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Hexamethylene amiloride engages a novel reactive oxygen species- and lysosome-dependent programmed necrotic mechanism to selectively target breast cancer cells

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ABSTRACT

Anticancer chemotherapeutics often rely on induction of apoptosis in rapidly dividing cells. While these treatment strategies are generally effective in debulking the primary tumor, post-therapeutic recurrence and metastasis are pervasive concerns with potentially devastating consequences. We demonstrate that the amiloride derivative 5-(*N*,*N*-hexamethylene) amiloride (HMA) harbors cytotoxic properties particularly attractive for a novel class of therapeutic agent. HMA is potently and specifically cytotoxic toward breast cancer cells, with remarkable selectivity for transformed cells relative to non-transformed or primary cells. Nonetheless, HMA is similarly cytotoxic to breast cancer cells irrespective of their molecular profile, proliferative status, or species of origin, suggesting that it engages a cell death mechanism common to all breast tumor subtypes. We observed that HMA induces a novel form of caspase- and autophagy-independent programmed necrosis relying on the orchestration of mitochondrial and lysosomal pro-death mechanisms, where its cytotoxicity was attenuated with ROS-scavengers or lysosomal cathepsin inhibition. Overall, our findings suggest HMA may efficiently target the heterogeneous populations of cancer cells known to reside within a single breast tumor by induction of a ROS- and lysosome-mediated form of programmed necrosis.

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Introduction

While the past several decades have witnessed unparalleled advances in our understanding of the cellular and molecular mechanisms regulating cancer initiation and progression, substantial challenges remain with respect to cancer treatment efficacy. Significantly, commonly employed chemotherapeutics often fail to distinguish between tumor and normal tissue, inducing the indiscriminate death of rapidly dividing cells such as those of the digestive tract, bone marrow, mucous membranes and hair follicles [1]. In addition, cancer cells are particularly resistant to apoptotic death induced by conventional and targeted therapies [1], engaging pro-survival pathways such as phosphatidylinositol-3 kinase (PI3K)/Akt and extracellular regulated kinase (Erk; [2]) while inhibiting pro-apoptotic pathways [3]. Thus, agents that can distinguish normal from cancer cells, and that induce forms of cell death distinct from apoptosis and independent of cell cycling, may offer a superior therapeutic approach to improving patient outcomes.

The FDA-approved potassium-sparing diuretic amiloride was originally developed as a specific inhibitor of the epithelial sodium channel (ENaC) of renal collecting ducts [4]. When administered to cells at moderate (5–50 μM) to high (~0.5 mM) concentrations, amiloride can also inhibit sodium-hydrogen exchange (NHE; [5]), drug efflux channel activity [6] and urokinase receptor (uPAR) activity and expression [7–9], and perturb cellular metabolism via loss

Abbreviations: HMA, 5-(*N*,*N*-hexamethylene) amiloride; DCFH-DA, carboxy-2′,7′-dichloro-dihydro-fluorescein diacetate; ROS, reactive oxygen species; PI3K, phosphatidylinositol-3 kinase; PKB, protein kinase B; Erk, extracellular regulated kinase; ENaC, epithelial sodium channel; NHE, sodium hydrogen exchanger; uPAR, urokinase receptor; UCD 38B, 5-benzylglicinal amiloride; NAc, N-acetyl cysteine; TBHP, tert-Butyl hydrogen peroxide; zVADfmk, z-VAD.fmk Z-Val-Ala-Asp (OMe) fluoromethylketone; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute medium; EGF, epidermal growth factor; MEBM, mammary epithelial basal media; FBS, fetal bovine serum; LDH, lactate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; RIPK, receptor-interacting kinase; LC3, light chain 3; MLKL, mixed lineage kinase domain like; AIF, apoptosis-inducing factor; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; mTOR, mechanistic target of rapamycin; mTORC1, mTOR complex 1; v-ATPase, vacuolar H+ adenosine triphosphatase.

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of ATP [10] and calcium and potassium transport [5]. Given these effects, it is not surprising that high concentrations of amiloride act in an anticancer capacity. Notably, amiloride reduced the growth of hepatoma, mammary adenocarcinoma and prostate tumor cells *in vivo*, inhibited the formation of mutagen-induced lesions in the liver, pancreas, and colon, and suppressed the establishment of metastatic lesions (reviewed in [11]). Collectively, these observations are consistent with the cytostatic and anti-migratory effects of amiloride when employed at concentrations known to inhibit NHE1 and other cell surface ion channels, as well as the extracellular protease urokinase plasminogen activator (uPA; [11]).

Interestingly, it has been reported that very high concentrations of amiloride (~0.5 mM) induce the death of glioma cells, and that this cytotoxicity cannot be mimicked by independently inhibiting NHE1 with cariporide [12]. Expanding on these observations, we and others have demonstrated that the amiloride derivative 5-benzylglicinal amiloride (UCD38B) is cytotoxic toward cultured glioma [12,13] and breast cancer cells [14]. In both cases, UCD38B cytotoxicity was attributed to the induction of a caspase-independent, non-apoptotic cell death resembling programmed necrosis [13,14]. Further investigation suggested that UCD38B-induced cytotoxicity toward glioma cells correlates with the mistrafficking of components of the uPA system following drug treatment [15]. Taken together, these data point to UCD38B as a potential treatment for refractory cancers. However, the high dose of UCD38B (>100 μM) required to efficiently elicit specific anticancer effects precludes its clinical utility.

Our previous observations point to a strong correlation between the cytotoxicity of amiloride derivatives and their ability to access the cell interior [13,14], suggesting that cytotoxic amilorides may act on one or more intracellular targets to trigger programmed necrotic cell death. Our observations also suggest that modification of the amine at the C(5) carbon of amiloride with hydrophobic moieties (Fig. 1A) augments cytotoxic potency, likely by elevating cell permeability of the derivatives. Here we demonstrate that the highly hydrophobic analog 5-(*N*,*N*-hexamethylene) amiloride (HMA) acts at micromolar concentrations to induce the programmed necrotic death of cultured breast cancer cells via a novel lysosome- and reactive oxygen species (ROS)-dependent mechanism. Critically, we observe that HMA rapidly and selectively kills breast tumor cells relative to non-transformed cells derived from a variety of tissue types, and does so in a manner independent of the cell cycle. Taken together, our observations underscore the potential of HMA as a novel anti-cancer therapeutic that can specifically attack poorly proliferative tumor cell subpopulations by inducing their programmed necrotic death.

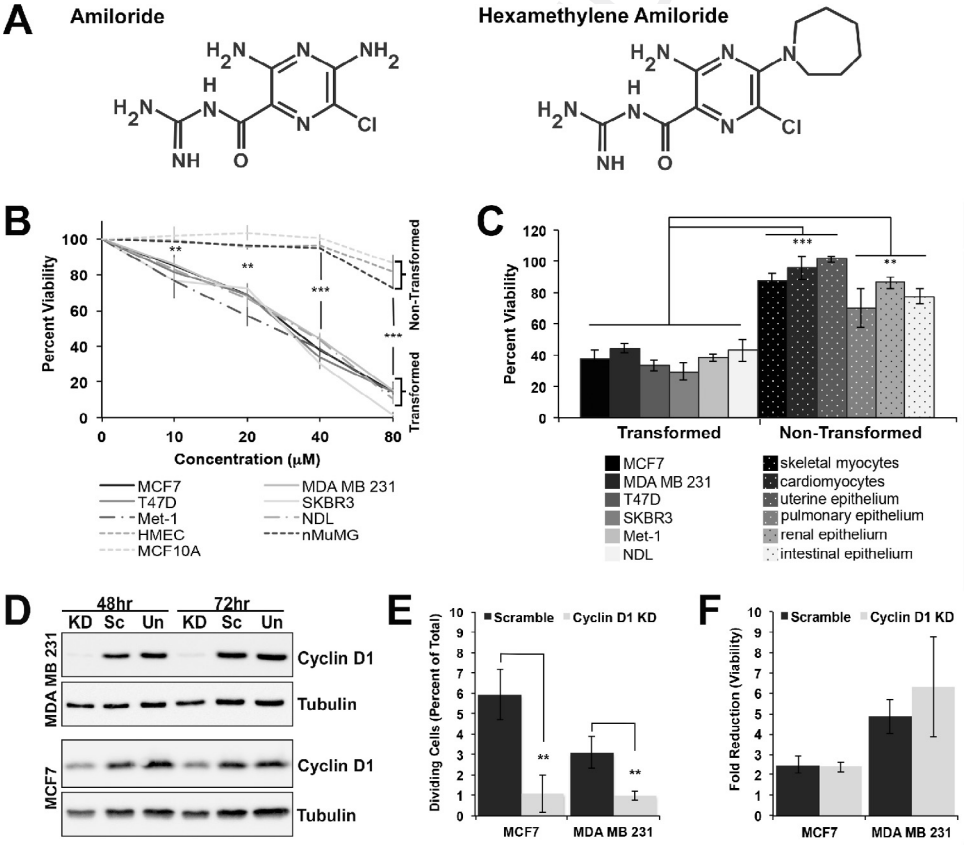

Fig. 1. HMA specifically depletes breast cancer cells irrespective of their molecular profile or proliferative status. (A) The chemical structures of amiloride and HMA are depicted. (B) The viabilities of human and mouse non-transformed and transformed cell lines treated for 24 hrs with varying concentrations of HMA were assessed by trypan blue exclusion. Data are presented as averages of at least four independent biological trials and expressed as a percent of vehicle control ± SEM. (C) The indicated non-transformed primary cells were exposed for 24 hrs to either vehicle or 40 μM HMA, and their relative viabilities were compared to tumor cell lines from panel B. Data are presented as averages of at least three biological replicates and expressed as a percent of control ± SEM. HMA did not significantly reduce the viability of non-transformed cells ($p > 0.05$ for all tissue types). (D) MDA-MB-231 and MCF7 cells were left untransfected (Un), or were transfected for 48 hrs or 72 hrs with either scrambled (Scr) or cyclin D1 knockdown (KD)-directed siRNA oligonucleotides. Lysates were immunoblotted for cyclin D1 and tubulin loading control. (E) Mitotic MCF7 or MDA-MB-231 cells in anaphase or telophase were determined by morphologic features following DAPI staining after transfection with scrambled or cyclin D1-directed siRNAs. Data are presented as the average mitotic percentage of the total cell population for three replicate experiments ± SEM. (F) Following control or siRNA-mediated cyclin D1 knockdown, cells were exposed to vehicle or 40 μM HMA for 24 hrs, and the total number of viable cells was assessed by trypan blue exclusion. Data are presented as the fold reduction in cell viability ± SEM as determined from three biological replicates. $^{**}p < 0.01$; $^{***}p < 0.001$.

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