Selective Toxicity of MKT-077 to Cancer Cells Is Mediated by Its Binding to the hsp70 Family Protein mot-2 and Reactivation of p53 Function


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Abstract

MKT-077, a cationic rhodacyanine dye analogue has been under preclinical cancer therapeutic trials because of its selective toxicity to cancer cells. Its cellular targets and mechanism of action remain poorly understood. Here we report that MKT-077 binds to an hsp70 family member, mortalin (mot-2), and abrogates its interactions with the tumor suppressor protein, p53. In cancer cells, but not in normal cells, MKT-077 induced release of wild-type p53 from cytoplasmically sequestered p53-mot-2 complexes and rescued its transcriptional activation function. Thus, MKT-077 may be particularly useful for therapy of cancers with wild-type p53.

Introduction

MKT-077 (formerly known as FJ-776) is a water soluble delocalized lipophilic cation/rhodacyanine analogue (related to rhodamine 123) dye that exhibits significant antitumor activity in a variety of in vitro and in vivo model systems. Because of its positive charge, it can pass through the hydrophobic barriers of cell membrane lipid bilayers and is preferentially retained inside mitochondria (high negative charge). The higher mitochondrial membrane potential of carcinoma cells accounts for its preferential retention and cytotoxicity (1–4), and this has led to its use in preclinical and clinical cancer therapeutic trials (5, 6). The molecular mechanism of the growth arrest induced by MKT-077 and its selective toxicity to cancer cells and its cellular targets have not been elucidated thus far. It has been proposed that in addition to the reversible impairment of mitochondrial function (1), MKT-077 has different cellular targets in normal versus malignantly transformed cells (7). On the basis of the higher sensitivity of human bladder carcinoma, EJ (carrying an oncogenic c-Ha-Ras mutant) and v-Ha-Ras-transformed NIH 3T3 cells as compared with their normal counterparts, oncogenic Ras mutant proteins have been credited for its selective toxicity (7). We report here that MKT-077 binds to an hsp70 family protein, mortalin (mot-2). There are two mortalin (mot) proteins in mouse that differ in two amino acids in the COOH-terminus (8). The present study, we have investigated the binding of MKT-077 to a hsp70 family member, mortalin (mot) because it shows differential cellular localizations in normal and immortal/transformed cells (9).

Results and Discussion

By MKT-077 Sepharose affinity chromatography, two cellular proteins, actin (M, 45,000) and hsc70 (M, 75,000) were previously identified as possible MKT-077 targets in ras-transformed cells (7, 12). In the present study, we have investigated the binding of MKT-077 to a hsp70 family member, mortalin (mot) because it shows differential cellular localizations in normal and immortal/transformed cells (9). MKT-077-conjugated Sepharose beads pulled down mortalin (detected by mortalin-specific antibody; Ref. 9) from cellular lysates (Fig. 1a). We next sought to define the binding region of MKT-077 on mortalin. Various deletions of V5-tagged mortalins were expressed in COS 7 cells to perform pull-down assays with control or MKT-077-related compounds confirmed this to be the pathway for their action. Furthermore, the absence of mot-p53 interactions in normal cells accounts, at least in part, for the selectivity of MKT-077 for cancer cells.

Materials and Methods

Cell Culture and MKT-077 Treatments. Cells were cultured in Dulbecco’s modified Eagle’s MEM supplemented with 10% fetal bovine serum. MKT-077 [1-ethyl-2-[[3-ethyl-5-(3-methylbenzothiazolin-2-ylidene)]-4-oxothiazolidin-2-ylidinemethyl]] pyridinium chloride or its derivatives were added to the culture medium for either 48 h (growth assays) or 24 h (reporter assays).

MKT-077 Pull-Down and Coimmunoprecipitation Assays. Cell lysates (300 μg) were incubated with MKT-077-Sepharose beads for 90 min at 4°C with slow rotation. MKT-077 complexes were washed six times with NP40 lysis buffer [20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 1% NP40], boiled in SDS loading buffer, resolved on SDS-PAGE and then Western blotted with indicated antibodies. COS 7 cells either untreated or MKT-077-treated or transfected with expression plasmids (Invitrogen) encoding various V5-tagged mortalin deletion mutants were used for p53-mot immunoprecipitation assays. Polyclonal anti-p53 (CM-1; Novocastra) antibody was used for p53-immunoprecipitation followed by Western detection of endogenous mortalin by monoclonal antimitortalin (mHSP70; Affinity Bioreagents, Inc.) or V5-tagged mortalin by anti-V5 tag antibody (Invitrogen).

Immunostaining. Cells were double-stained with monoclonal anti-p53 (PAb421; Calbiochem) and polyclonal antimitortalin (9) antibodies, and visualized by secondary staining with FITC-conjugated sheep antimouse IgG and Texas Red-conjugated donkey antirabbit IgG (Amersham Corp.). The cells were examined under Fluoview Confocal Laser Scanning Microscope (Olympus Corp.). The individual mortalin and p53 images were seen as red and green fluorescence, respectively.

Reporter Assay. Cells were stably transfected with the p53-responsive luciferase reporter plasmid, PG13-luc (kindly provided by Dr. Bert Vogelstein, Howard Hughes Medical Institute, Baltimore, MD). Isolated clones were treated with MKT-077 compounds for 24 h. Luciferase assays (Dual-Luciferase Reporter Assay System; Promega) were performed, and the values were calculated per microgram of the protein as determined by Bradford protein assay.

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KMT-077 ABROGATES mot-p53 INTERACTIONS

Fig. 1. KMT-077 binds to mot protein, and the region of mot that is required for this interaction is contained within amino acids 252–310. a. KMT-077 pull-down assay. COS 7 cell lysates were incubated with control or KMT-077 Sepharose beads for 90 min at 4°C followed by six washings with NP40 lysis buffer. Bound proteins were resolved on SDS-PAGE and analyzed by Western blotting with anti-mortalin antibody. b. COS 7 cells were transfected with expression plasmids encoding V5-tagged mortalins. Cell lysates were incubated with KMT-077 Sepharose beads. KMT-077-bound mortalin fragments were detected by anti-V5 tag antibody. KMT-077 pulled down exogenous mortalin mutants as indicated and the binding region on mortalin was localized to amino acid residues 252–310. kDa, M, in thousands.

Fig. 2. KMT-077 dissociates mot from p53, resulting in increased p21\textsuperscript{WAF1} levels. a. Western blotting of KMT-077 treated cells with anti-mot, -p21\textsuperscript{WAF1}, and -actin antibodies. Increases in mot, and p21\textsuperscript{WAF1} levels were detected subsequent to KMT-077 treatment. Actin probing was used as a loading control. b. cells treated with indicated concentrations of KMT-077 (Input seen in a) were used for immunoprecipitation of p53 protein with polyclonal anti-p53 antibody. p53-immunocomplexes were analyzed for the presence of mot by Western blotting with monoclonal anti-mot antibody. Immunoprecipitated p53 was detected by a monoclonal anti-p53 antibody. Mot was not detected in p53 immunocomplexes from cells treated with high concentrations of KMT-077.
tion of MKT-077, FJ-5744, or FJ-5826 (Fig. 3c and data not shown). Untreated cells showed nuclear p53 in only about 10% cells. Notably, cellular distribution of mot in MKT-077-treated transformed cells reverted back to that of normal type (Fig. 3c). When MRC-5 cells were treated with equivalent concentrations of MKT-077, they exhibited an increase in mot similar to that in the transformed cells, but there was no change in the subcellular distribution of p53 (Fig. 3c). Taken together with the above data, abolition of the cytoplasmic retention of p53 by mot-2 and restoration of its transcriptional activation function, thus, emerges as a mechanism for MKT-077-induced selective growth arrest in transformed cells.

Differential cellular distributions of mortalin in normal and immortal-transformed cells has been described previously (9). Induction of senescence in transformed cells by introduction of a single chromosome (15), chromosome-fragments, and genes (16) or chemicals (17) has led to the reversion of their nonpancytosolic distribution pattern of mortalin to the pancytosolic type characteristic of normal cells. A similar alteration in mortalin-staining pattern in transformed cells, as a result of treatment with MKT-077 compounds (Fig. 3c), indicated that the growth arrest of these cells may involve induction of a senescence-like phenotype. This has also been suggested by another study in U937 cells in which telomere erosion (18) was observed in response to treatment with MKT-077 derivative, FJ5002. In normal cells, pancytosolic mortalin and wild-type p53 do not colocalize/interact (10). In this regard, shift of the mortalin-staining pattern from nonpancytosolic to pancytosolic suggested abrogation of mot-p53 interactions, which is well supported by the biochemical analyses described above. Because in normal cells pancytosolic mortalin and p53 do not interact, the action of MKT-077 by this pathway accounts, at least in part, for its selectivity for cancer cells. Consistent with this, MKT-077 treatment did not induce nuclear translocation of p53 in MRC-5 cells (Fig. 3c).

Given this pathway of action for MKT-077 class of compounds, it can be anticipated that it will be more effective for tumors such as neuroblastomas, breast carcinomas, and teratocarcinomas, which have wild-type p53 that is inactivated by cytoplasmic sequestration. Taking together our data on the rescue of p53 function by MKT-077 with the reports that MKT-077 and its analogue FJ-5002 cause inhibition of telomerase activity (18) and that p53 inactivates telomerase (19), it is likely that these effects are linked. Furthermore, considering the proposed role for mortalin as one of the chaperonins, it is expected to interact temporarily with other proteins and aid in their correct folding and cellular localization functions. The binding of MKT-077 to mortalin, thus, may also cause effects independent of that of the p53 pathway described here. We have recently detected an association of mortalin with an hsp90 family member, GRP94.3 Notably, in this regard, the chaperonin function of hsp90 has been shown to be essential for telomerase activity (20). Therefore, it would be interesting to investigate whether the binding of MKT-077 to mortalin can affect the function of chaperonin complexes that are critical for the determination of cell growth and arrest. In our cell-growth assays, SV40-immortalized skin fibroblasts, GM847 (EC$_{50}$ 1.02 μM) and 4NQO-transformed liver fibroblasts, SUSM-1 (EC$_{50}$ 1.02 μM) are telomerase negative and possess alternative telomere lengthening (ALT) mechanism (21) were sensitive to MKT-077, which suggests that telomerase is not a requirement for MKT-077-induced growth arrest of cancer cells. Similarly, A1698 (EC$_{50}$ 0.69 μM) with mutant p53 was sensitive, which implied that other pathways, e.g., actin bundling (7, 12), may operate independent of that of wild-type p53. Elucidation of these additional pathways may facilitate the development of effective cancer therapeutics. In summary, we have shown a novel pathway of MKT-077-induced growth arrest. By occupying the p53-binding region of mot, it causes abrogation of mot-p53 interactions, nuclear translocation, and rescue of wild-type p53 function. Lack of mot-p53 interactions in normal cells contributes to its selective toxicity to cancer cells.

References


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