Tyrphostin AG825 Triggers p38 Mitogen-activated Protein Kinase-dependent Apoptosis in Androgen-independent Prostate Cancer Cells C4 and C4-2

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Abstract

Prostate cancer (PCa) progression is aided by abnormal autocrine growth factor loops. We screened for small cell-permeable inhibitors of receptor tyrosine kinases that could block their signaling and trigger cell death in PCa cell lines. We found that the human epidermal growth factor receptor (HER)-2/neu inhibitor tyrphostin AG825 is preferentially toxic to PCa cells that are phenotypically androgen independent. These effects were dose and time dependent in the human LNCaP, C4, and C4-2 cell line models of progression and correlated with the inhibition of HER-2/neu phosphoactivation and its down-regulation. In addition, we show that the inhibition of HER-2/neu signaling with AG825 triggers an imbalance between extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase activation, which leads to p38-dependent apoptosis. Inhibition of HER-1 with Compound 56 had no effect. These findings suggest that the androgen-independent C4 and C4-2 cells can be killed by selectively inhibiting their HER-2/neu signaling pathway and provide insights into the mechanism of action of AG825 in PCa cells.

Introduction

The success of androgen ablation therapy in PCa is often cut short by the emergence of androgen ablation-refractory (A-I) disease (1, 2). Recent findings suggest that during progression to the A-I state, PCa cells acquire abnormal autocrine GF loops and decrease their rate of cell death (2). This malignant progression fosters the emergence of A-I growth. Members of the HER family and their ligands have long been implicated in the progression of PCa (2, 3). For example, HER-2/neu and HER-3 as well as transforming growth factor-α-I growth. Members of the HER family and their ligands have long been implicated in the progression of PCa (2, 3). This malignant progression fosters the emergence of A-I disease (1, 2).

Materials and Methods

Cell Culture and Treatment Conditions. LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA), and the LNCaP C4 and C4-2 sublines were obtained from UroCor (Oklahoma City, OK). Cells were used during their 27th to 43rd passage and maintained at 37°C as monolayers in a humidified atmosphere containing 5% CO2. Parental LNCaP cells were cultured in RPMI 1640 (Celox, St. Paul, MN) containing 10% FBS (Life Technologies, Inc., Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone. The LNCaP C4 and C4-2 sublines were grown in T media (8) with 5% FBS (Life Technologies, Inc.) and antibiotics as described above. Media were changed routinely every 72 h. All chemical inhibitors were purchased from Calbiochem (La Jolla, CA), prepared in DMSO at 1000×, and kept at −20°C and away from light. All treatments were carried out under dark conditions as follows: cells were grown to 60–70% confluence (log growth); and AG9, AG825, Compound 56, or DMSO (vehicle) was added to the final concentrations indicated. When MEK (20 μM PD98059) or p38 MAP kinase (5 μM PD169316) inhibitors were used, the MAP kinase inhibitor was added simultaneously with AG825 (50–75 μM).

Morphological Cell Death Assay. At the indicated times, attached and floating cells were pooled and fixed with a solution of 1.5% formaldehyde, 40% methanol, and 10% acetic acid. Bis-benzimide (Sigma Chemical Co.) was added to a final concentration of 1 μg/ml and the cells were incubated for 10 min at room temperature. Ten-μl aliquots were placed on slides and viewed under UV and/or phase-contrast illumination (Carl Zeiss Axioshot). Cells were scored manually (~600 nuclei/sample from three high-powered field quadrants). Apoptotic cells/nuclei exhibited signs of intensely fluorescent condensed chromatin and/or nuclear fragmentation. Necrotic cells (with signs of nuclear DNA hypofluorescence) exhibited cell lysis, pyknosis, or karyolysis. Both apoptotic and necrotic cells were counted as dead and expressed as a percentage of the total number of cells counted in each sample. In general, the total cell death seen in LNCaP cells, ~80% was necrotic, and ~20% was apoptotic. In the C4 and C4-2 sublines, the total cell death was composed of ~90% apoptotic and ~10% necrotic cell death. Data presented are from three independent experiments performed in duplicate.

Western Immunoblotting. All steps for protein sample preparation were carried out at ice temperature by lysing cells (pooled adherent and floating cells) in radioimmunoprecipitation assay buffer [1% PBS without calcium or magnesium (pH 7.4), 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM Na3VO4] and passing the lysate three times through a 27-gauge needle and vortexing, followed by a 15-min centrifugation at 14,000

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The abbreviations used are: PCa, prostate cancer; A-I, androgen-independent; ERK, extracellular signal-regulated kinase; GF, growth factor; HER, human EGF receptor; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; PI3K, phosphatidylinositol 3’-kinase; RTK, receptor tyrosine kinase; FBS, fetal bovine serum.
rpm in a microcentrifuge at 4°C. Equal amounts of protein (20–50 μg) from cell lysate supernatants were denatured in sample buffer, subjected to lithium dodecylsulfate-neutral polyacrylamide gel electrophoresis (LDS/NuPAGE) on 10% or 4–12% gels (Novex, San Diego, CA), and transferred to nitrocellulose membranes (Bio-Rad). The blots were probed with specific antibodies (as per the supplier’s instructions) to Akt, phospho-Akt (Ser-473), doubly phospho-ERK1/2 (Thr-202/Tyr-204), or phospho-p38 MAP kinase (Thr-180/Tyr-182; all from New England Biolabs) and ERK2 (1:5,000; Upstate Biotechnology, Inc., Lake Placid, NY). For immunoblotting of HER family members, 100-μg protein aliquots were used, and the transfer was performed in 15% methanol-containing transfer buffer for 5 h (Novex mini-cell apparatus). The anti-phospho-HER-1 (Y1173) clone 9H2 (Upstate Biotechnology, Inc.) was used at 0.5 μg/ml and incubated for 12 h at room temperature. The anti-phospho-HER-2 (Y1248) Ab-18, clone PN2A, and the anti-HER-2 Ab-15, clone 3B5 from NeoMarkers (Fremont, CA), were used at 0.2 μg/ml and incubated for 12 h at room temperature. The anti-HER-1 antibody (a kind gift from Dr. Nita J. Maihle; Mayo Foundation) was used at a 1:3,000 dilution of the hybridoma supernatant and incubated for 2 h at room temperature. Appropriate secondary horseradish peroxidase-conjugated antibodies were used (1:5,000; incubation, 1 h at room temperature), and immunoreactivities were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Results

Effects of AG825 and Compound 56 Treatment on LNCaP, C4, and C4-2 Cells. In an effort to target autocrine GF loops in PCA cell lines, we focused on the HER family of RTKs. We screened small, cell-permeable inhibitors that preferentially inhibit the tyrosine kinase activity of HER-1 and HER-2/neu for cytotoxicity against these cells under their optimal growth conditions (7, 9). As shown in Fig. 1A, the tyrophostin AG825 (HER-2/neu IC50 = 0.35 μM) dramatically decreased the percentage of surviving cells as judged by the presence of morphological features of apoptosis (Fig. 1A, inset) in C4 and C4-2 after 24 h of treatment (LD50 ~35 and 55 μM, respectively). In the parental LNCaP cells, a comparable decrease in the percentage of surviving cells required higher drug concentrations (LD50 ~100 μM). However, LNCaP cell death was due mainly to necrosis (~80%) as judged by cell lysis and/or karyolysis (data not shown). Neither the inactive tyrophostin AG9 nor Compound 56 (HER-1 IC50 ~6 pm) had any effect on cell growth or viability in any of the cell lines examined (Fig. 1A; data not shown). Thus, AG825 is preferentially toxic to the A-1 C4 and C4-2 cells over the parental androgen-sensitive cell line LNCaP.

Inhibition of HER-1 and HER-2 in LNCaP Cell Lines by Compound 56 and Tyrophostin AG825. To determine if indeed Compound 56 and tyrophostin AG825 were inhibiting their putative targets, we assessed expression and the phosphoactivated state of HER-1 and HER-2/neu in control and treated cells. To this end, we used phosphorylation-specific antibodies to detect the activated forms of HER-1 and HER-2/neu. Because activation of RTKs leads to autotransphosphorylation (4, 5), the presence of phosphorylated RTK protein at canonical tyrosines equates with the activation state of the respective RTK. Under these experimental conditions, 50 μM AG825 was required for consistent HER-2/neu inhibition as determined by the phospho-specific antibodies. Therefore, cells were treated for 24 h with 50 μM AG825, AG9, or Compound 56, and whole cell lysates were immunoblotted for phosphoactive or whole HER-1/neu or HER-2/neu proteins. As shown in Fig. 1B, HER-2/neu activation was specifically inhibited in AG825-treated cells, which was not the case with AG9- or Compound 56-treated cells, confirming the specificity and bioactivity of AG825. Likewise, HER-1 activation was inhibited by Compound 56 treatment but not by AG825 or AG9 treatment, demonstrating the bioactivity and specificity of Compound 56. Importantly, neither Compound 56 nor the inactive tyrophostin AG9 affected cell viability (Fig. 1A; data not shown). Treatment of cells with AG825 induced a dramatic decrease in immunodetectable HER-2/neu protein as well as a modest decrease in HER-1 levels. The latter effects were dose and time dependent (Fig. 1B; data not shown). Nevertheless, prolonged exposure of these blots did not reveal a phospho-HER-2/neu signal in AG825-treated samples. These changes in immunodetectable proteins may be due to inhibition-dependent degradation of HER-2/neu and other potentially associated GF receptors such as HER-1, as has been shown in other cells (10). These findings suggest that the cytotoxicity of AG825 is specific, and its preferential effects on the aggressive C4 and C4-2 cells correlate with inhibition and diminished levels of HER-2/neu.

HER-2/neu Inhibition: Effects on the PI3K-Akt, ERK1/2, and p38 MAP Kinases. Two major pathways by which RTKs convey their signals involve PI3K and Ras (5). To investigate the potential effects of RTK inhibition on these two major downstream effectors, exponentially growing cell monolayers were treated for 24 h and subjected to immunoblot analysis for Ser-473 phosphoactive Akt, an indicator of PI3K activity (5). As shown in Fig. 2, A and B, neither AG825 nor Compound 56 affected Akt phosphoactivation despite the inhibition of the respective RTKs and the ongoing cell death. The conditions used for these studies maintained serum in the growth
media of these cells. Thus, it is conceivable that the presence of serum GFs maintained activation of the PI3K-Akt signaling pathway through other GF receptors, precluding detectable changes under these conditions.

We next examined the Ras to MAP kinase cascades to determine whether they were affected by these drugs. The ERK1/2 MAP kinase was analyzed because of its importance for growth and survival in many cell types (11). The p38 MAP kinase cascade was examined because of its importance in the mediation of stress signals (12), and this cascade could become important to cells under the stress of RTK inhibition. On analysis with phosphorylation, active state-specific antibodies to ERK1/2 and p38 MAP kinases demonstrated that in the parental LNCaP cells, p38 MAP kinase activation by AG825 was accompanied by ERK1/2 activation (Fig. 2B). Cell death in the LNCaP cells was predominantly necrotic (~80%) and usually took place at much later times (36–48 h) and to a lesser extent than that in C4 or C4-2 cells (~40% versus ~80%; Figs. 1 and 2; data not shown).

We next considered the time course of MAP kinase activation with AG825 treatment. To this end, cells were treated as described above and harvested at various times for Western blotting analysis. To obtain a semiquantitative measure of the temporal changes in ERK1/2 and p38 activation, the immunoreactivity bands were assessed by densitometry from two independent experiments and are depicted in Fig. 2D as relative levels of either MAP kinase activation signal. As seen in Fig. 2, C and D, C4 and C4-2 cells consistently showed p38 activation between 6 and 24 h of treatment with AG825, whereas ERK1/2 MAP kinase activation was low or absent. This was not the case for LNCaP cells, which showed a simultaneous ERK1/2 hyperactivation whenever p38 MAP kinase was activated (Fig. 2, B–D). Neither the inactive tyrphostin AG9 nor Compound 56 had any effect on cell death, PI3K-Akt activity, or ERK1/2 or p38 hyperactivation seen with AG825 in C4 or C4-2 cells (Fig. 2). In addition, differences in detectable phosphoactivities were not due to differences or changes in MAP kinase expression levels during AG825 treatment as is shown for ERK2 protein levels, which also serve as a loading control. Taken together, these data suggest that the effects of AG825 are specific and trigger a p38 kinase hyperactivation that is not coupled to ERK1/2 activation in C4 and C4-2 cells as it is in LNCaP cells. These events correlated with the greater cell death seen in C4 and C4-2 cells. Moreover, this effect was not seen in LNCaP cells. These findings are consistent with previous observations by others in rat PC-12 cells, where the dynamic balance between ERK1/2 and c-Jun-NH2-terminal kinase-p38 pathways is important in determining whether PC-12 cells lived or died by apoptosis (13).

Inhibition of p38 but not ERK1/2 Activation Blocks Apoptosis Attributable to AG825 Treatment in C4 and C4-2. The above-mentioned findings suggested that inhibiting p38 activation would block apoptosis and that inhibiting ERK1/2 activation might enhance this process. To test these possibilities, we used a pharmacological inhibitor of p38 (PD169316; Ref. 12). Likewise, activation of ERK1/2 was blocked with the MEK inhibitor PD98059 (14). As shown in Fig. 3, inhibition of p38 activation but not ERK1/2 activation blocked apoptosis in C4 or C4-2 cells at 24 h of AG825 treatment. A slight
increase in cell death was seen in cells cotreated with AG825 plus the MEK inhibitor (PD98059; Fig. 3B). As expected, treatment of cells with AG825 alone or in combination with MEK or p38 inhibitors led to inhibition of HER-2/neu phosphoactivation (Fig. 3A). Combined inhibition of MEK and HER-2/neu consistently led to higher levels of p38 activation. Of note, inhibition of ERK1/2 or p38 activation did not inhibit HER-2/neu kinase activity, whereas combined inhibition of MEK and HER-2/neu consistently led to higher levels of p38 activation. Thus, the necrotic cell death seen in LNCaP cells at change the amount of necrotic cell death seen in parental LNCaP cells.

Discussion

During androgen-deprived conditions, autocrine GF support may increase in cell death was seen in cells cotreated with AG825 plus the MEK inhibitor (PD98059; Fig. 3B). As expected, treatment of cells with AG825 alone or in combination with MEK or p38 inhibitors led to inhibition of HER-2/neu phosphoactivation (Fig. 3A). Combined inhibition of MEK and HER-2/neu consistently led to higher levels of p38 activation. Of note, inhibition of ERK1/2 or p38 activation did not change the amount of necrotic cell death seen in parental LNCaP cells (data not shown). Thus, the necrotic cell death seen in LNCaP cells at higher concentrations of AG825 (Fig. 1A) may have resulted from nonselective means. Regardless of the mechanism of this necrotic death, the fact that this PCa cell line also died due to AG825 treatment (but not AG9 or Compound 56 treatment) underscores the potential utility of this drug or similarly acting drugs in blocking HER-2/neu signaling and affecting the survival of PCa cells. Taken together, these findings suggest that the AG825-mediated apoptotic cell death in A-I C4 and C4-2 cells is dependent on overactivation of p38 MAP kinase. In addition, cytotoxicity in these cell lines is modestly enhanced when ERK1/2 activation is blocked.

In summary, our findings show that AG825 treatment of LNCaP parental LNCaP cell line. Cell death of the latter was predominantly necrotic under the same conditions. The cytotoxic effects correlated specifically with the inhibition of HER-2/neu signaling and HER-2/neu down-regulation because inhibition of HER-1 with Compound 56 exhibited no such effects. It is unclear whether these effects are preferentially cytotoxic to the A-I sublines. In certain cell types that overexpress HER-2/neu, its inhibition preferentially sensitizes them to apoptotic stimuli compared with non-HER-2/neu-overexpressing cells (16, 17). It is conceivable that malignant cells expressing HER-2/neu may develop a hyperdependence on this GF. There appears to be no gross difference in the expression levels of HER-2/neu in the LNCaP, C4, and C4-2 cell lines. However, immunofluorescence-based detection of HER-2/neu in C4 and C4-2 cells has revealed a greater percentage (18–27%) of cells with predominately plasma membrane-localized HER-2/neu as compared with parental LNCaP cells (1–3%). Thus, localization may play a role in the effects seen in C4 and C4-2 cells. In this context, the down-regulation of HER-2/neu with AG825 treatment may compromise trophic signals via HER-2/neu in C4 and C4-2 cells, thereby leading to p38-dependent apoptosis.

The concentrations of AG825 necessary to produce a biological effect in these studies were relatively high (≥10 μM). However, tyrophostins such as AG825 have poor aqueous solubility. This makes it difficult to know the actual concentrations of freely solubilized AG825 in the cell-media cultures, which would be expected to influence its bioavailability. In addition, our studies do not rule out the possibility of other unknown potential AG825 targets that may be involved in these cells. A difference in the repertoire of kinases expressed and used by C4 and C4-2 cells compared with parental LNCaP cells could contribute to the differences seen in HER-2/neu inhibition sensitivity.

Detection of HER-2/neu expression levels in primary or metastatic PCa has been variable (18, 19). However, what has been consistent is its association with aggressive and progressing disease (18). For example, HER-2/neu expression in A-I LAPC-4 xenograft sublines has been shown to be functionally important to the A-I phenotype (19). Moreover, its expression can endow A-I growth in LNCaP cells (19). Although HER-2/neu overexpression alone is unlikely to account for PCa progression in all cases, the fact that it alone can promote A-I growth suggests that it is a critical player. Furthermore, overexpression may not be required because HER-2/neu has been shown to be required for cytokine signaling such as interleukin 6 in PCa cells (20). Thus, multiple GF signaling cascades may benefit HER-2/neu expression even at low levels. Therefore, targeting HER-2/neu kinase activity may have a major impact on the growth and survival of PCa cells by affecting a myriad of signaling events.

Previous studies have shown elevated expression of several MAP kinase cascade members (MAP kinase phosphatase 1, ERK1, c-Jun-NH2-terminal kinase, and p38) that may enhance the net MAP kinase activity in PCa tumors (21, 22). A correlation was shown between increased levels of ERK1 and ERK2 phosphoactivation, Gleason score, and progression to A-I growth in PCa tumors (22). Such findings suggest important roles for the MAP kinases in PCa progression. In this context, our findings suggest potential strategies to target MAP kinases in PCa cells in a way that has a major impact on their survival or death. Furthermore, chemosensitization or additive effects may be obtained with HER-2/neu inhibition in combination with other cytotoxic agents, as has been shown in other systems (16, 17).

In summary, our findings show that AG825 treatment of LNCaP cell lines inhibits HER-2/neu signaling, promotes HER-2/neu down-

4 Unpublished data.
regulation, and results in a higher rate of cell killing in LNCaP cells as compared with the A-I C4 and C4-2 cells. The preferential cytotoxicity correlated with an imbalance in ERK1/2 and p38 activation and was sensitive to p38 inhibition. Given the implicated role of HER-2/neu in aggressive PCa tumors and of MAP kinases in PCa progression (18–22), our findings illustrate the potential utility of small chemical inhibitors against this GF receptor in PCa cells, pointing to additional therapeutic options.

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References

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