Research Article

Eradication of Multiple Primary and Metastatic Melanoma Types in Vitro by Human Recombinant Dnase1

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Abstract

To address treatment-resistant metastatic melanoma, we previously engineered a form of human recombinant deoxyribonuclease-1 (hrDNase1) with a demonstrated killing efficiency of 70-100% in the Mel-Juso human melanoma cell line. hrDNase1 was previously shown to eliminate Mel-Juso cells, an apoptosis-resistant cell line, through a mechanism that resembles apoptosis. In this study, we endeavored to amplify the killing efficiency of hrDNase1 in Mel-Juso cells by increasing its resistance to actin, its major inhibitor. Also, we sought to elucidate whether the cytotoxic properties of hrDNase1 extend to other primary and metastatic human melanoma cell types. To this end, we generated an array of hrDNase1 constructs with various amino acid substitutions in the actin binding site. Structural modifications that conferred hrDNase1 with the greatest cytotoxicity included the removal of its signal peptide, the addition of a nuclear localization signal at the N-terminus, and an A114F substitution at the actin binding site. Under improved transfection conditions, hrDNase1 killing efficiency was increased from 70-100% to 98-99% in Mel-Juso cells. Moreover, the selected gene construct achieved a killing efficiency of 97-100% in the G361, M14, A2058 and A378 cell lines with only half of the previously reported therapeutic dose. These results demonstrate that hrDNase1 is a potential therapeutic that can kill human melanoma cells in vitro regardless of their metastatic potential..

Keywords: DNase1; Actin; Melanoma; Cancer; Gene-therapy

Introduction

Conventional therapeutics currently utilized to treat primary and metastatic melanoma include radiotherapy, chemotherapy and genetic-based modalities such as ipilimumab and Vemurafenib [1-3]. However, melanoma often responds to these treatment types by eliciting a series of anti-apoptosis defense mechanisms that promote apoptosis resistance and continued tumor propagation. In order to bypass the intrinsic defense mechanisms inherent in treatment-resistant melanoma, we recently engineered a human recombinant deoxyribonuclease1 (hrDNase1) that has the capacity to trigger cell death from the bottom end of the apoptosis signaling cascade. In this way, we have successfully bypassed all upstream anti-apoptosis defense mechanisms in melanoma cells

by activating an apoptosis-like process at the level of the DNA-degrading nuclease and by replacing the native apoptosis executioner nuclease.

The wild-type (wt) isoform of DNase1 is not directly activated by the apoptosis signaling cascade and has no access to nuclear DNA and is unable to trigger apoptosis [4]. We previously introduced three modifications into the genetic structure of wtDNase1 to confer the enzyme with the ability to digest nuclear DNA and activate cell death (Figure 1) [5]. First, we removed the signal peptide (SP), which directs DNase1 into the secretory pathway and outside of the cell [6]. Second, we added a nuclear localization signal (NLS) to enable DNase1 to be transported across the nuclear envelope, where it can gain access to genomic DNA [7]. Finally, we mutated the DNase1 actin binding site to prevent actin, a major intracellular inhibitor, from binding to and inactivating DNase1 [8]. The resultant hrDNase1 is not secreted from the cell, can transverse into the nucleus (Figure 1), and, by causing direct DNA damage, can initiate a cytotoxic process that closely resembles apoptosis in 70-100% of UVB-, chemo- and apoptotic-resistant Mel-Juso human melanoma cells [5,9,10]. Furthermore, to address the question of whether hrDNase1 constructs induced cytotoxicity directly by their catalytic activity, a mutation was introduced into the catalytic site of DNase1 [5]. The substitution increased melanoma cell survival from ~ 32% to 100%, suggesting that the hrDNase1 constructs directly impact cytotoxicity [5].
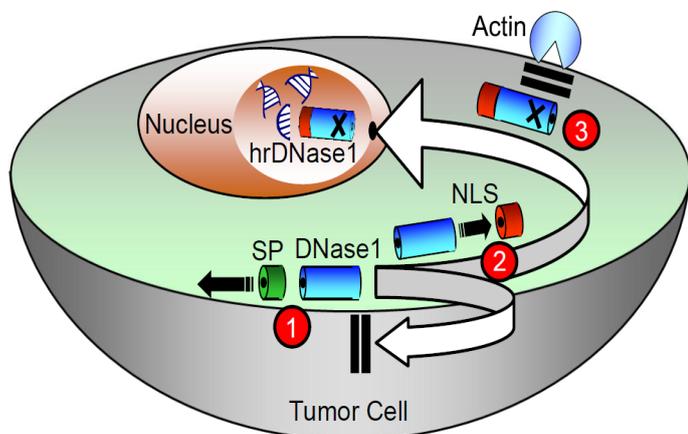

Figure 1. Diagrammatic Portrayal of the Key Properties of hrDNase1 Gene Constructs. (1) The signal peptide (SP) was removed to prevent hrDNase1 secretion from the cell or compartmentalization of hrDNase1 within lysosomes. (2) A nuclear localization signal (NLS) was added to transport hrDNase1 across the nuclear membrane and into the nucleus. (3) The actin binding site was mutated to confer hrDNase1 with resistance to actin-mediated deactivation.

Since the efficacy of an anti-cancer therapy is determined, in large, by its killing efficiency, we aimed to increase the cytotoxic capacity of hrDNase1by increasing the amount available for enzymatic reaction. This was accomplished by improving its resistance to actin – a major intracellular inhibitor of DNase1 that is abundantly present in the nucleus and cytoplasm [11-13]. We previously conferred hrDNase1 with partial resistance to actin by substituting one of the six amino acids (A114F) that constitute the actin binding pocket of DNase1. Although the A114F substitution has been reported to increase the potency of wtDNase1 by approximately five-fold, [14] Ulmer et al. showed that an A114R substitution decreased actin binding by over 10,000 fold compared to wtDNase1, and increasd the ability of DNase1 to digest DNA in the sputum by up to 50 fold [15]. Similarly, the Y65R substitution has been found to decrease actin binding by more than 1,000 fold compared to wtDNase1 [15]. These data suggest that replacing A114F with A114R, introducing Y65R substitution, or combining both amino acid substitutions may confer hrDNase1 with greater resistance to actin-mediated inhibition. To test this notion, we generated an array of hrDNase1 constructs with the above substitutions at the actin binding site, and selected the hrDNase1 construct with the highest killing efficiency in Mel-Juso human melanoma cells for further study.

In this study, we show that under optimized transfection conditions, DNase1 that lacks an SP, is fused with an N-terminal NLS, and is modified with an A114F at the actin binding site induces the highest cell killing efficiency. In addition, we demonstrate that the high cytotoxic activity of this engineered hrDNase1 is maintained across melanoma cell types regardless of their primary or metastatic origin. Finally, we show that, contrary to what has been suggested by in vitro enzymatic assays, [15] an A114R mutation does not confer hrDNase1 with greater killing efficiency than A114F.

Materials and Methods

Materials: Dulbecco's phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), and 0.5% Trypsin-EDTA were purchased from Invitrogen (Grand Island, NY). RPMI-1640 medium and fetal bovine serums (FBS) were purchased from Gibco Life technologies (Grand Island, NY). McCoy medium and L-glutamine (200 mmol) were purchased from HyClone (Logan, Utah). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO).

Constructs: We previously described the preparation of wtDNase1 (C03), C09, C11 and C13 hrDNase1 gene constructs [5]. Constructs C11 and C13 were generated as follows: (i) removal of the SP through deletion of the 22 amino acids upstream of the DNase1 start codon; (ii)

introduction of point mutations (A114F) in the DNase1 actin binding site; and (iii) addition of an SV40 NLS (PPKKKRKV) [7] to the C-terminus of construct C11, or the N-terminus of construct C13 [5]. The ten newly generated hrDNase1 constructs (C36 to C45) and associated primer sets are described in Figure 3 and Table 1, respectively. All site-directed mutations were generated using the QuikChange® Lightning Site-Directed Mutagenesis Kit (#210518; Stratagene, Cedar Creek, TX) following the manufacturer's instructions. Plasmid DNA was extracted from bacterial clones using either a maxiprep (Invitrogen) or miniprep kits (Qiagen, Germantown, MD) kit as per manufacturers' instructions and sent for sequencing (Applied Genomics Technology Center (AGTC), Detroit, MI). The chloramphenicol acetyltransferase (CAT) reporter plasmid (pcDNA/GW-47/CAT-GFP) served as a control for measurement of transfection efficiency. Alanine (A) at position 114 of DNase1 was mutated to Arginine (R) in construct C09 (Figure 2) to produce constructs C36 and C37, respectively, using 114CGC-forward/114CGC-reverse and 114CGT-forward/114CGT-reverse primer sets. The 65CGC-forward/65CGC-reverse primer set was used to substitute tyrosine (Y) with arginine at DNase1 position 65 (Figure 2) in constructs C09, C11, C36 and C37, to generate constructs C38, C40, C42 and C44, respectively. The 65CGT-for/65CGT-rc primer set was used to substitute tyrosine with Arginine at DNase1 position 65 in construct C09, C11, C36 and C37, to generate constructs C39, C41, C43 and C45 (Table 1, Figure 3). The nucleotide composition of each newly generated hrDNase1 construct was confirmed by sequencing.
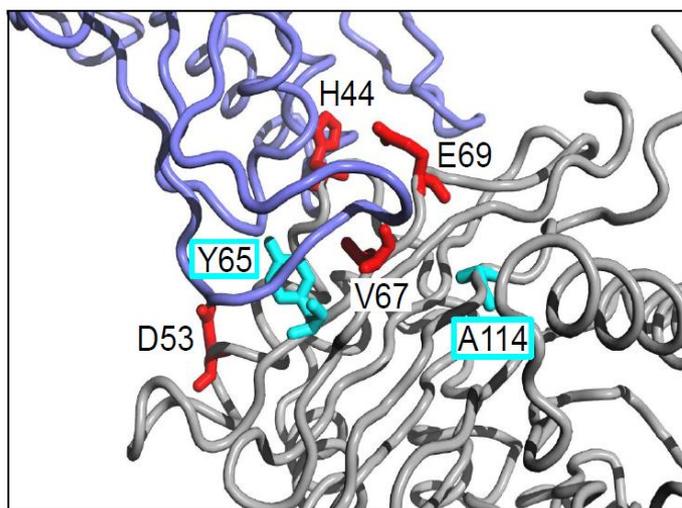
Table 1. Primer sequences used to generate the hrDNase1 gene constructs

Primer	Sequence (5'-3')
65CGT	5'-gcaccagacacctatcacCGTgtggtcagtgagccactg-3'
65CGT-rc	5'-cagtggctcactgaccacACGgtgataggtgtctggtgc-3'
114CGT	5'-caccttcaaccgagagccaCGTattgtcaggttcttctccc-3'
114CGT-rc	5'-gggagaagaacctgacaatACGtggctctcggttgaaggtg-3'
65CGC	5'- caccagacacctatcacCGcgtggtcagtgagccac -3'
65CGC-rc	5'- gtggctcactgaccacgCGgtgataggtgtctggtg -3'
114CGC	5'- caccttcaaccgagagccaCGcattgtcaggttcttctcc -3'
114CGC-rc	5'- ggagaagaacctgacaatgCGtggctctcggttgaaggtg -3'

Introduced mutations are shown in upper case and underlined.

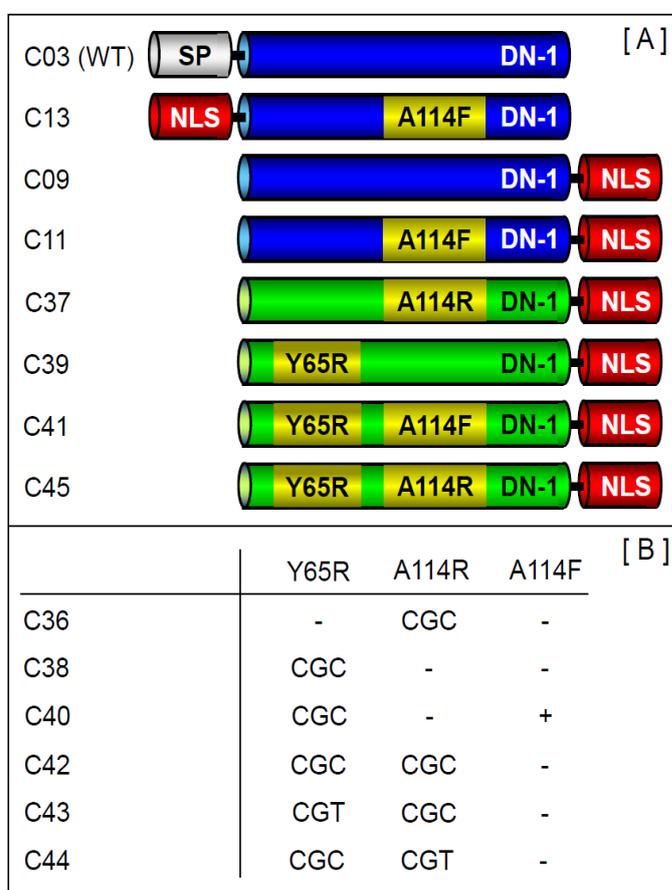|	Y65R	A114R	A114F
C36	-	CGC	-
C38	CGC	-	-
C40	CGC	-	+
C42	CGC	CGC	-
C43	CGT	CGC	-
C44	CGC	CGT	-

Figure 3. Schematic Representation of the Designed hrDNase1 Gene Constructs. (A) The composition of the human recombinant Deoxyribonuclease-1 (hrDNase1) proteins tested in this study. Previously generated hrDNase1 constructs are depicted in blue, and newly generated hrDNase1 constructs are depicted in green. Amino acid substitutions that have been introduced into the actin binding site are depicted on a yellow background within hrDNase1 constructs. In the constructs illustrated here (C37, C39, C41 and C45), Arginine (R) is coded for by the CGT codon. The remainders of the hrDNase1 constructs generated for the present study are depicted in Figure 3b. Wild-type DNase1 (WT); signal peptide (SP); DNase1 gene (DN-1); nuclear localization signal (NLS). (B) The codon sequence of Arginine and the

Figure 2. Amino Acid Composition of the Actin Binding Site of wild-type (wt) DNase1. Actin is depicted in purple, DNase1 is depicted in gray, and the six amino acids that comprise the actin binding site are depicted in red and turquoise. The two amino acids (Tyrosine-65 (Y65) and Alanine-114 (A114)), which are involved in actin binding and replaced by Arginine (R), are highlighted in turquoise. Based on the human wtDNase1 structure (1ATN) from the Protein Data Bank (http://www.rcsb.org/pdb/) PyMol molecular visualization system (http://www.pymol.org/) was utilized to prepare this model of wtDNase1.

associated substitutions at the actin binding site of the newly generated hrDNase1 gene constructs (C36, C38, C40, C42, C43 and C44) are summarized in chart form; (+) indicates the addition of an A114F substitution.

Cell Culture and Transfection: The human primary melanoma cell lines, Mel-Juso (DSMZ, Braunschweig, Germany) and M14 (National Cancer Institute, Frederick, MD), were cultured in RPMI-1640 medium. The human metastatic melanoma cell lines, A375 and A2058 (American Type Culture Collection (ATCC), Manassas, VA), were cultured in DMEM medium. The human primary melanoma cell line, G361 (ATCC, Manassas, VA), was cultured in McCoy's medium. All media were supplemented with 10% FBS and 1% L-glutamine. Cells were grown to confluence and re-seeded for a period of at least 24 h prior to transfection. Cultures were maintained at 37°C in humidified 5% CO2 air.

Colony Forming Assay (CFA) for Cell Viability: Cells were plated in six-well plates at a density of 600 cells per well, in triplicate. To identify the hrDNase1 construct that exhibited the highest cytotoxicity in the Mel-Juso cell line, cells were transfected 24 h later with 0.3 μg of hrDNase1-containing vector or control vector. Both Cat-GFP and C03 plasmids were employed as procedural controls. In addition, constructs C11, C13 and C36 through C45 were tested individually. Experiments were performed in five replicates. Since the construct C13 yielded the highest killing efficiency in the Mel-Juso cell line, it was used in five melanoma cell lines (A375, M14, G-361, Mel-Juso and A-2058). Cells were transfected with various concentrations of a control or hrDNase1-containing construct, 24 h post plating. The following C13 plasmid DNA concentrations were tested in each cell line: 0.3 μg, 0.6 μg, 1 μg and 2 μg. Experiments were performed four times for 0.3 μg, 0.6 μg and 1 μg doses, and only once for the 2 μg dose since the cytotoxicity level of C13 at 1 μg and 2 μg did not differ biologically (Figure 6). All transfections were conducted using LipofectamineTM LTX and PlusTM reagent (Invitrogen, Carlsbad, CA). Two modifications to the protocol to optimize transfection efficiencies in Mel-Juso cell were made: (i) replacing the serum-free medium with medium containing 5% FBS; and (ii) doubling and tripling the recommended incubation times for the PlusTM reagent and LipofectamineTM LTX, respectively. After 4 h, 2 ml of fresh medium containing 10% FBS and 1% L-glutamine was added to each well. After one week, old medium was exchanged for fresh medium containing 10% FBS and 1% L-glutamine. Transfected cells were allowed to grow for 12-14 days post plating, fixed with absolute ethanol for 30 min., stained with 1% crystal violet in distilled water, and colonies counted (one colony ≥ 30 cells).

Determination of Transfection and Killing Efficiencies in Colony Forming Assays:

Twenty-four hours post-transfection, the total number of GFP-positive and - negative cells transfected with CAT-GFP in six-well plates were counted in ten sequential fields under fluorescence microscopy using the Olympus IX71 Inverted Microscope (Olympus, Centervalley, PA) and SlideBook 4.2 software (Olympus, Denver, CO) at 400 X magnification using an ocular grid consisting of a simple square lattice of 100 test points [5,16]. Transfection efficiency was calculated as the percentage of cells expressing GFP in the total population of Cat-GFP transfected cells [5]. Killing efficiency was determined by comparing relative survival rates in hrDNase1-treated cells to mock-treated cells [17].

Statistics: Data were transformed using a square root transformation in order to meet the assumptions of the statistical methods. Linear mixed effects models were used in which replicate and experiment were denoted as random effects. Post-hoc comparisons were made using Bonferroni's procedure to maintain the type I error rate at $p < 0.05$. Model fit was assessed graphically and by examination of residuals.

Results

Cell killing is independent of codon usage at the A114R and Y65 substitutions

According to Ulmer et al. (1996), [15] the two amino acid substitutions thought to confer the highest resistance to actin were reported to be A114R (>10,000 fold) and Y65R (>1,000 fold; Figure 2) [15]. Using various combinations of these two substitutions, we produced four hrDNase1 construct prototypes (C37, C39, C41 and C45; Figure 3a), in which Alanine (A) was replaced with Arginine (R) at hrDNase1 positions 65, 114, or both via the incorporation of a CGT codon. Due to redundancy in the coding process, Arginine can be coded for by six different codon sequences [18]. To test whether codon usage would affect hrDNase1 cell killing efficiency we created an additional six constructs containing a CGC codon at amino acid positions 65 and 114 as alternatives to CGT in the above hrDNase1 prototypes. Constructs C36, C38 and C40 served as alternatives to constructs C37, C39 and C41, respectively, whereas constructs C42, C43 and C44 served as alternatives to construct C45 (Figure 3b). Each prototype and its alternative differed only by a single point mutation, and exhibited the same degree of cytotoxicity (each p > 0.99 except C45 vs C42 p = 0.22; data not shown).

A114F, A114R and Y65R substitutions introduced into the actin binding site each confer hrDNase1 with maximal resistance to actin-mediated deactivation in melanoma cells

No difference in cell survival was observed between all three controls employed throughout this study: untreated-, mock treated- and Cat-GFP-treated cells (each p > 0.99; Figure 5). All tested hrDNase1 constructs (C11, C13, C37, C39, C41 and C45; Figure 5) decreased cell survival significantly compared to wtDNase1 (p = 0.002 to p < 0.001). These hrDNase1 constructs differed from wtDNase1 in regards to the presence or absence of an SP, the position of the NLS and point mutations in the actin binding site, thus, confirming our previous findings that these modifications bestow hrDNase1with the ability to efficiently kill cancerous cells. Also, A114F, A114R and Y65R (Figure 3) alone or in combination provided hrD-Nase1 with the ability to resist actin-mediated deactivation. No difference in cell survival was observed between the newly generated hrDNase1 constructs: C37, C39, C41 and C45 (p > 0.99; Figure 5). These findings show that hrDNase1 exhibits the same degree of resistance to actin whether it contains the A114R (C37) or Y65R (C39) substitution within its actin binding site. Moreover, combining both the Y65R and A114R substitutions in construct C45 also did not substantially improve hrDNase1 killing efficiency.

N-terminus-NLS and A114F substitution in construct C13 yielded the highest killing efficiency in melanoma cells

Construct C11 decreased cell survival significantly more than C39 (p = 0.002) and C45 (p = 0.001), but not more than C37 (p = 0.58) or C41 (p > 0.99; Figure 5). Resistance to actin in vitro did not significantly improve with A114R substitution (C37), nor did actin resistance increase when a second amino acid critical for actin binding was mutated (Y65R; C41) relative to the A114F substitution. Construct C13 decreased cell survival significantly more than constructs C37, C39, C41 and C45 (each p < 0.001), although C13 cytotoxicity was only marginally higher when compared to that of C11 (p = 0.07; Figure 4A and Figure. 5). However, in contrast to C11, C13 reduced cell survival more than C37 and C41 (Figure 5). These results indicate that hrDNase1-directed cytotoxicity is optimal when an NLS is fused upstream of the hrDNase1 start codon. Also, the marginal increase in C13 killing efficiency relative to C11, as well as the superior cytotoxicity of C13 compared to C37 (A114R) and C41 (Y65R and A114; Figure 5), suggest that altering the position of the NLS and amino acid composition of the actin binding site potentially change the actin binding site and/or hrDNase1 catalytic activity.
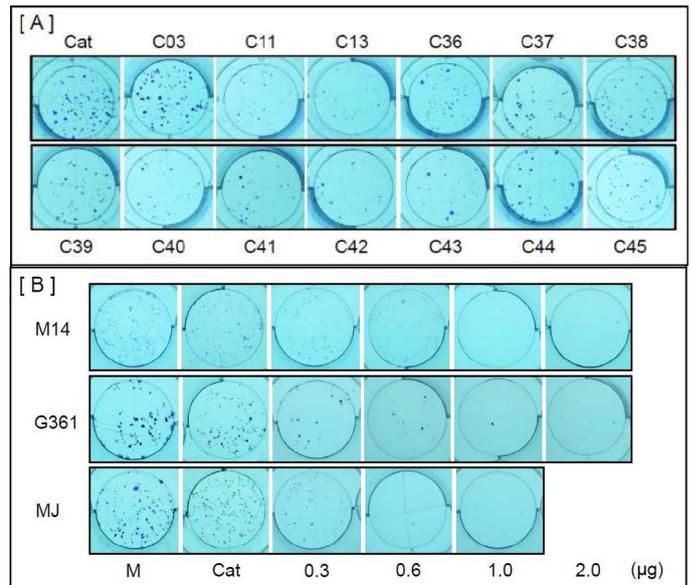

Figure 4. Determination of Human Recombinant DNase1 (hrDNase1) Cytotoxicity in Melanoma cells by a Colony Forming Assay. Survival of melanoma cells was measured as described in 'Materials and Methods'. (A) Representative results demonstrating the relative cytotoxic effects of wtDNase1 (C03) and twelve hrDNase1 constructs (0.3 µg) in Mel-Juso human melanoma cells. C13 hrDNase1 construct demonstrated the highest killing efficiency. (B) Representative results from three (M14, G361, Mel-Juso) of the five human melanoma cell lines treated with the indicated concentrations of C13 hrDNase1 construct. Experiments were replicated four times with all concentrations except once with 2 µg. The results for M14 and G361 were obtain represent experiment conducted with C13 at 0.3 µg, 0.6 µg, 1 µg, and and 2 µg doses, and the representative results for MJ represent an experiment conducted with C13 at 0.3 µg, 0.6 µg and 1 µg doses (see Material and Methods). Remnants of the marker used for counting are visible in some of the wells. Human recombinant DNase1 (hrDNase1); chloramphenicol acetyltransferase (CAT); Mel-Juso (MJ).
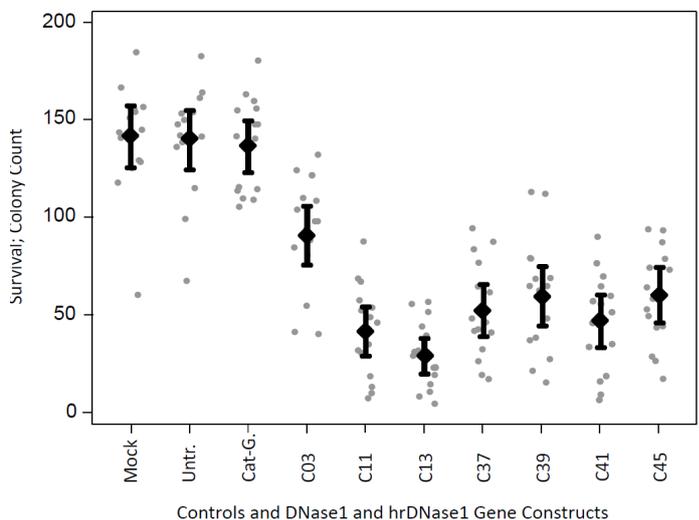

Figure 5. Comparison of Cytotoxicity in Six Actin-Resistant, Human Recombinant DNase1 (hrDNase1) Constructs by a Colony Forming Assay in Mel-Juso Human Melanoma Cells. Survival of Mel-Juso human melanoma cells was measured 12–14 days following treatment with a series of hrDNase1 gene constructs, as described in the 'Materials and Methods' section. All actin-resistant hrDNase1 gene constructs (C11, C13, C37, C39, C41 and C45) significantly decreased melanoma cell survival compared with wild-type DNase1 (P<0.0001). C13 decreased cell survival significantly more than C37, C39, C41

and C45 (p < 0.001), and only slightly more than C11 (p = 0.05). Values represent colony counts (>30 cells) in five experiments that were performed in triplicates. Closed diamonds represent means, and lines represent 95% confidence intervals, n=15. Untreated (Untr.); wild-type DNase1 (WT).

Under optimized transfection conditions, hrD-Nase1 (C13) eliminates 97-100% of all primary and metastatic melanoma cell types

All five human melanoma cell lines were efficiently eliminated by the C13 hrDNase1 construct. M14, G361, Mel-Juso and A2058 were almost equally sensitive to 1 µg and 2 µg of

C13 (Figure 6). However, A375 demonstrated higher sensitivity to C13 than the remainder of the melanoma cell lines (p < 0.001). A375 cell survival decreased to 1% with 0.6 of µg C13, and to 0% with 1.0 µg of C13 (Figure 6). With regards to procedural controls, no difference in cell survival was observed between untreated-, mock treated- and Cat-GFP-treated cells (p > 0.99) in all five melanoma cell lines. Furthermore, the impact of C13 on melanoma cell survival was found to be dose dependent in all cell lines tested. C13 at 0.3 µg decreased cell survival by ∼ 2.4 to 7.8 fold (p < 0.001) compared to cell survival percentages obtained with Cat-GFP; 0.6 µg of C13 decreased cell survival by ∼ 4.5 to 28.6 fold (p < 0.001) compared to those with 0.3 µg; and 1 µg of C13 decreased cell survival by ∼ 1 to 3.5 fold (p = 0.03) compared to those obtained with 0.6 µg (Figure 6). C13 at 1 µg decreased the colony count by 97-100%, and similarly, a dose of 2 µg of C13 decreased the colony count by 98-100%. Though the difference in cell survival between 1 µg and 2 µg was found to be statistically significant (p = 0.01), the difference was not large enough to be considered biologically significant (Figure 6). Taken together, these results suggest that the substantial killing capacity (97-100%; Figure 4B and Figure 6) exhibited by hrDNase1 is maintained across melanoma cell types, regardless of their primary or metastatic origin.
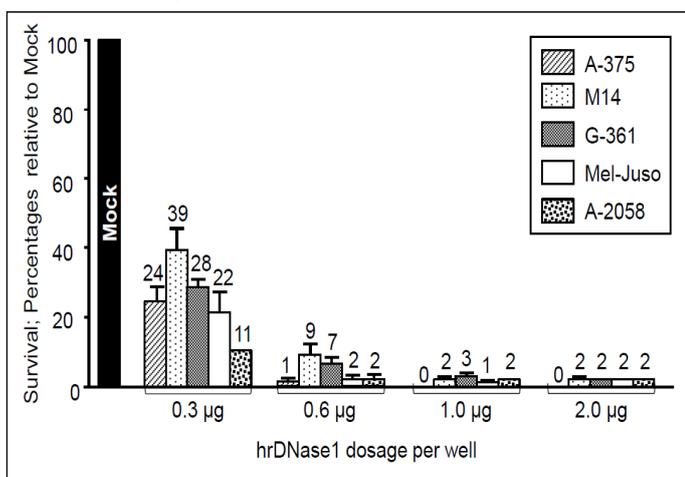

Figure 6. Determination of Human Recombinant DNase1 (hrD-Nase1) Cytotoxicity by a Colony Forming Assay for Construct C13

in Five Human Primary and Metastatic Melanoma Cell Lines. Survival of melanoma cells was measured 12–14 days after treatment with the hrDNase1 gene construct, C13, as described in the 'Materials and Methods' section. No statistically significant difference was found between Mock and Cat-GFP treated cells (p > 0.99). The impact of hrDNase1 on survival was determined to be dose-dependent in all cell lines. Though the difference in C13 cytotoxicity was computed to be statistically significant between 1 µg and 2 µg doses for all cell lines tested (p=0.01), the biological outcome was essentially identical for both doses. Approximately, 97-100% of all cell types were eliminated with a dose of 1 µg of construct C13.

C13 hrDNase1 construct killing efficiency exceeds transfection efficiency

Transfection conditions were optimized for Mel-Juso cells, since this cell line was originally employed in the 2011 Rosner et al. study to determine the cytotoxic capacity of the first generation of hrDNase1 constructs (C11, C13) [5]. Transfection conditions optimized for the Mel-Juso cell line were subsequently applied to all other cell lines tested due to the following:

(i) To allow for better comparison of hrDNase1 cytotoxicity between human melanoma cell lines in the present study; and (ii) To eliminate factors pertaining to transfection conditions, which could serve as potential confounders. Resultant transfection efficiencies were determined to be 71% (A2058), 70% (Mel-Juso), 65% (A375), 47% (G361) and 29% (M14), whereas the lowest cytotoxicity was determined to be 97% for construct C13 (at 1 µg; Figure. 6). Based on this comparison, it is evident that killing efficiency exceeded transfection efficiency for all melanoma cell types tested. The discrepancy between killing and transfection efficiency was found to be greatest for C13 in the M14 cell line, as 98% cytotoxicity was achieved at low (29%) transfection efficiencies.

Discussion

Following the improvement of transfection conditions in the Mel-Juso human melanoma cell line in this study, we aimed to engineer and identify the hrDNase1 construct that displays the greatest cytotoxic capacity in vitro. Towards this end, we sought to further increase hrNDase1 cytotoxicity by introducing point mutations into the actin binding site, which ultimately enabled hrDNase1 to better resist actin-mediated deactivation. Our findings revealed that construct C13 displayed the greatest cytotoxicity in multiple melanoma cell lines compared to alternative hrDNase1 isoforms including C11, whose killing efficiency was previously reported to be 70-100% in Mel-Juso human melanoma cells [5]. Next, we endeavored to determine whether the cytotoxic effects of hrDNase1 (C13) were limited to the Mel-Juso cell line only, or extended to additional primary and metastatic human melanoma types.

Optimized transfection efficiency increases hrDNase1 cytotoxicity in Mel-Juso human melanoma cells

In a prior study, we determined the killing efficiencies of C11 and C13 hrDNase1 constructs to be 70-100% and 40%, respectively in Mel-Juso human melanoma cells [5]. In the present study, the killing efficiency of C13 increased to 98-99% using half of the previously reported dose (1 µg; Figure 6). Though the transfection efficiency of hrDNase1 was measured at 24 h post-transfection in both studies, transfection conditions were optimized for Mel-Juso cells in the current study, as evidenced by an increase in transfection efficiency from 20% to 70% [5]. Therefore, it is thought that the increase in C13 killing efficiency reflects an increase in transfection efficiency.

The sequences of arginine codon substitutions at hrDNase1 positions 65 and 114 do not impact hrDNase1 killing efficiency

Ulmer et al. reported that DNase1 substitutions A114R and Y65R substitutions conferred actin resistance, however, they did not specify which codon sequence(s) were utilized to code for arginine substitutions at DNase1 positions 65 and 114 [15]. Therefore, our selection and incorporation of the CGT codon at either or both locations may not correspond with the codon sequences used by Ulmer et al.. Provided that changes to the codon sequence can potentially impact transcription levels [19,20] putative differences between the arginine sequences utilized by Ulmer et al. and the present study may have resulted in different levels of hrDNase1 expression. However, our results show that the codon sequence of arginine at amino acid locations 65 and 114 (CGT in C37, C39, C41 and C45) or CGC in C36, C38, C40, C42, C43 and C44) had a negligible impact on hrDNase1 cytotoxicity values (Figure 6), thereby effectively ruling out expression bias as a confounding variable.

hrDNase1 killing efficiency does not improve with the substitution of Phe-114 with Arg-114 or Arg-65, but can be increased by fusing an NLS to the N-terminus of hrDNase1

hrDNase1 constructs containing either an A114R or Y65R substitution in the actin binding site demonstrated cytotoxicity levels that were relatively equivalent to one another. These findings were unexpected, given that A114R was reported by Ulmer et al. [15] to decrease the binding of actin to DNase1 by a magnitude of 10 fold relative to Y65R. In addition, the ability of DNase1 to digest DNA in human sputum has been directly linked to the extent to which DNase1 is inhibited by actin [15]. Moreover, the A114R substitution has been shown to increase DNase1 potency by 50 fold, whereas the A114F substitution only improves DNase1 cytotoxic activity by

a factor of 5 fold compared to wtDNase1 [14,15]. As a result, it was anticipated that a 10 to 50 fold increase in hrDNase1 killing efficiency would be observed for constructs C36 to C45 (Figure 3). Instead, the cytotoxicity levels induced by C11 (A114F) were equivalent to the levels induced by C37 (A114R), and were greater than the levels induced by C39 (Y65R; Figure 5). Construct C11 (A114F) is similar to constructs C37 (A114R) and C39 (Y65R) in that all three carry an NLS at the C-terminus of hrDNase1. Considering that hrDNase1 does not display cytotoxic effects without the concurrent mutation of its actin binding site [5] the above data suggest that A114F leads to a maximal reduction in actin binding. Consequently, increasing hrDNase1 killing efficiency through additional substitutions at the actin binding site, as suggested by Ulmer et al. [15] cannot be accomplished in our system using A114R and Y65R mutations. Also, since improving transfection efficiency was sufficient to increase hrDNase1 killing efficiency to 97-100% for construct C13 in multiple human melanoma cell lines, it may not be necessary to increase hrDNase1 potency. Lastly, C13 cytotoxicity was determined to be significantly higher than the cytotoxicity of all newly generated hrDNase1 constructs (C36 to C45), and marginally higher than C11 cytotoxicity. Since C13 differed from C11 only in the localization of the NLS relative to the position of hrDNase1, it is likely that an NLS located at the N-terminus of hrDNase1 causes less interference at the catalytic site compared to an NLS located at the C-terminal end. The present study suggests that 2% of four of the five tested melanoma types (M14, Mel-Juso, A2058 and G361) may escape elimination when treated with 2 µg of hrDNase1. Further isolation and characterization of this sub-population is warranted, to determine whether these cells are inherently resistant to hrDNase1 treatment.

Underestimation of transfection efficiency values coupled with the aggressive nature of hrDNase1-induced cytotoxicity may partially account for discrepancies between transfection and killing efficiencies

Transfection efficiency was quantified at 24 h post-transfection, while killing efficiency was calculated at the conclusion of the 12-14 day incubation period. Given that the transfection medium was replaced seven days following transfection, it is plausible that hrDNase1 uptake in melanoma cells continued after the 24 h time point. Hence, the most likely explanation for the discrepancy between killing and transfection efficiencies is that both parameters were measured at different time points. However, exposure time to transfection media alone is not sufficient to account for the high killing efficiency obtained by C13 in this study, especially in light of the following consideration. The killing efficiency of construct C13 was previously shown to be 40% in Mel-Juso cells exposed to the plasmid for a period of one week, [5]

whereas C13 killing efficiency was measured at 98-99% in the present study (Figure 6). Given that the transfection efficiency in the previous study was only 20% in Mel-Juso cells compared to the 70% transfection efficiency achieved in the current study; this suggests that the optimization of transfection conditions played a major role in increasing C13 killing efficiency. Other factors may have also contributed significantly to the underestimation of transfection efficiency values. Transfection efficiency was measured as the percentage of cells strongly expressing GFP in the population of Cat-GFP transfected cells. The limiting factor regarding sensitivity to GFP fluorescence detection is the autofluorescence property of living cells and not the methodology utilized for detection of GFP fluorescence [21,22]. To avoid the false positive scoring of non-transfected autofluorescent cells, only cells with strong GFP fluorescence were counted. Transfected cells, whose GFP signal intensity was equal to the background [21] or, alternatively, whose GFP expression was below the designated cut-off level, were not included in the count. Thus, potentially the exclusion of these cell sub-populations couldcontributed to underestimation of transfection efficiency.

In addition, the extensive cytotoxic capacity of hrDNase1 may have also contributed to the discrepancy between transfection and killing percentages. In contrast to GFP, which has no impact on cell survival, hrDNase1 has cytotoxic properties. Hence, it is possible that the number of hrDNase1 molecules required to trigger cell death was lower than the number of GFP molecules required to produce a strong GFP signal ($\sim 10^4$ to 10^5 GFP molecules per cell) [21,23]. It follows that the percentage of surviving cells was determined to be lower in cultures transfected with hrDNase1 than it was in cultures exposed to GFP. Therefore, due to the biological impact of hrDNase1 on melanoma cell survival, transfection efficiency appears to be underestimated in hrDNase1-transfected cultures compared to those transfected with a GFP control.

The "Bystander" effect does not account for the apparent difference between cell transfection and hrDNase1 killing efficiencies

Killing efficiency that exceeds transfection efficiency is typically observed with the "bystander" effect—a phenomenon that often occurs during treatment with suicide gene therapy. In particular, features of the "bystander" effect have been thoroughly investigated in the Herpes Simplex Virus-thymidine kinase (HSVtk)/gancyclovir (GCV) prodrug system [24,25]. In this type of suicide gene therapy targeted cells are transduced with a gene that encodes a non-mammalian

enzyme (HSVtk). The prodrug (GCV) administered at a later stage is phosphorylated to a DNA-toxic triphosphate metabolite by the viral thymidine kinase and cellular kinases [26]. The cytotoxic GCV metabolites are incorporated into cellular DNA inducing chain termination that eventually triggers cell death. GCV metabolites also cross into adjacent non-transduced cells through intercellular channels, thereby destroying the neighboring cells [27]. In this way, the "bystander" effects allows for 60-90% or more of a given cell population to be eliminated when only 10-30% of cells have been transduced [28]. With regards to the present study, the "bystander" effect likely does not account for the differences noted between transfection and killing efficiencies, due to the following arguments: First, hrDNase1 damages nuclear DNA directly, and does not rely on the generation of a toxic metabolite through prodrug exposure [5]. Thus, upon transfection with hrDNase1, no toxic metabolite is generated or available for penetration into neighboring non-transfected cells. Second, during "bystander" effect, particles diffuse between cells through cannexon protein channels. Termed gap junctions, these protein channels span the cell membranes of two adjacent cells, thereby forming a direct connection between neighboring cytoplasms [29]. McMasters et al. [30] demonstrated that the "bystander" effect does not occur in the absence of functional gap junctions in human colon tumor cells [30]. Gap junctions allow for the passive diffusion of a variety of molecules smaller than 1 kDa, [27] as well as linearized peptides that have a molecular weight of up to 1.8 kDa (8-14 amino acids) [31]. In the HSVtk/GCV system, the toxic metabolite derived from GCV diffuses freely to neighboring cells through gap junctions due to its small size of < 0.5 kDa [32]. In direct contrast, hrDNase1 cannot cross freely between cells through gap junctions as a result of its large size ($\sim$ 30-34 kDa, 268 amino acids; size range reflects glycosylation state) [5,33] hrDNase1 without a "bystander" effect may avoid adverse effects of prodrug suicide gene therapy that are related to killing of neighboring normal cells [34].

In Summary

We have demonstrated that hrDNase1 is an efficacious modality for eliminating various types of human melanoma cell lines of primary and metastatic origions. In addition, we have shown that hrDNase1 killing efficiency can be enhanced through optimizing the transfection procedure. Additionally, hrDNase1-mediated cytotoxicity does not increase with the introduction of A114R or Y65R substitutions in the actin binding site; rather, the A114F substitution yields a maximal decrease in actin binding.

Conflict Of Interest

The authors declare no conflict of interest.

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