PRECLINICAL EVALUATION OF NOVEL TRIPHENYLPHOSPHONIUM SALTS WITH BROAD-SPECTRUM ACTIVITY

Abstract # 2542

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Abstract

Proliferation under selective pressures exerted by an unstable microenvironment, requires tumor cells employ adaptive mechanisms that confer growth advantage. Adaptation to adverse conditions results in cellular phenotypes that typify neoplastic transformation and offer unique opportunities for selective targeting of cancer cells. Agents that target tumor cell mitochondria with high selectivity hold clinical significance due to the adaptive, modulatory and essential role of this organelle in cancer cell energy production, metabolism and apoptosis. To this end, we have identified a series of novel, mitochondriotropic phosphonium salts, (TP compounds), that have shown broad-spectrum anti-cancer and anti-angiogenic activity in preclinical evaluation. A high-throughput MTT-based screen of over 10,000 drug-like small molecules for anti-proliferative activity identified the phosphonium salts TP187, 197 and 421 and numerous close analogues as having IC_{50} concentrations in the sub-micromolar range. TP treatment induced cell cycle arrest, lowered oxygen consumption, and increased mitochondrial superoxide production. Administered as single agents in a mouse model of human breast cancer, TP compounds significantly decreased tumor growth with no observed toxicities. Protein microarray data demonstrated significant down-regulation of integrin and growth factor mediated signaling pathways governing key processes including cancer cell survival, proliferation and tumor angiogenesis. At low micromolar concentrations, TP compounds prevented integrin-mediated cell adhesion to fibronectin and vitronectin coated substrates and tumor cell haptotaxis on vitronectin coated boyden chambers. Similar concentrations of TP compound also prevented growth factor induced endothelial cell tube formation in 3-D culture using basement membrane extracts. Taken together, these results suggest that as mitochondria-targeted agents, TP compounds act to inhibit tumor cell proliferation and angiogenic capacity.

Methods

Cell viability assays were performed in indicated cell lines using MTT and colony formation assays. DNA content was measured by flow cytometry on TP treated cells fixed and permeablized with ethanol and stained with propidium iodide/RNase A. Animal studies were performed in female nude mice injected subcutaneously with MDA-MB-435 breast cancer cells. Treatments were administered 5 times weekly at a dosage of 10 mg/kg body weight. TP421, TP197 and Mitotracker Green FM staining were visualized by fluorescence microscopy to determine uptake and cell morphology. Mitochondrial membrane potential and superoxide production were measured by flow cytometry using JC-1 membrane potential sensitive dye and Mitosox Red superoxide indicator respectively. Oxygen consumption and glycolytic capacity were measured using the Seahorse XF-24 analyzer. Proteomics analysis was performed on the IPA platform using data generated from kinexus antibody microarray of TP421 treated MDA-MB-435 cells. Tube formation assays were performed in PAECs and HUVECs. Further studies of angiogenic capacity were performed in HUVEC.

Conclusions

TP compounds are cytotoxic in a panel of human cancer cell lines, acting to arrest cell cycle progression in cancer cell lines. TP compounds inhibit tumor growth *in vivo* through decreasing tumor cell proliferation and induction of caspase-3 cleavage in a mouse xenograft model. TP compounds localize to the mitochondria, alter mitochondrial morphology, oxygen consumption and glucose utilization, increases superoxide production and decrease mitochondrial membrane potential. TP 421, TP197 and several close analogues exhibit anti-angiogenic properties. Proteomics analysis of microarray data suggests TP421 and analogues affect proteins involved in key cellular functions required for angiogenesis and tumor cell survival.

Acknowledgments

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Results

TP compounds are cytotoxic in a cancer cell panel

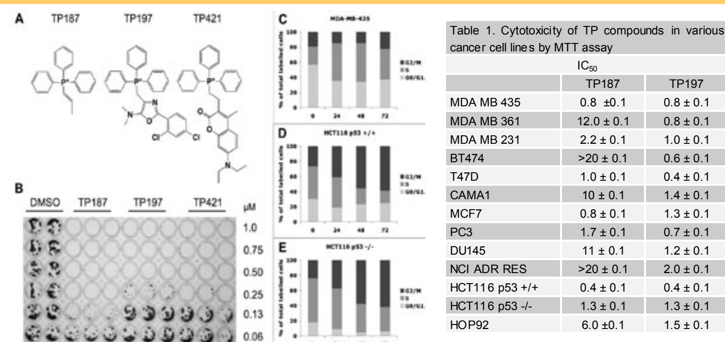


Table 1. Cytotoxicity of TP compounds in various cancer cell lines by MTT assay		
	IC_{50}	
	TP187	TP197
MDA MB 435	0.8 ±0.1	0.8 ± 0.1
MDA MB 361	12.0 ± 0.1	0.8 ± 0.1
MDA MB 231	2.2 ± 0.1	1.0 ± 0.1
BT474	>20 ± 0.1	0.6 ± 0.1
T47D	1.0 ± 0.1	0.4 ± 0.1
CAMA1	10 ± 0.1	1.4 ± 0.1
MCF7	0.8 ± 0.1	1.3 ± 0.1
PC3	1.7 ± 0.1	0.7 ± 0.1
DU145	11 ± 0.1	1.2 ± 0.1
NCI ADR RES	>20 ± 0.1	2.0 ± 0.1
HCT116 p53 +/+	0.4 ± 0.1	0.4 ± 0.1
HCT116 p53 -/-	1.3 ± 0.1	1.3 ± 0.1
HOP92	6.0 ±0.1	1.5 ± 0.1

Figure 1. TP compounds decrease cell proliferation and induce cell cycle arrest independent of p53 status. (A) Structures of lead compounds TP187, 197 and 421. (B) Colony formation assay in HCT116 p53 +/+ cells treated continuously for 7 days with TP compounds at the noted concentrations. DNA content was analyzed in propidium iodide stained MDA-MB-435 (C), HCT116 p53 +/+ (D) and HCT116 p53 -/- (E) cell lines treated for up to 72 h with 1 μM TP compounds.

In vivo evaluation of TP efficacy and safety

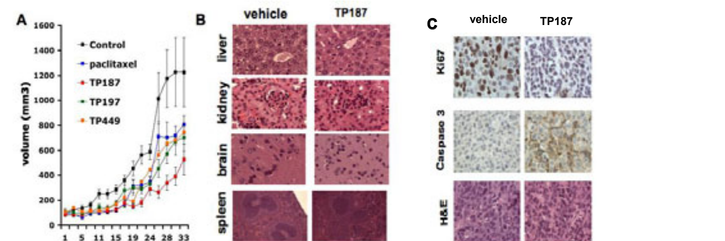


Figure 2. TP compounds suppress tumor growth, decrease cell proliferation and induce caspase 3 cleavage in mouse xenograft models with no systemic toxicities. Panel A. Graph shows average tumor volumes amongst the treatment groups over the course of the experiment. Tumor volumes were measured three times weekly, error bars represent SEM. Panel B. Representative micrographs of H&E stained organ tissue collected from vehicle and TP187 treated mice. Panel C. Immunohistochemical staining was performed on tumor sections collected from vehicle and TP187 treated mice. Ki67 staining (upper) was significantly decreased in TP187 treated mice compared to control. Caspase 3 cleavage (middle) was increased in TP187 treated tumor sections versus vehicle treated tumors. An H&E staining of tumor sections is shown in the bottom panel. Micrographs are representative of three separate experiments.

Mitochondrial localization of TP compounds

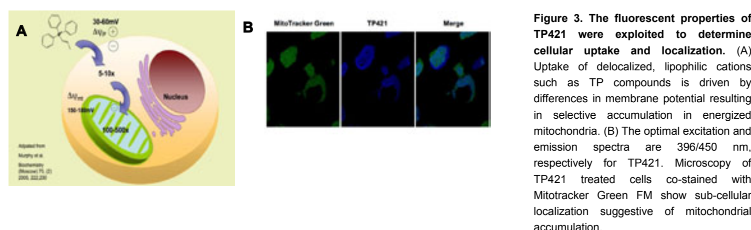


Figure 3. The fluorescent properties of TP421 were exploited to determine cellular uptake and localization. (A) Uptake of delocalized, lipophilic cations such as TP compounds is driven by differences in membrane potential resulting in selective accumulation in energized mitochondria. (B) The optimal excitation and emission spectra are 396/450 nm, respectively for TP421. Microscopy of TP421 treated cells co-stained with Mitotracker Green FM show sub-cellular localization suggestive of mitochondrial accumulation.

TP compounds alter mitochondrial function

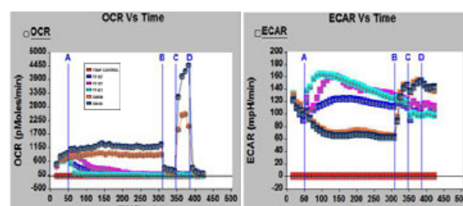


Figure 4. Oxygen consumption is decreased in TP treated MDA-MB-435 cells. OCR (left) and ECAR (right) were measured using the XF 24 Extracellular Flux Analyzer. Addition of TP compounds resulted in a decrease in OCR with a simultaneous increase in ECAR. The observed decrease in OCR was not affected by addition of inhibitors of ATP synthase, uncoupling agents or complex I inhibition. [Port A: TP compound (5μM), Port B: Oligomycin (0.005 mg/mL), Port C: FCCP (1μM) Port D: Rotenone (1μM).

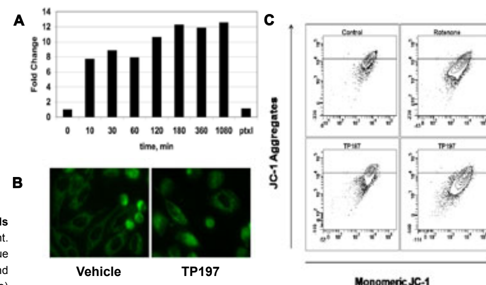
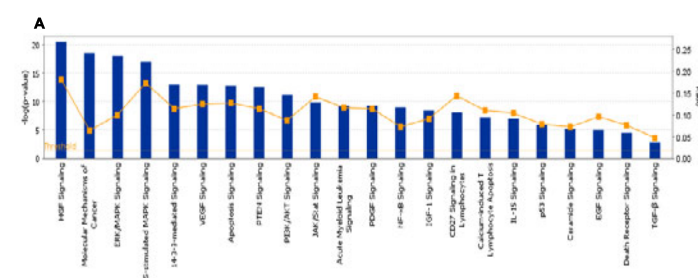


Figure 5. Mitochondrial superoxide production is an early and sustained event in TP action. MDA-MB-435 cells were treated with 5 μM TP197 and superoxide production was measured at various timepoints using MitoSOX Red, a fluorogenic indicator of mitochondrial superoxide production. (A) Compared to vehicle and paclitaxel controls, TP197 caused a marked increase in superoxide production. (B) Decrease in oxygen consumption results in morphological changes in the mitochondria. MDA-MB-435 cells treated with 1 μM TP197 for 30 min were stained with Mitotracker green dye prior to visualization under fluorescent microscope. (C) Mitochondrial membrane potential decreases following TP treatment. MiaPaCa cells were treated with 1 μM TP compound or rotenone (complex I -inhibitor) for 6 h prior to loading with JC-1 membrane potential sensitive dye. Under normal conditions JC-1 forms aggregates within the mitochondria that emit fluorescence in the red range. Upon loss of mitochondrial membrane potential, JC-1 dye enters the cytosol where it exists in the monomeric state emitting fluorescence in the green range.

Proteomic analysis of TPs on cancer signaling pathways



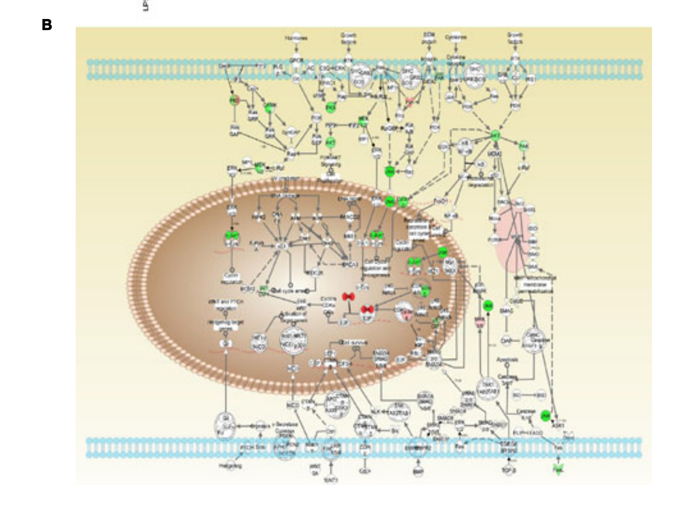


Figure 6. TP–treated cells exhibit significant alterations in signaling pathways critical to cancer cell growth and survival. Kinexus Antibody microarray was performed on MDA-MB-435 cells treated with 1 μM TP421 for 24h. Compared to control, TP421 caused significant alterations in abundance and phosphorylation levels of proteins critical for cell proliferation. (A) Statistical ranking of pathways altered in response to treatment. (B) TP421 treatment acts on multiple pathways driving cancer progression.

TP compounds inhibit angiogenic capacity

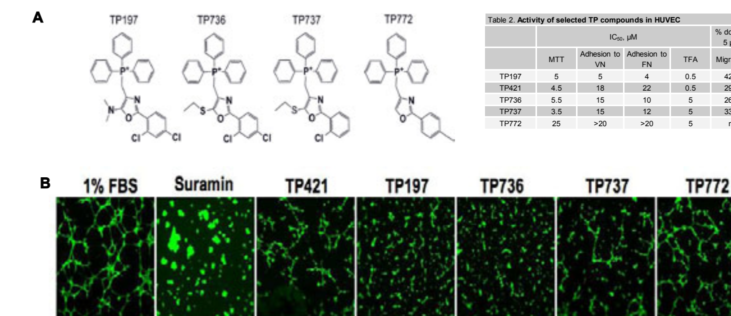


Table 2. **Activity of selected TP compounds in HUVEC**					
	IC_{50}, μM				% dcrs @ 5 μM
	MTT	Adhesion to VN	Adhesion to FN	TFA	Migration
TP197	5	5	4	0.5	42.1
TP421	4.5	18	22	0.5	29.9
TP736	5.5	15	10	5	26.2
TP737	3.5	15	12	5	33.3
TP772	25	>20	>20	5	nt

Figure 7. TP compounds exhibit anti-angiogenic properties. (A) Representative structures of TP compounds exhibiting activity in angiogenesis models. (B) TP compounds inhibit endothelial cell tube formation in basement membrane extracts. Porcine Aortic Endothelial Cells (PAEC) failed to form organized tube-like structures when seeded on basement membrane extract in the presence of 5 M TP compound (TP197, TP421, TP736, TP737 and TP772). These results are similar to that observed with suramin, an angiogenesis inhibitor. Table 2. TP compounds show moderate cytotoxicity, inhibit integrin dependent MDA-MB-435 breast cancer cell adhesion to and migration on vitronectin and fibronectin substrates and inhibit tube formation in human umbilical vein endothelial cells (HUVEC).

A predicted role for growth factor-mediated signaling pathways

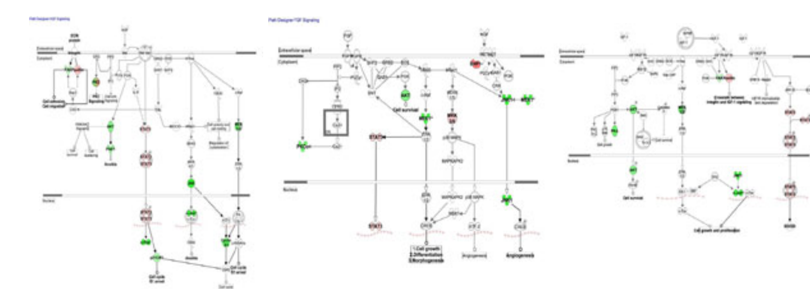


Figure 8. Schematic representations of growth factor receptor-mediated signaling pathways found to be significantly altered in response to TP421 treatment. Molecules affected by treatment have been overlaid on pathways, red indicates up-regulation and green indicates down- regulation of selected proteins.