Therapeutic Potential of T-oligo and its Mechanism of Action
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Abstract
Traditional chemotherapy is the first treatment option for the majority of cancer patients, but due to harsh and toxic side effects, more targeted therapies are needed. T-oligo is an oligonucleotide homologous to the 3’ overhang of the telomere. It induces several DNA damage and anti-cancer responses similar to experimental telomere loop disruption, including senescence, apoptosis and differentiation in malignant cells. To explore T-oligo’s anticancer potential, a panel of 6 malignant melanoma cell lines was treated with T-oligo. Melanoma cell lines with functional p53 or p73 exhibited cell death ranging from 11.80% to 31.73% after T-oligo treatment, with MU and MM-AN melanoma cells expressing the maximum response. There was no significant response in p53 and p73 null RPM-EP cells. Based on these results, MM-AN cells were chosen as a model system to study T-oligo’s effects in vitro and in vivo. To further elucidate its mechanism of action, pro-apoptotic and differentiation markers typically up regulated in responsive melanoma cell lines were studied in RPM-EP cells. FACS analysis and immune fluorescence studies confirmed uptake of fluorescein labeled T-oligo in both cell lines. Western blotting and confocal microscopy studies indicated upregulation of γH2AX after T-oligo treatment in both cell lines. In RPM-EP cells, expression of p73 and TRP-1 was not detected, nor was there up regulation of E2F1 and Tyrosinase. For in vivo experiments, SCID mice were injected with MM-AN cells to form tumors on their flanks, which were later treated with T-oligo and complementary oligo using Alzet pumps. Results demonstrated a 98% reduction in tumor size, as well as up regulation of differentiation markers important for anti-tumor immune responses. This study provides novel evidence which further establishes p53/p73 as crucial downstream signalling proteins and important players in T-oligo mediated anti-cancer effects in melanoma. Our results clearly demonstrate that T-oligo may be an effective and novel therapeutic option for melanoma.

Keywords: Melanoma; Tyrosinase; Malignant transformations; Telomerase

Introduction
This year 76,690 individuals in the U.S. will be diagnosed and 9,480 will die from melanoma [1]. Current chemotherapies used in the treatment of metastatic melanomas fail to show specificity for malignant transformations and hence demonstrate toxicity against normal cells, as well as stem cells [2]. Molecularty targeted therapies with low toxicity against telomerase, a protein known to be over expressed in malignant cells, have been established, however, drug resistance is still a major concern [3]. In this study, we explored a potent and less toxic approach for melanoma therapy using T-oligo, an oligonucleotide homologous to the 3’ telomere overhang. Telomeres are protective structures composed of tandem nucleotide repeats (TTAGGG) at the ends of chromosomes, which allow cells to distinguish between healthy and damaged chromosome ends [4]. Disruption of the telomere overhang leads to DNA damage responses (DDRs) [4], and ultimately apoptosis and/or senescence [2,5-7], making targeted telomere disruption an attractive anti-cancer therapeutic option [8]. T-oligo, an 11-base oligonucleotide, has been proposed to prevent telomere stabilization [9], and induces apoptosis and differentiation in malignant cells with little or no effect on normal cells [2,10,11]. Furthermore, T-oligo can be used in cancer cells to study the different mechanisms of DDRs caused by telomere disruption [5,10]. Thus, T-oligo is being investigated as a novel cancer therapeutic. It has been shown that p53 plays a major role in the intrinsic and extrinsic apoptotic pathways [12,13] and p53 wild-type cell lines undergo increased apoptosis compared to p53 mutant lines [13]. Although MM-AN cells do not have active p53, they do contain its homologue p73, which is believed to compensate in part for the induction of apoptosis normally mediated by p53 [5]. E2F1 is a transcription factor that plays important roles in regulating the action of tumor suppressor proteins and directly activates p73 [14]. Previously, our group has shown strong evidence suggesting that these aforementioned proteins cooperate to induce DDRs after T-oligo treatment in MM-AN melanoma cells [5]. However, their roles in cells insensitive to T-oligo treatment, such as RPM-EP cells, have not yet been established.

Phosphorylation of γH2AX is one of the key events associated with DDRs [15]. Eller et al. [16] have demonstrated that cells treated with T-oligo contain foci that co-localize with telomeres. We have previously demonstrated that T-oligo up regulates differentiation markers tyrosinase, MART-1, TRP-1, and HMB-45 (gp-100) in vitro [2,9]. These proteins are tumor associated antigens (TAAs) that induce differentiation, and are potential targets for melanoma immunotherapy [2,17-19]. In the present investigation, the efficacy of T-oligo was studied on a panel of 6 melanoma cell lines, since melanoma cells express heterogeneity [20]. We also examined the uptake of T-oligo in MM-AN and RPM-EP melanoma cells, as well as the induction of DDRs after treatment in vitro. Furthermore, we investigated the efficacy of T-oligo after delivery in vivo using Alzet pumps. Additionally, T-oligo’s effect on several differentiation markers/TAAs, such as tyrosinase, MART-1, TRP-1 and gp-100 was studied in vivo. Our results further characterize T-oligo’s signalling mechanism and establish key downstream signalling targets involved in mediating T-oligo induced anticancer effects.

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Materials and Methods

Cell lines and cell culture

MM-AN, MU, PM-WK, MM-RU, MM-MC and RPM-EP melanoma cells were obtained by explant culture [21], and were grown in MEM (Cat No: MT-10-010-CM, Thermo Fisher Scientific, Pittsburg, PA) supplemented with 10% (v/v) fetal bovine serum (Cat No: S11150, Atlanta Biologicals, Lawrenceville, GA) and 1% (v/v) antibiotic/antimycotic (Cat No: 15240 Invitrogen, Grand Island, NY).

Antibodies

Antibodies used in this study include anti-tyrosinase monoclonal antibody (clone T311, Nova-castra, New Castle upon Tyne, UK), anti-TRP-1 monoclonal antibody (cloneTa99, Signet, Dedham, MA, USA), anti-gp-100 monoclonal antibody (MO 634, Dak Corporation, Carpinteria, CA, USA), anti-MART-1/Melan-A monoclonal antibody (clone M2-7C10, Signet, Dedham, MA, USA), Pan Melanoma cocktail (Biocare, Tempe, AZ), anti-phospho-H2AX (pSer-139) antibody (Millipore, Billerica, MA) and β-actin antibody (Sigma-Aldrich, St. Louis, MO). Antibodies were used according to manufacturer instructions.

Oligonucleotides

We designed two oligonucleotides with 5′ phosphate groups and phosphodiesther linkages: T-oligo, which is homologous to the 3′ overhang sequence (pGTTAGGGTTAG), and c-oligo, which is a complementary sequence (pCTAACCTAAC) (Midland Certified Reagent Co., Midland, TX, USA).

Evaluation of cell death

All melanoma cell lines were grown in MEM with 5% FBS and treated with 40 µM of oligonucleotides T-oligo or c-oligo or an equal volume of water (diluent) for 72 hours. Cells were then stained with propidium iodide and analyzed by Becton Dickinson FACS Scan and Cell Quest software. Percentage cell death was evaluated by determining the sub G0/G1 DNA content.

Intracellular uptake of T-oligo by melanoma cells

MM-AN and RPM-EP melanoma cells were treated with either diluent (water) or 40 Mm of fluorescein isothiocyanate (FITC) labeled T-oligo for 4 hours, and then fixed with 4% paraformaldehyde in PBS (Electron Microscopy Sciences, Hatfield, PA) for 15 minutes. Fluorescence was measured on Becton Dickinson FACS Scan and analyzed by Cell Quest software. For confocal microscopy analysis, melanoma cells were plated on Glass chamber slides (Lab-Tek, Naperville, IL) and treated for 4 hours with FITC-labeled T-oligo (Midland Certified Reagent Co., Midland, TX, USA). The nucleus was stained as described earlier [22]. Melanoma cells were fixed in 4% paraformaldehyde in PBS, mounted with Slow fade reagent (Molecular Probes, Eugene, OR), and stored at 4°C in the dark. T-oligo uptake by cells was visualized with a confocal microscope (Olympus IX81).

Western blotting analysis

MM-AN melanoma cells were treated with 40 µM of either T-oligo, c-oligo or an equal volume of diluent (water) for 48-72 hours, as described above. Cells were lysed as described previously [23,24] in buffer containing 20 mM Tris (pH, 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 0.42% NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 10 mM protase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cell lysates were separated by SDS-PAGE electrophoresis under reducing conditions and then transferred to immobilization membranes (Bio-Rad Laboratories, Hercules, CA). An anti-β-actin antibody was used as a loading control. The membranes were then probed with antibodies against γH2AX, E2F1, p73, tyrosinase and TRP-1, and blots were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Rockford, IL). Densitometry of western blot bands was performed using NIH Image J software.

Immunofluorescence staining

10,000 MM-AN cells were plated on chamber slides and allowed to adhere for 24 hours. Cells were then treated with 40 µM of T-oligo for 48 hours, fixed with 4% paraformaldehyde, and immunofluorescence was performed with anti-γH2AX (pSer139) antibody and FITC labeled secondary antibody, using anti-rabbit DyLight 488 secondary antibody (Green, Thermo Fisher Scientific) and 4′,6-diamidino-2-phenylindole (DAPI, blue) for nuclear staining (Vector Shield, vector labs, Burlingame, CA), and analyzed on a confocal microscope (Olympus IX81) [25].

Animal experiment

Two million MM-AN melanoma cells were injected into the flank of SCID mice to produce subcutaneous tumors, which were allowed to develop for one week. When tumors were visible, mice were treated with T-oligo or c-oligo (10 mice in each group), which were delivered by Alzet pumps (DURECT Corporation, Cuperino, CA) surgically implanted into the animal near the tumor site. Mice were treated with 420 µg of T-oligo delivered daily via continuous infusion for week. Following the final dose, mice were left untreated for two weeks. The mice were then euthanized via CO2 inhalation and tumor sizes were measured with digital callipers, and their volumes were determined. Tumors were fixed using 4% formalin and stained with H and E. The melanoma tumors, immunohistochemistry was performed as described earlier [2,24]. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago and animal treatments were done according to institution approved protocols. Care was taken to ensure animals did not suffer discomfort, distress or pain.

Immunostaining of differentiation markers in tumors

Immunostaining for differentiation markers MART-1, TRP-1, and gp-100 was performed as described previously [24]. Following resection of tumors from the SCID mice, samples were formalin fixed paraffin-embedded (FFPE) and sectioned. FFPE sections 5 incubated with non-immune rabbit serum served as negative controls. Tumors were subsequently graded by two investigators on a 0-3 scale [26].

Results

T-oligo induced cell death in melanoma

Cell Lines Induction of cell death was studied in six melanoma cell lines: MM-AN, MU, PM-WK, MM-RU, MM-MC and RPM-EP. Five of the six cell lines were found to undergo apoptosis in response to T-oligo (Table 1). Cell death rates ranged from 11.8% to 33.24% at 72 hours in T-oligo responsive cell lines, with MU and MM-AN cells showing the maximum response. RPM-EP was the only unresponsive cell line, which was unresponsive. Our earlier studies indicate that T-oligo does not induce cell death in normal human melanocytes, with
cell death at 72 hours ranging between 1-2% [2] in both T-oligo and control groups. These results indicate that T-oligo induces cell death specifically in melanoma, and not their normal counterpart.

Uptake of T-oligo

To study the uptake of T-oligo, the responsive MM-AN cells and the unresponsive RPM-EP cells were both treated with diluent or 50 µM of T-oligo labeled with FITC for 4 hours and then analyzed by FACs scan. MM-AN and RPM-EP cells treated with T-oligo showed uptake of T-oligo into the cell (Figure 1A), indicating that T-oligo is able to enter the cells freely. These results indicate that the inability to uptake T-oligo was not the cause of unresponsiveness in RPM-EP cells. Uptake of FITC labeled T-oligo in MM-AN melanoma cells was also confirmed by confocal microscopy and nuclear localization of T-oligo was clearly seen (Figure 1B). Similar results were observed in RPM-EP cells.

Effect of T-oligo on γH2AX

DNA damage characteristically leads to phosphorylation of ATM on Ser1981, which subsequently phosphorylates H2AX on Ser139. This phosphorylation leads to modification of chromatin structure, and is thought to be a critical step in propagating DDRs and activating cell-cycle checkpoints [27]. Therefore, T-oligo’s effect on phosphorylation of H2AX was also studied. The induction of γH2AX foci were also observed on a confocal microscope in MM-AN melanoma cells (Figure 1C). No γH2AX foci were seen after treatment with diluent or c-oligo. As seen by western blotting, treatment of MM-AN melanoma cells with 40 µM T-oligo resulted in a 9-20 fold increase of phosphorylation of H2AX at 48-72 hours (Figure 1D), indicating that T-oligo induces a DNA damage signal.

T-oligo mediated DDRs were not induced in RPM-EP melanoma cells

DDR's induced by T-oligo are believed to be mediated through p53/p73 and E2F1 [2,9]. We have previously reported that cells which lack detectable levels of p53 have increased expression of p73 and E2F1 preceding T-oligo induced apoptosis [2]. Furthermore, it was shown that siRNA knockdown of p73 in p53 null melanoma cells resulted in a 50% reduction in apoptosis [5], suggesting a significant role for p73 in T270 oligo induced DDRs. The in vivo, p73 null RPM-EP cells did not exhibit cell death after treatment with T-oligo (Figure 2A and 2B). As expected, expression of E2F1 was not upregulated nor was p73 detectable (Figure 3A). Additionally, we explored modulation of melanocyte differentiation-specific antigens in response to T-oligo treatment. Progressive melanoma often exhibits a loss of melanocyte differentiation antigens, which are the targets of immunotherapy-mediated treatments of melanoma [28]. The expression of these antigens is associated with decreased tumorigenicity and slower cell proliferation [22,29,30]. Previous reports by our group have demonstrated up regulation of tyrosinase and TRP-1 in several melanoma cell lines after T-oligo treatment [2,9]. Furthermore, an increasing number of reports suggest that these TAAs are regulated by p53 and p73 [27,31]. Therefore, we demonstrated that RPM-EP cells failed to induce upregulation of either TRP-1 or tyrosinase differentiation markers, following T-oligo treatment (Figure 3B). This evidence shows for the first time the requirement of p73 for expression of differentiation antigens and DDRs induced by T-oligo in a p73 null cell line. These data further substantiate the importance of the p53 protein family in mediating T-oligo antitumor effects.

Effect of T-oligo on tumors in SCID mice

T-oligo was delivered systemically in SCID mice using Alzet pumps and its effects on pre-existing tumors were studied. T-oligo delivered by Alzet pumps inhibited the growth of melanoma tumors in SCID mice by 98% (Figure 4A and 4B), and 60% of treated mice had clinically no detectable tumors as seen by H and E staining in fixed tumors (Figure 4C). Tumors were identified and validated as human melanoma with a pan melanoma cocktail, which stains for gp-100, MART-1 and tyrosinase which are all important melanoma markers (Figure 4C).

Up regulation of differentiation markers by T-oligo

Treatment with T-oligo displayed a 2.5 fold increase in intensity of staining for MART-1 expression, a 3.4 fold increase in intensity of TRP-1, and a 2.0 fold increase in intensity of gp-100 in MM-AN melanoma tumors (Figure 4D). These data demonstrate that T-oligo induces expression of differentiation markers in vivo.

Discussion

The incidence of melanoma has increased by approximately 2.8% annually since 1981 in the United States [32]. Despite extensive ongoing research, there are no effective therapeutic treatment options for melanoma. Moreover, the 10-year survival rate for patients with metastatic melanoma is less than 10% [33]. Therefore, it is critical that better therapeutics with improved efficacy are developed for melanoma. Although several studies have demonstrated T-oligo’s high therapeutic potential in multiple malignant cell lines, its mechanism of action is not fully understood. We and others suggest that its effects are modulated through a combination of signaling pathways which may differ across cell types. Several proteins, including ATM/p95/Nbs1, chk2, p53/p73 and cdk2, are considered substantial players in T-oligo’s signaling pathways [2,9,34,35]. In the present study, we present additional evidence that p73 has an essential role in mediating DDRs induced by T-oligo in melanoma. Additionally, we investigated the therapeutic efficacy of T-oligo in vitro and in vivo studies, and demonstrate nuclear uptake of T-oligo, the induction of cell death in melanoma cell lines and the reduction of malignant melanoma tumors in mice after T-oligo treatment. Genomic integrity and transmission are regulated by high fidelity mechanisms [36-38]. When these mechanisms fail [39], DDRs are activated. The ATM gene plays a major role in the proper functioning of these DDRs [39] and phosphorylates several protein targets, such as checkpoint kinase (Chk2) and p53 [40]. It has been shown that T-oligo treatment results in DDRs which are mediated by ATM kinase and its effector proteins [6]. In contrast, other studies suggest that T-oligo induced DDRs are modulated independently of ATM/p53 in several cell lines [34]. However, we propose that in cells lacking detectable quantities of p53, T-oligo-induced DDRs progress...
Figure 1: Uptake of T-oligo and its effects on MM-AN melanoma cells. MM-AN cells were treated with either diluent or 50 µM of FITC labeled T-oligo for 4hrs and uptake of T-oligo was studied. A. FACS analysis demonstrated the uptake of T-oligo by MM-AN cells and RPM–EP cells. B. Confocal microscopy of MM-AN cells showing nuclear localization of FITC-T-oligo. The nucleus was stained with propidium iodide and colocalization of the two fluorescent molecules was seen as a yellow color. C. MM-AN melanoma cells were treated with T-oligo or c-oligo for 48 hours, fixed with paraformaldehyde, and immunostaining was performed with a γH2AX monoclonal antibody and a secondary antibody linked with FITC. γH2AX foci were seen mainly in cells treated with T-oligo. Very few foci could be detected in cells treated with c-oligo DMM-AN melanoma cells were treated with T-oligo, c-oligo, or an equal volume of diluent for the indicated times and samples were collected for western blotting for detecting γH2AX (Phospho Ser-139 H2AX). γH2AX was upregulated at 48-72 hours after treatment with T-oligo.

Figure 2: Unresponsiveness of RPM-EP cells to T-oligo treatment. A. FACS analysis was carried out using propidium iodide staining after treatment with 40 µM of either T-oligo or c-oligo and diluent for 96 hours. Percentage cell death was evaluated by determining the sub-G0/G1 DNA content. No difference in cell death was observed between the treatment conditions. B. RPM-EP cells were treated with 40 µM of either T-oligo or c-oligo and diluent for 72 hours and TUNEL assay was performed. Since no shift in peaks was seen after treatment, there was no induction of apoptosis after T-oligo or c-oligo treatment.
Figure 3: RPM-EP cells were unresponsive to T-oligo treatment. RPM-EP cells were treated with 40 µM of either diluent, T-oligo or c-oligo for 48 and 72 hours. Western blot analysis was performed to evaluate the expression of E2F1, p73, tyrosinase and TRP-1. A. No change in expression of E2F1 was observed and p73 was not detected. B. No change in expression of tyrosinase was observed and TRP-1 was not detected. β-actin was used as the loading control.

Figure 4: Reduction of tumor size and upregulation of differentiation markers with T-oligo treatment: One week after injection of MM-AN cells, SCID mice with visible melanoma tumors on the flanks were treated with 420 µg of T-oligo or c-oligo daily for one week and then left untreated for two weeks. A. T-oligo treated mice showed very small residual tumors in comparison to animals treated with c-oligo. B. Animals (n=10) treated with T-oligo showed a 98% reduction in tumor volume as compared to c-oligo. The differences in tumor size in these studies were statistically significant (p<0.0001). C. H and E staining of animals treated with T-oligo and c-oligo showed that animals treated with c-oligo had large tumors. Tumors were absent in 60% of animals treated with T-oligo. Melanoma tumors were identified by their positive staining with the pan melanoma cocktail. D. Sections from FFPE tumors from SCID mice were prepared and immunostaining procedures were performed. FFPE sections incubated with non-immune rabbit serum served as negative controls. T-oligo treatment increased expression of MART-1, TRP-1 and gp-100.
through its homologue p73. In addition, histone H2AX is one of the important targets phosphorylated by ATM [41]. These investigations and our studies suggest that T-oligo treatment induces activation of the ATM pathway which leads to the phosphorylation of histone H2AX, resulting in DNA fragmentation and cell death. To further verify the importance of p73 in T-oligo’s signaling mechanism, we investigated its role in RPM-EP cells which lack detectable levels of p53 or p73. Treatment with T-oligo did not demonstrate any detectable DDRs, including induction of cell death or apoptosis and no expression of p73 or upregulation of E2F1 expression. Furthermore, there was no up regulation of TAA antigens, TRP-1 or tyrosinase. Although previous reports have shown similar results in MM-AN melanoma cells treated with p73 siRNA, apoptosis was only inhibited by 50% in these cells [5]. This may be attributed to incomplete knockdown of p73 following siRNA treatment. The results presented in this study are novel, since RPM-EP are known to have no detectable expression of p53/p73, providing evidence of a critical role for p53/p73 in the induction of T-oligo mediated DDRs.

Alzet pumps were successfully used as a novel delivery system for T-oligo. Administration via the Alzet pumps allows for continuous infusions of T-oligo, maintaining concentrations within therapeutic levels for the duration of the experiment [42]. Our results demonstrate a 98% reduction in tumor volume, thus increasing T-oligo’s efficacy, as compared to alternate routes of delivery, as shown by us in previous studies [2]. This novel route of delivery broadens our understanding of T-oligo’s potential clinical efficacy. Our studies demonstrate that T-oligo treatment up regulates expression of melanoma differentiation markers/TAAs gp-100, TRP-1 and MART-1 [2,43]. gp-100 has been shown to be highly specific for metastatic melanoma in lymph nodes [44], while MART-1 has been reported to be specific for both primary and metastatic melanoma [44]. TRP-1 is a 75-kDa melanosomal glycoprotein (gp 75), which is most abundant in melanocytes and melanoma [45]. TRP-1 has been shown to be involved in melanogenesis, in the prevention of inhibition of melanocytic cell death [46], and is also shown to be highly expressed in malignant melanomas [47]. Pioneering research by Rosenberg et al. [48] and Boon [49] research groups indicated cytotoxic T cells target self, non-mutated proteins like MART-1, gp-100 and TRP-1, which are common to both normal melanocytes as well as melanoma. It is suggested that the over expression of TAA antigens in melanoma cells elicits immune responses by low-avidity TAA-specific T cells [50-52]. Interestingly, clinical trials with TAs have been shown to be promising. In a phase 3 clinical trial, it has been shown that a vaccine against the gp-100 antigens increases progression free survival in patients with advanced melanoma [53]. In a phase 1/2 clinical trial with AdVARM1T1-transduced dendritic cell vaccine, it was shown to be safe and immunogenic in patients with metastatic melanoma [54]. Further, it has been shown that epitopes of TRP-1 antigen induced long lasting antitumor immune responses [55]. In the present study, we suggest that T-oligo stimulates the antitumor immune response by increasing the expression of TAA antigens, which play a pivotal role in inhibiting proliferation in melanoma. In summary, our results suggest treatment with T-oligo increases phosphorylation of histone H2AX, which results in apoptosis of cancer cells and up regulation of tumor associated antigens in vivo. These responses then lead to antitumor immune responses against melanoma tumors, thus preventing the growth and progression of melanoma. Furthermore, we demonstrate the requirement of p73 in T-oligo induced DDRs. These results indicate that T-oligo could be a potential therapeutic for melanoma. Further studies are being carried out in our laboratory to improve the delivery and stability of T-oligo as a targeted nano particle, which could enhance the efficacy of T-oligo as a therapeutic agent in melanoma and other cancers.

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