activation in prostate cancer cells. Together, our findings suggest that induction of the BH3-only protein Hrk is a critical step in 2-ME activation of the JNK-induced apoptotic pathway targeting mitochondria by liberating pro-apoptotic protein Bak.
Introduction

2-Methoxyestradiol (2-ME) is produced in vivo by catechol-O-methyltransferase-mediated O-methylation of 2-hydroxyestradiol (1). Although it is initially considered to be an inactive end-product of estrogen metabolism, 2-ME has emerged as a potential anti-tumor agent for several types of cancer including prostate cancer (2). The molecular network of 2-ME action is a complex process that involves various pathways and despite extensive investigation, the precise mechanisms for anti-tumor activity of 2-ME are not fully elucidated (2, 3).

Jun N-terminal kinase (JNK) activation plays an important role in the mitochondrial apoptotic pathway induced by 2-ME (3). Following 2-ME exposure activated JNK is translocated to the mitochondria, where it initiates a decrease in membrane potential and subsequent release of cytochrome c (cyt c) into the cytosol leading to caspase activation (4-6). Although the mechanism by which JNK mediates cyt c release is not fully understood, several lines of evidence indicate that the Bcl-2 gene family may be the potential targets of JNK (7). In the unstimulated state, the anti-apoptotic proteins Bcl-2 and Bcl-xL neutralize the function of pro-apoptotic proteins Bax and Bak, which constitute the pore or channel that permeabilize mitochondria (8). 2-ME-mediated JNK activation regulates the inhibitory function of anti-apoptotic proteins by phosphorylation in prostate cancer cells (9, 10). JNK also modulate the activities of pro-apoptotic BH3-only proteins of the Bcl-2 family, especially Bid, Bim, Bmf and Bad, at the posttranslational level and by doing so, BH3-only proteins can engage Bax and Bak to release cyt c by inactivating the anti-apoptotic proteins (7).
Harakiri (Hrk) belongs to the human BH3-only protein subgroup of the pro-apoptotic Bcl-2 family and was identified by its ability to bind Bcl-2 and Bcl-xL in a yeast two-hybrid screen (11). Exogenous expression of Hrk activates cell death and this is repressed by over-expression of Bcl-2 and Bcl-xL (11, 12). Bax and mitochondrial protein p32 are suggested as direct effector molecules for Hrk action (13, 14). However, despite these studies, the precise molecular mechanism involved in Hrk-mediated cell death remains largely unknown.

It has been suggested that Hrk down-regulation contributes to the development and progression of cancers (15). Hrk expression is frequently lost in colorectal and gastric cancer (16), glioblastoma (17), primary central nervous system lymphoma (18) and prostate cancer (19) due to aberrant methylation of its promoter region. Hrk inactivation is also inversely correlated with apoptosis indices in tumors (17-19). Hrk is located in chromosome 12q13 (20) where loss of heterozygosity is often observed in several types of human tumors (21-23). Moreover, Hrk over-expression suppresses cell growth in prostate, breast, and ovarian cancer cells (24). Despite its potential significance, functional role of Hrk has not been defined in cancer.

In this study, we found that 2-ME induces Hrk in a JNK-dependent manner in prostate cancer cells. We also show that Hrk is involved in the 2-ME-induced apoptotic pathway by activating caspase through Bak-mediated cyt c release. Therefore Hrk, a newly identified target of 2-ME action, is a critical downstream effector of JNK dependent mitochondrial apoptotic signaling pathway of 2-ME in prostate cancer cells.
Materials and Methods

Cell lines and reagents

Human prostate carcinoma cell lines (LNCaP, PC-3, and DU-145) were obtained from the ATCC. All cell lines were authenticated at ATCC prior to purchase by standard DNA typing. Eagle’s minimum essential medium (EMEM), RPMI 1640, Opti-MEM® and penicillin/streptomycin mixtures were obtained from the UCSF Cell Culture Facility. Fetal bovine serum (FBS) was a product of Atlanta Biologicals. 2-ME and SP600125 were obtained from Sigma and chemical structures are shown in Fig. 6.

Cell culture

The LNCaP and PC-3 cell lines were grown in RPMI 1640. The DU-145 cell line was cultured in EMEM. All culture medium contained 10% FBS and 100 μg/ml penicillin/streptomycin mixture. All cell lines were maintained at 37°C in a humidified atmosphere composed of 5% CO₂/95% air.

Apoptosis assay

Cells were stained with an Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (7-AAD) (BD Biosciences) as described by the manufacturer and analyzed by a Cell Lab Quanta™ SC MPL (Beckman Coulter). Both early (Annexin V-positive, 7-AAD-negative) and late (Annexin V-positive, 7-AAD-positive) apoptotic cells were included in cell death determinations.

Quantitative RT-PCR
Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and was converted into cDNA by using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. To assess gene expression, cDNAs were amplified with the TaqMan® Gene Expression Assays and TaqMan® Fast Universal PCR Master Mix using the 7500 Fast Real-Time PCR System (Applied Biosystems). To investigate the expression of genes key to apoptosis, the Human Apoptosis RT² Profiler™ PCR Array (SABiosciences) were used as per manufacturer’s instructions.

**Western blot**

Whole cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) containing protease inhibitor cocktails (Roche). For sub-cellular fractionation, cytosolic and membrane proteins were isolated with a Sub-cellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific). Immunoblotting was carried out according to standard protocols with antibodies against Hrk (Sigma), cytochrome c, phospho-JNK, JNK, c-Jun, Bcl-xL, Bcl-2, Bak, XIAP (Cell Signaling Technology), phospho-c-Jun (Ser 63/73), JNK1 (Santa Cruz Biotechnology), and FLAG (OriGene). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (Santa Cruz) were used to confirm equal loading.

**Caspase enzymatic activity assay**

Caspase-3 activation was measured using a Caspase-3 Assay Kit as described by the supplier’s instructions (BD Biosciences).
Transfection

Cells were transfected with pCMV6-ENTRY vector expressing the C-terminally FLAG-tagged human Hrk cDNA and empty pCMV6-ENTRY vector as a control (OriGene) using Fugene HD Transfection Reagent (Roche) according to the manufacturer’s protocol. For small interfering RNA transfection, siRNA duplexes (20 nM) or universal scrambled negative control (OriGene) was transfected using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) as described by the manufacturer’s instructions. Target specificity and knockdown efficiency were evaluated by real-time PCR with three different sets of siRNA duplexes at different concentrations.

Immunoprecipitation

Cell lysates were prepared in lysis buffer (20 mM Tris-pH 7.4, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 % glycerol) containing 1% CHAPS (Cell Signaling Technology), supplemented with protease inhibitor cocktails (Roche). Immunoprecipitation was performed with a Pierce® Direct IP Kit (Pierce) according to the manufacturer’s instructions.

Assessment of mitochondrial membrane potential

2-ME-treated cells were stained with CMXRos (Mitotracker Red) (Molecular Probes) in PBS for 20 min at 37°C, and analyzed by a Cell Lab Quanta™ SC MPL (Beckman Coulter).

Statistical analysis

8
Values are presented as the mean ± standard error of mean (SEM) based on results obtained from at least three independent experiments. Statistical significance was evaluated by conducting a two-tailed unpaired Student’s *t*-test using GraphPad PRISM Software. A *P* value of <0.05 was regarded as statistically significant.
Results

Hrk is induced by 2-ME in prostate cancer cells.

Dose-dependent apoptosis assays show that 2-ME was effective at a concentration of 1 μM (Fig. 1A) and modest degrees of apoptotic cell death were noted after 12 h of 2-ME exposure reaching maximal levels after 72 h (Fig. 1B). Based on these results, androgen-dependent LNCaP and androgen-independent PC-3 cells, and 1 μM of 2-ME were used in subsequent experiments.

To identify 2-ME signaling pathways, we looked for changes in gene expression in LNCaP cells exposed to 2-ME using the Human Apoptosis RT² Profiler PCR Array. Among several affected genes, we detected an increase of TP53 gene expression similar to a previous report showing its induction by 2-ME (25). Also, Hrk was induced 6.7-fold over controls (Table 1). A significant increase in Hrk mRNA expression was detected as early as 1 h after 2-ME exposure in both LNCaP and PC-3 cells (Fig. 1C). An increase in Hrk protein expression was also noted after 6 h of 2-ME exposure reaching maximum levels after 36 h in LNCaP and 24 h in PC-3 cells (Fig. 1D).

We also evaluated whether mRNA expression of other BH3-only proteins such as Bim, Puma and Noxa are influenced by 2-ME. Induction of Puma and Noxa was at modest levels compared to Hrk in LNCaP cells (Supplementary Fig. S1). Since Puma and Noxa are p53-dependent genes, we examined induction of these genes in PC-3 and DU-145 cells, which have mutated p53. Unlike LNCaP cells, which have wild-type p53, Puma and Noxa were not increased by 2-ME in PC-3 and DU-145 cells. Bim was induced only in PC-3 cells (Supplementary Fig. S1).
**JNK signaling induces Hrk in prostate cancer cells.**

2-ME caused an increase in the level of phospho-JNK without detectable changes in total JNK levels (Fig. 2A). This was followed by c-Jun phosphorylation, paralleling the increase in JNK phosphorylation. The c-Jun protein level was significantly increased, the probable consequence of auto-regulation (Fig. 2A).

Although JNK regulation of Hrk levels has been studied in cultured neurons and pancreatic β-cells (26-28), it is unknown in cancers. Therefore, we examined whether JNK activation is required for Hrk up-regulation in prostate cancer cells using JNK inhibitors. SP600125, a potent JNK inhibitor, suppressed Hrk induction by 2-ME (Fig. 2B). As a further test of specificity, we performed selective knockdown of the JNK pathway using a siRNA. JNK1 siRNA significantly reduced the level of endogenous JNK1 protein (Fig. 2C), and resulted in a dramatic decrease in Hrk mRNA induction (Fig. 2D). Additionally, c-Jun siRNA efficiently suppressed endogenous c-Jun (Fig. 2E) and led to inhibition of Hrk mRNA induction in prostate cancer cells (Fig. 2F). These results indicate that Hrk is regulated by JNK signaling in prostate cancer cells.

**Hrk up-regulation is required for 2-ME-induced apoptotic cell death.**

To investigate functional significance of Hrk in 2-ME-mediated apoptosis of prostate cancer cells, we performed knockdown experiments using Hrk specific siRNA. Transfection with two different Hrk siRNAs resulted in a dramatic reduction in endogenous levels of Hrk mRNA (Fig. 3A) and suppressed 2-ME-mediated induction of Hrk mRNA and protein compared with a control siRNA (Fig. 3A and B). Hrk knockdown did not affect cell viability under basal conditions, but significantly reduced
the cytotoxic effect of 2-ME (Fig. 3C and Supplementary Fig. S2). Also Hrk siRNA-1, which had a more significant knockdown effect than siRNA-2 caused less 2-ME-induced cell death than siRNA-2 (Fig. 3A-C).

Since 2-ME potently induces apoptosis through the mitochondrial apoptotic pathway (29) and Hrk is predominantly located in the mitochondria (Supplementary Fig. S3), we examined the effect of Hrk knockdown on the mitochondrial apoptotic events triggered by 2-ME. As shown in Figure 3D, 2-ME caused a significant increase of CMXRos negative cells indicating the reduction of mitochondrial membrane potential but Hrk knockdown reduced this change. In a parallel experiment, no difference was observed in total cell number by 2-ME treatment (Supplementary Fig. S4). This result excludes the possibility that the decrease in CMXRos-stained cells is not a secondary result caused by a reduction in mitochondrial number but rather the result of a decrease in membrane potential following 2-ME exposure. Hrk knockdown also diminished 2-ME-induced cyt c release into the cytosol from mitochondria (Fig. 3E) and subsequent caspase-3 activation (Fig. 3F). Collectively, these results indicate that Hrk plays an important functional role in 2-ME-induced prostate cancer cell apoptosis by targeting mitochondria.

Hrk triggers apoptotic cell death by displacing Bak from Bcl-xL sequestration.

Hrk physically interacts with Bcl-2 and Bcl-xL, but not Bax or Bak (11). Hrk-induced cell death is repressed by over-expression of Bcl-2 and Bcl-xL (11, 12). Therefore, we hypothesized that Hrk triggers apoptosis by neutralizing Bcl-2 and/or Bcl-xL rather than directly activating Bax and/or Bak. Reduction of 2-ME-induced cell death with knockdown of Bak (Fig. 4A and B) but not Bax (Supplementary Fig. S5) indicates that
Bak activation is a 2-ME downstream apoptotic signal. Moreover, Bak knockdown diminished 2-ME-induced cyt c release into cytosol (Fig. 4C) and subsequent caspase-3 activation (Fig. 4D) consistent with the results obtained by Hrk knockdown (Fig. 3E and F). In prostate cancer cells, Bak binds with Bcl-xL, but not with Bcl-2 (Supplementary Fig. S6). These results led us to examine whether Hrk affected the association between Bak and Bcl-xL.

While the levels of Hrk interacting with Bcl-xL were increased by 2-ME, the level of Bak associated with Bcl-xL was diminished (Fig. 4E). This change is abolished by Hrk knockdown (Fig. 4F). We subsequently evaluated whether over-expression of Hrk affects the interaction of Bak with Bcl-xL. Consistent with 2-ME treatment, the level of Bak associated with Bcl-xL was reduced in Hrk transfectants compared with the vector control (Fig. 4G). Together, these data demonstrate that Hrk contributes to 2-ME-mediated apoptosis by liberating Bak from the complex with Bcl-xL.

BH3 domain of BH3-only proteins mediates its association with anti-apoptotic proteins and is required for cell death induction (30). To determine whether the BH3 domain of Hrk is critical for 2-ME-mediated prostate cancer cell apoptosis, we engineered a mutant form of Hrk lacking the BH3 domain (Hrk \( \Delta BH3 \)). Substantial amounts of both wild-type and mutant Hrk proteins were expressed in transiently transfected prostate cancer cells (Supplementary Fig. S7A). Absence of the BH3 domain dramatically reduced the ability of Hrk to induce apoptotic cell death (Supplementary Fig. S7B) and to interact with Bcl-xL (Supplementary Fig. S7C). As a consequence, there was no change in the level of Bak associated with Bcl-xL by Hrk \( \Delta BH3 \) over-expression (Supplementary Fig. S7D). These results indicate that the BH3 domain-mediated
interaction with Bcl-xL is critical for the pro-apoptotic activity of Hrk in prostate cancer cells.

**Hrk-mediated Bak activation represses XIAP levels.**

To investigate additional downstream target molecules of Hrk activation, we evaluated gene mRNA levels altered by 2-ME (Table 1) in cells over-expressing Hrk (Fig. 5A). From the genes analyzed, we found that only XIAP mRNA was significantly decreased by 40% compared to cells transfected with vector control (Fig. 5B). We also verified the decrease in XIAP mRNA expression by 2-ME (Fig. 5C). Furthermore, XIAP protein expression was significantly reduced by both 2-ME exposure (Fig. 5D) and Hrk over-expression (Fig. 5E). Next, we evaluated XIAP levels in cells transfected with Bak siRNA after 2-ME treatment to assess whether Hrk activation of Bak is required for the regulation of XIAP level. As shown in Figure 5F, Bak knockdown abolished the reduction of XIAP protein in cells treated with 2-ME. Therefore, these data indicate that Hrk participates in the 2-ME-mediated down-regulation of XIAP by activating Bak in prostate cancer cells.
Discussion

In this study, we identified the BH3-only protein, Hrk, as a new 2-ME target gene, acting downstream of the JNK signaling pathway in prostate cancer cells. Hrk is a particularly interesting candidate because: 1) it belongs to the pro-apoptotic Bcl-2 gene family (11, 12), 2) it is down-regulated in prostate cancer and loss of Hrk expression is closely associated with decreased apoptosis in high-grade prostate tumors (19), 3) its expression is regulated by JNK (Fig. 2) which is a downstream molecule of 2-ME (3, 26), 4) unlike other BH3-only proteins such as Bim, Puma, and Noxa, 2-ME-mediated Hrk induction is not affected by androgen dependency or p53 status of cells (Supplementary Fig. S1).

Although it has been well described that JNK activation is involved in the 2-ME-mediated cytotoxic effect in cancer cells, the upstream components regulating JNK activation are still unknown. Davoodpour et al. suggests that Smad7, which is stabilized by 2-ME, is required for 2-ME-induced JNK activation (6). In addition, death-associated protein kinase 1 (DAPK1), which was induced by 2-ME in our study (Table 1) also regulate JNK signaling through protein kinase D1 as described by other investigators (31). Therefore, DAPK1 and Smad7 may be downstream signaling components of 2-ME that activate the JNK cascade. Further investigation regarding the interaction between DAPK1 and Smad7 will be needed to delineate the precise molecular mechanisms of 2-ME-mediated JNK activation.

JNK activation occurs rapidly following 2-ME treatment and plays an important role in the 2-ME-mediated mitochondrial apoptotic pathway (3). Although JNK-induced cyt c release is a critical step for downstream apoptotic signaling, the mechanism is unclear. 2-
ME-induced JNK phosphorylation and subsequent translocation to the mitochondria was proposed as a possible explanation (4). Based on the findings that JNK mitochondrial signaling modulated the activities of BH3-only proteins at the posttranslational level in other cell types by different apoptotic stimuli (7), it is highly possible that BH3-only proteins might also be involved in 2-ME activation of JNK-mediated cyt c release. In this study, we have shown that the BH3-only protein Hrk is a key molecule that links JNK signaling to mitochondria targeted by the interaction of Bak with Bcl-x\textsubscript{L} to induce apoptosis accompanied by cytosolic release of cyt c.

How BH3-only proteins trigger the activation of Bax or Bak has been a central issue in the regulation of apoptosis by the Bcl-2 gene family (8). BH3-only proteins could promote activation of Bax or Bak via their ability to inactivate anti-apoptotic Bcl-2 family members. Alternatively, Bax or Bak may be activated via direct association with certain BH3-only proteins. We observed that in addition to Hrk, 2-ME up-regulates other BH3-only proteins Bim, Puma, and Noxa (Supplementary Fig. S1). Bim and Puma can directly activate Bax and Bak, and they also sequester all anti-apoptotic Bcl-2 family members (8). Noxa neutralizes only Mcl-1 and A1, and promotes Mcl-1 degradation (32).

Despite the idea that Hrk might promote cell death by inhibiting the protective activity of Bcl-2 and Bcl-x\textsubscript{L} (11), the precise mechanism of Hrk has not been extensively studied. The results of this study suggest a mechanism for Hrk-induced apoptotic cell death by regulating the interaction of Bak with Bcl-x\textsubscript{L}. In untreated prostate cancer cells, Bcl-x\textsubscript{L} sequesters active forms of Bak; upon 2-ME treatment, transcriptionally activated Hrk displaces Bak from the heterodimer with Bcl-x\textsubscript{L}, thus releasing Bak (Fig. 4E and G). This promotes Bak oligomerization on the mitochondrial membrane and results in cyt c
release followed by induction of apoptosis. Thus our data supports the indirect activation or displacement model to explain how Hrk activates Bak in addition to the results showing no association of Bim and Puma with Bak after 2-ME treatment in prostate cancer cells (I. Chang; unpublished data). However, this does not entirely rule out the possibility that the direct activation model might also be involved in this mechanism. For instance, Bim and Puma up-regulated by 2-ME may independently cause Bax or Bak activation. It is also possible that Bim and Puma may interact with Bax instead of Bak. Kuwana et al. observed that Hrk appeared to activate Bax directly although it was modest and the significance of the activation was uncertain (33). Therefore, whether 2-ME-mediated induction of BH3-only proteins activates Bak or Bax directly or indirectly remains a matter of discussion and needs further clarification.

Based on previous findings, Hrk may regulate apoptotic cell death via several different mechanisms. Rizvi et al. reported that C6 ceramide induces not only Hrk expression through JNK phosphorylation but also its translocation to mitochondria where Hrk interacts with the mitochondrial protein p32 and the BH3-only protein Bad. These protein interactions eventually lead to mitochondria dysfunction and cell death in human corneal stromal fibroblasts (34). It will therefore be of interest to determine whether 2-ME also induces translocation of Hrk to mitochondria to interact with p32 and Bad, and plays a role in Hrk pro-apoptotic activity in prostate cancer cells. Unlike Bim, Puma, and Bid, other BH3-only proteins selectively neutralize only a subset of anti-apoptotic Bcl-2 proteins. Thus, a combination of several BH3-only proteins binding to complementary subsets is required to promote apoptosis; for example, Bad binding to Bcl-2, Bcl-xL and Bcl-w, plus Noxa binding to Mcl-1 and A1. In vitro competitive binding assays have
shown that Hrk has high binding affinity to Bcl-w and A1 in addition to Bcl-x\textsubscript{L} but not to Mcl-1 (35). Although binding to Bcl-2 is controversial (35), Hrk was originally identified as a Bcl-2 binding protein (11) and we have also verified its interaction with Bcl-2 in prostate cancer cells (I. Chang; unpublished data). Therefore, in addition to Bad, Hrk and Noxa can completely neutralize anti-apoptotic proteins. Intriguingly, anti-apoptotic proteins Bcl-2, Bcl-x\textsubscript{L} and Mcl-1 are significantly expressed and confer resistance to apoptotic signaling in prostate cancer cells (36). Hrk depletion causes partial reduction of 2-ME-induced apoptosis (Fig. 3C). This can be explained by the incomplete knockdown of Hrk but also indicates the possibility that Hrk activation is not entirely responsible for induction of apoptosis and other mechanisms are involved. Although 2-ME causes diverse apoptotic signaling, it is an interesting possibility that both Hrk and Noxa are involved in 2-ME-mediated prostate cancer cell death.

XIAP is often over-expressed in malignant cells and elevated levels of XIAP increase resistance to apoptosis (37). It has been demonstrated that 2-ME reduces the levels of XIAP in prostate cancer cells (38, 39). However, the underlying mechanism is largely unknown. In this study, we found that Bak activation is essential for 2-ME-mediated XIAP reduction and Hrk plays an important role in the process by Bak activation and subsequent cyt \textit{c} release. It has been reported that 2-ME also induces a second mitochondrial-derived activator of caspase (Smac) (4). Therefore, in addition to cyt \textit{c}-mediated caspase activation, the release of Smac from mitochondria presumably mediated by Bak activation may facilitate the processing of caspase-3 by competitive binding to XIAP and liberate the bound caspases. The increased caspase activation could further inactivate XIAP by proteolysis.
It is well known that anti-apoptotic proteins Bcl-2 and Bcl-x_L are substantially expressed and confer resistance to apoptotic signaling in prostate cancer cells (36). 2-ME activation of JNK phosphorylates Bcl-2 and Bcl-x_L to suppress their anti-apoptotic function (9, 10). Aside from this mechanism, Hrk induction is a newly discovered inhibitor of Bcl-x_L, and an initiator of apoptosis in human prostate cancer cells. Since Hrk expression is down-regulated in prostate cancers through promoter methylation (19), a combination of 2-ME with a DNA methylation inhibitor may enhance the Hrk-mediated cell death pathway. According to Lin et al., Hrk is the most effective suppressor of cancer cell growth compared to p53, BRAC1, and PTEN in prostate, breast and ovarian cancer cells which are estrogen-associated cancers expressing high levels of Bcl-2 and Bcl-x_L (24). Moreover, breast and ovarian cancer cells are very sensitive to 2-ME (2). Therefore, it would be of interest to determine whether Hrk is involved in the 2-ME-mediated apoptotic pathway in these types of cancer.

In summary, we have identified Hrk as a critical mediator of 2-ME-induced apoptosis in prostate cancer cells. Our study demonstrates that Hrk links 2-ME activation of JNK signaling to the mitochondrial apoptotic pathway and contributes to 2-ME-mediated XIAP reduction by releasing Bak from the complex with Bcl-x_L. This study provides useful insights into the molecular mechanisms underlying Hrk-mediated apoptosis and also into the future development of 2-ME-based therapeutic strategies for prostate cancer treatment.

Acknowledgements

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References


Table 1. Summary of apoptosis related-genes identified as significantly altered in LNCaP cells by 2-ME treatment for 12 h.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>9.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hrk</td>
<td>6.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DAPK1</td>
<td>3.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TNF</td>
<td>3.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TNFSF8</td>
<td>2.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CASP1</td>
<td>2.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>BIRC8</td>
<td>0.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IGF1R</td>
<td>0.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>XIAP</td>
<td>0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PYCARD</td>
<td>0.2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

aGenes identified as increased or decreased by 2-fold or greater are listed.

bFold change represents the ratio of signal in 2-ME-treated LNCaP cells relative to DMSO-treated LNCaP cells for each primer set.
Figure Legends

Figure 1. 2-ME induces apoptosis and Hrk activation.

(A and B) 2-ME induces apoptotic cell death. LNCaP (●), PC-3 (■) and DU-145 (▲) cells were treated with indicated concentration (A) or 1 μM (B) of 2-ME. Cell death was determined by double staining with Annexin V-FITC and 7-AAD at 48 h (A) or various time points (B) after 2-ME treatment.

(C) Induction of Hrk mRNA in cells treated with 2-ME for the indicated time interval. Hrk expression determined by quantitative RT-PCR is normalized to GAPDH and values are presented as fold increase relative to Hrk expression in DMSO-treated cells (0 h).

**P<0.01, ***P<0.001 compared to 0 h.

(D) Up-regulation of Hrk protein expression as determined by Western blot in cells treated with 2-ME for the indicated time interval.

Figure 2. JNK activation of c-Jun mediates Hrk up-regulation.

(A) JNK-mediated c-Jun activation by 2-ME in prostate cancer cells. Protein expression was determined by Western blot in cells treated with 2-ME for the indicated time interval.

(B) JNK inhibitor, SP600125 attenuates Hrk up-regulation. Hrk expression was determined by quantitative RT-PCR in cells exposed to 2-ME in the absence or presence of 10 μM SP600125 for 24 h. Hrk expression is normalized to GAPDH and values are presented as fold increase relative to gene expression in untreated cells. ***P<0.001
(C) Suppression of endogenous JNK1 by siRNA knockdown. JNK1 expression was determined by Western blot in cells transfected with non-specific (NS) control or JNK1 siRNA for 24 h.

(D) JNK knockdown represses Hrk induction. Hrk expression was determined by quantitative RT-PCR in cells transfected with NS or JNK1 siRNA for 24 h and then treated with 2-ME for 12 h. Hrk expression is normalized to GAPDH and values are presented as fold increase relative to gene expression in NS siRNA transfected cells. ***P<0.001

(E) Suppression of endogenous c-Jun by siRNA knockdown. C-Jun expression was determined by Western blot in cells transfected with NS or c-Jun siRNA for 24 h.

(F) C-Jun knockdown represses Hrk induction. Hrk expression was determined by quantitative RT-PCR in cells transfected with NS or c-Jun siRNA for 24 h and then treated with 2-ME for 12 h. Hrk expression is normalized to GAPDH and values are presented as fold increase relative to gene expression in NS siRNA transfected cells. *P<0.05; **P<0.01

Figure 3. Hrk knockdown prevents 2-ME-mediated apoptotic cell death.

(A) Suppression of the 2-ME-mediated Hrk induction by Hrk siRNA. Hrk expression determined by quantitative RT-PCR in cells transfected with NS or Hrk siRNAs for 24 h and then treated with 2-ME for 12 h. Hrk expression is normalized to GAPDH and values are presented as fold increase relative to Hrk expression in NS siRNA transfected cells. *P<0.05; **P<0.01; ***P<0.001
(B) Suppression of the 2-ME-mediated Hrk protein induction by Hrk siRNA. Hrk expression was determined by Western blot in cells transfected with NS control or Hrk siRNAs for 24 h and then treated with 2-ME for 12 h.

(C) Hrk knockdown prevents 2-ME-mediated apoptotic cell death. Cells were transfected with NS or Hrk siRNAs for 24 h and then treated with 2-ME for 48 h. The number of apoptotic cells was determined by double staining with Annexin V-FITC and 7-AAD.

**P<0.01

(D) Hrk knockdown prevents 2-ME-mediated decrease in mitochondrial membrane potential. Cells were transfected with NS or Hrk siRNA-1 for 24 h and then treated with 2-ME for 6 h. Reduction in mitochondrial membrane potential was determined by monitoring uptake of CMXRos. *P<0.05; **P<0.01

(E) Hrk knockdown represses 2-ME-mediated cyt c release. Cells were transfected with NS or Hrk siRNA-1 for 24 h and then treated with 2-ME for 12 h. Lysates of cytosolic (C) and membrane (M) fractions were subjected to Western blot.

(F) Hrk knockdown impedes 2-ME-mediated caspase activation. Caspase enzymatic activity was assessed with whole cell lysates from cells transfected with NS or Hrk siRNA for 24 h and then treated with 2-ME for 12 h. **P<0.01

Figure 4. Hrk disrupts the association of Bak with Bcl-xL.

(A) Suppression of endogenous Bak by siRNA knockdown. Bak expression was determined by Western blot in cells transfected with non-specific (NS) control or Bak siRNA for 24 h.
(B) Bak knockdown prevents 2-ME-mediated apoptotic cell death. Cells were transfected with NS or Bak siRNA for 24 h and then treated with 2-ME for 48 h. The number of apoptotic cells was determined by double staining with Annexin V-FITC and 7-AAD. *P<0.05

(C) Bak knockdown represses 2-ME-mediated cyt c release. Cells were transfected with NS or Bak siRNA for 24 h and then treated with 2-ME for 12 h. Lysates of cytosolic (C) and membrane (M) fractions were subjected to Western blot.

(D) Bak knockdown impedes 2-ME-mediated caspase activation. Caspase enzymatic activity was assessed with whole cell lysates from cells transfected with NS or Hrk siRNA for 24 h and then treated with 2-ME for 12 h. *P<0.05; **P<0.01

(E) 2-ME-mediated Hrk activation attenuates Bak association with Bcl-xL. Lysates from cells treated with DMSO or 2-ME were immunoprecipitated with anti-Bcl-xL or control IgG antibody and immunoblotted with anti-Bak or -Hrk antibody. Immunoblots of whole cell lysates with anti-Bak antibody represents equal amount of Bak expression in cells.

(F) Hrk knockdown prevents disruption of the interaction between Bak and Bcl-xL.

Cells were transfected with Hrk siRNA-1 and then treated with DMSO or 2-ME for 12 h. Lysates were immunoprecipitated with anti-Bcl-xL or control IgG antibody and immunoblotted with anti-Bak antibody.

(G) Hrk over-expression diminishes Bak association with Bcl-xL. Cells were transiently transfected with either empty vector (pCMV6) or vector containing Hrk cDNA (Hrk) for 24 h. Lysates were immunoprecipitated with anti-Bcl-xL or control IgG antibody and immunoblotted with anti-Bak antibody. Immunoblots of whole cell lysates with anti-
FLAG and -Bak antibodies represent Hrk over-expression and equal amount of Bak expression in cells, respectively.

**Figure 5. Hrk over-expression represses XIAP levels.**

**(A)** Hrk over-expression in LNCaP cells. Hrk expression was determined by Western blot in cells transiently transfected with either empty vector (pCMV6) or vector containing Hrk cDNA (Hrk) for 24 h.

**(B)** XIAP expression is decreased in Hrk over-expressing LNCaP cells. Gene expression was determined by quantitative RT-PCR in cells transiently transfected with either empty vector (pCMV6) or vector containing Hrk cDNA (Hrk) for 24 h. Gene expression is normalized to GAPDH and values are presented as fold change relative to gene expression in pCMV6-transfected cells. ***P<0.001

**(C)** 2-ME reduces endogenous XIAP levels. XIAP mRNA levels determined by quantitative RT-PCR in LNCaP cells treated with 2-ME for 18 h. XIAP expression is normalized to GAPDH and values are presented as fold change relative to expression in DMSO-treated cells. ***P<0.001

**(D and E)** Decrease in XIAP protein levels by 2-ME (D) and Hrk over-expression (E). XIAP protein level was determined by Western blot in cells treated with 2-ME (D) or transiently transfected with either empty vector (pCMV6) or vector containing Hrk cDNA (Hrk) for 24 h (E).

**(F)** Bak knockdown reverses 2-ME-mediated XIAP reduction. XIAP expression was determined by Western blot in cells transfected with NS or Bak siRNA for 24 h and then treated with 2-ME for 12 h.
Figure 6. Chemical structures of 2-Methoxyestradiol (A) and SP600125 (B).
Figure 1

A

![Graph showing apoptosis cell (%) against concentration (µM)]

B

![Graph showing apoptosis cell (%) against time (h)]

C

![Bar graph showing relative Hrk mRNA expression against time (h)]

D

![Western blot analysis showing Hrk and GAPDH expression against time (h)]
Figure 3

A

B

C

D

E

F

Relative Hrk mRNA expression

Hrk

GAPDH

Apoptotic cells (%)

Cyt c

β-actin

Cell death activity
Figure 6

A 2-Methoxyestradiol

B SP600125