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Can Osmolytes TMAO and Glycerol Rescue Destabilized Temperature-Sensitive Mutants of P53?

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ABSTRACT

Some 30-40% of oncogenic mutants of p53 are inactive because of low stability. Stabilization of such mutants is one route for novel anti-cancer drugs. The osmolytes glycerol and trimethylamine *N*-oxide stabilize all proteins by a combination of effects on solvent water and by direct interactions with proteins. It has been reported that protein instability defects in temperature-sensitive p53 mutants can be rescued in cell lines by the addition of low concentrations of those compounds. We measured the effects of glycerol and trimethylamine N-oxide on the thermal and kinetic stability of wild-type p53 and temperature-sensitive mutants *in vitro* and found that there were just negligible increases in stability at the concentrations reportedly used in cell lines. We found no rescue of mutants in H1299 cell lines at those reported concentrations. There was some stabilization at higher concentrations of glycerol and trimethylamine *N*-oxide *in* vitro but these concentrations were toxic to the cells. It would seem unlikely that the use of general osmloytes would be an effective strategy for the rescue of unstable mutants because even if they were effective at stabilizing p53, they would alter the proteostasis of the cell.

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Abbreviations

- T-p53 – Thermo-Stable p53 core domain in a frame work of 4 Stabilizing Mutations M133L, V203A, N239Y, and N268D
- ts – Temperature-Sensitive
- T_m- Apparent Melting Temperature
- $t_{1/2}$ - Denaturation Half-Life

Introduction

The tumor suppressor p53 is a key protein in the cell's defense against cancer. It is directly inactivated by mutation in some 50% of human cancers. Most of the oncogenic mutations occur in its core or DNA-binding domain, contained within the sequence of residues 94-292 [1, 2]. Many of the oncogenic mutants are non-functional simply because their stability is lowered so that the protein denatures very rapidly and is either too unstable to function at body temperature or is rapidly depleted by denaturation and aggregation. Around 30-40% of these oncogenic mutants are inactive at body temperature exhibiting a temperature sensitive (ts) protein folding defect [3]. Those mutant proteins are natively folded at temperatures below the permissive temperature. Recently, the structure of one such mutant, Y220C, has been solved and found to have a specific lesion that can be rescued by specific drugs [4, 5]. Since the native structures of those temperature-sensitive mutants are essentially identical at temperatures below the permissive, it is theoretically possible to find generic drugs that will stabilize wild type and ts mutants of p53 [1, 6-8].

Osmolytes, such as trimethylamine N-oxide (TMAO), accumulate in some cells as a protection against osmotic stress and also stabilize proteins against denaturation [9, 10]. They stabilize proteins *in vitro* against thermally induced denaturation through a variety of generic effects, including affecting the structure of water so that hydrophobic interactions are enhanced or by direct binding to proteins [11-14]. A model of preferential exclusion has been developed by to show that the proteins are stabilized and compacted by interacting unfavourably with such osmolyte solutes [12]. By this mechanism the solutes are excluded or driven away from the protein's surface resulting in a low solute concentration around the protein. This exclusion might be due to energetically unfavourable interactions with the functional groups of the peptide backbone as in the case of TMAO. Based on the Le Chatelier principle, this reduction in surface area of the protein upon solute exclusion, results in minimization of the thermodynamically unfavourable chemical activity. Consequently, it has an effect on increasing the resistance of the protein molecule to chemical or thermal denaturation (i.e. favouring the native over the denatured state).

It has been reported that the instability of temperature-sensitive p53 mutants can be corrected by the addition of the osmolytes glycerol at 0.6 M or TMAO at 75 mM to cell lines expressing the temperature sensitive mutants p53-A135V, which rescue the mutant p53 at non-permissive temperatures [15]. Such osmolytes would seem from these data to be suitable drugs for the stabilization of wild-type and ts mutants of p53.

As it seemed unusual, however, that the low concentrations of osmolytes used could have such a large stabilizing effect, we investigated directly the effect of TMAO and glycerol at various concentrations on the thermodynamic and kinetic stability of wild-type p53 core domain and the temperature-sensitive mutants Y220C and F270L *in vitro*. We also examined the effects in a cancer cell line H1299 that expresses the temperature sensitive Y220C p53 mutant.

Results and Discussions

Effects of TMAO and Glycerol on the Thermal Stability of P53

The temperature sensitive region of p53 is its core domain, which is the seat of virtually all of its oncogenic mutations. The stability and *T*m (apparent melting temperature) of individual core domains are essentially the same as that of the full-length protein, and core domain is generally used to measure the effects of agents and mutations on the stability of p53 [16]. The effective melting temperatures of the core domain were reproducibly determined from the denaturation curves measured using thermal scanning (Figure 1).

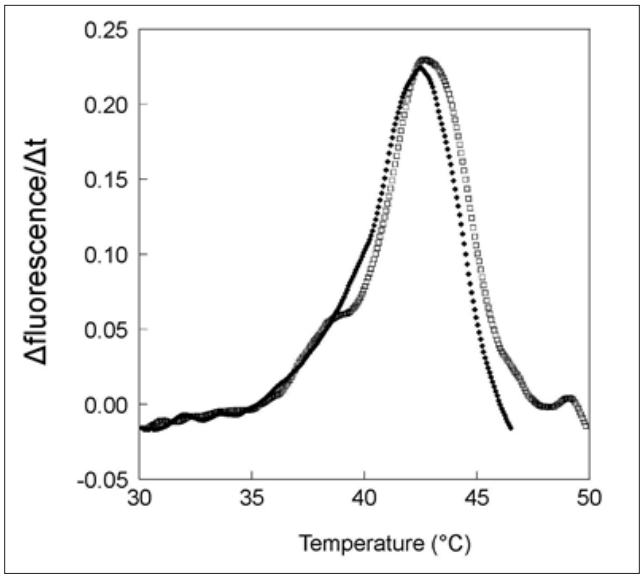

Figure 1: Measurement of *T*m of wild-type p53 from the rate of denaturation of the protein in the presence of Sypro orange dye. The slope of the increase in fluorescence of the dye (1:1000 v:v) is plotted against temperature for 10 µM p53 in 25 mM phosphate, 150 mM NaCl, 5 mM DTT at pH 7.2. Black dotted line represents p53 and the white dotted line represents p53 + 100 µM glycerol.

Shifts in *T*m are very reliably measured at the heating rate employed [5]. The increase of Tm of p53 on addition of increasing concentrations of osmolytes was negligible at the low concentration of 75 mM TMAO used by Welch et al. at about 0.2-0.3 °C for wild type and mutants (Figures 2) [15]. At the concentration of 0.6 M glycerol used in those studies (Figure 3), the increase in *T*m is only 0.6 oC. Temperature increases of about 4-7 oC are required to restore wild-type stability to highly destabilized common cancer mutants. This can be seen from the melting temperatures of common cancer mutants R249S and V143A which are estimated at 39 and 36 °C respectively compared to the wild-type with a *T*m of 43 °C [17].

A concentration of ~1 M TMAO was required to give an increase of 2.5-3 oC, which would be necessary for a physiologicaly relevant effect (Figure 2). Similarly, a concentration of 1.5-2 M glycerol is required to give a significant increase in *T*m (Figure 3). These data are in line with the effects of TMAO and glycerol on the stability of other proteins. For instance, Ishimaru and co-workers monitored the pressure-induced unfolding of wild-type p53 core domain in the presence of glycerol. High amounts of glycerol (above 10% v/v) were shown to be required to effectively stabilize p53 core domain to the extent of stability induced by binding of DNA and protect it from pressure-induced aggregation.

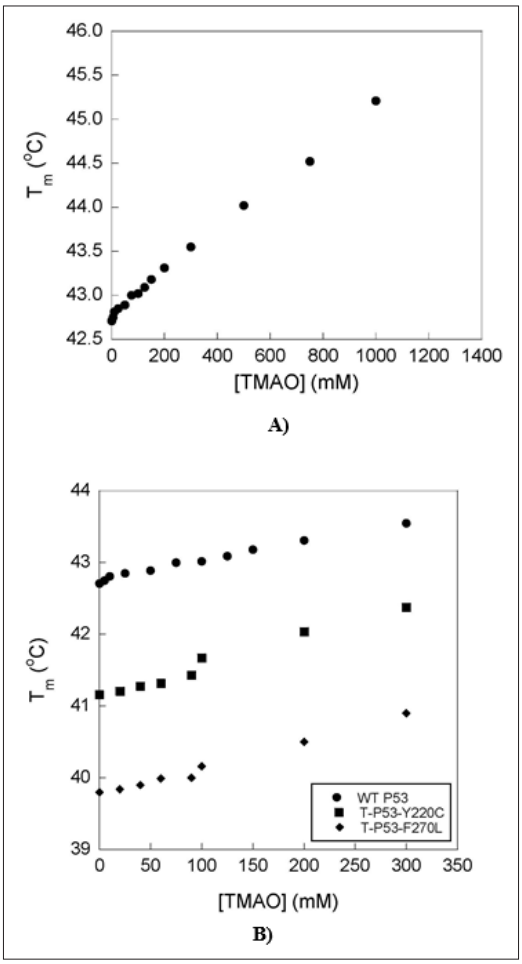

Figure 2: Effects of TMAO on the *T*m of wild-type and mutant p53. A) Wild-type p53. B) Wild-type and mutant p53 at lower concentrations of TMAO.

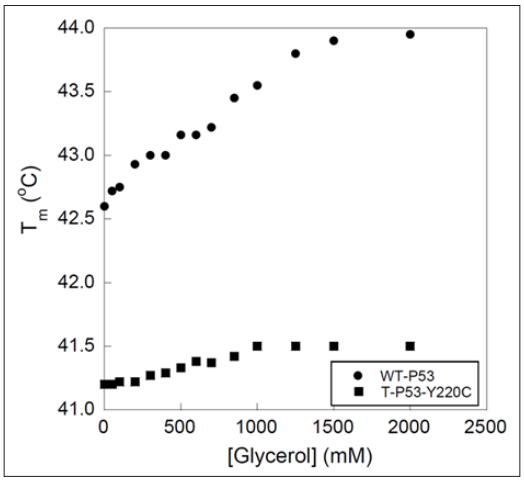

Figure 3: Effects of Glycerol on *T*m of wild-type p53.

Glycerol has been shown to be ineffective against MDH (malate dehydrogenase) at a concentration of 400 mM where it showed no change in the denaturation temperature and did not affect the unfolding curve significantly [18]. Whereas, in case of bovine α-Lactalbumin, only at a significant glycerol concentration of 10% (w/v), the apparent *T*m was seen to increase up by 2 °C [19]. Also it has been reported that a concentration as high as 500 mM of TMAO is needed to raise the *T*m of Tk-RNase HII by 5 °C (20).

Effects of TMAO and Glycerol on Kinetics of Denaturation of P53

As the half life of p53 at body temperature is low, we checked whether TMAO or glycerol could raise levels of p53 by lowering the rate of thermal denaturation, using wild-type protein [6]. The thermally induced denatured state of wild-type p53 core domain was monitored at 37 °C with increasing concentrations of TMAO or glycerol. There was essentially no change in the denaturation half life ($t_{1/2}$) observed for wild-type p53 between 0 and 75 mM TMAO (Figure 4, Table 1). The half life increased by about 50% in 600 mM glycerol.

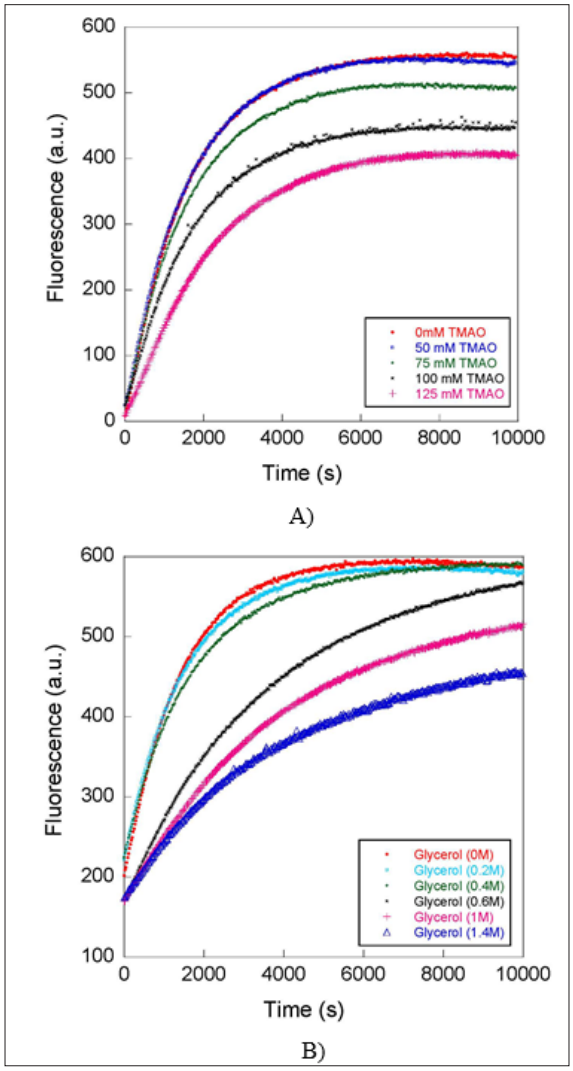

Figure 4: Denaturation kinetics of wild-type p53 core domain at 37° C, monitored using tryptophan fluorescence in 50 mM Hepes, pH 7.2, 1 mM Tris-2-carboxyethylphosphine at 340 nm at different concentrations of A) TMAO. B) glycerol.

Table 1: Observed unfolding rate constants of wild type p53 in the presence of TMAO or Glycerol, at 37 ˚C

a) TMAO (mM)	$t_{1/2}$ (37 °C) min
0	16.5
50	16.5
75	16.6
100	19.7
125	24.6
b) Glycerol (mM)	
0	15.8
200	17
400	20.6
600	24.5
1000	26.5
1400	27.9

Cell Viability Assays

We examined the effects of increasing concentrations of TMAO and glycerol on the viability of H1299 cells that lack p53 and an H1299 cell line that is stably transformed with the temperature sensitive p53-Y220C mutant [4]. We used an assay kit (Promega) that measures the conversion of a tetrazolium salt into a formazan product by living cells; the absorbance of the formazan is measured at 570 nm and the absorbance reading is directly proportional to the number of viable cells. At concentrations of up to 150 mM TMAO there was no significant difference between the viability of H1299 cells, and cells expressing the Y220C mutant after 24 h or 48 h (Figure 5). Stabilization by TMAO would be expected to reactivate the apoptotic capability of p53-Y220C. At concentrations of 1 M glycerol, we began to see reduced cell viability and by 1.5 M glycerol most of the cells were dead (compare with the 5 mM H2O2 control which kills all the cells). However, cell death was seen in both cell lines and was thus a non-specific effect of glycerol on the growth of the cells. Specifically, we did not see increased apoptosis of the cells expressing p53-Y220C compared with the controls.

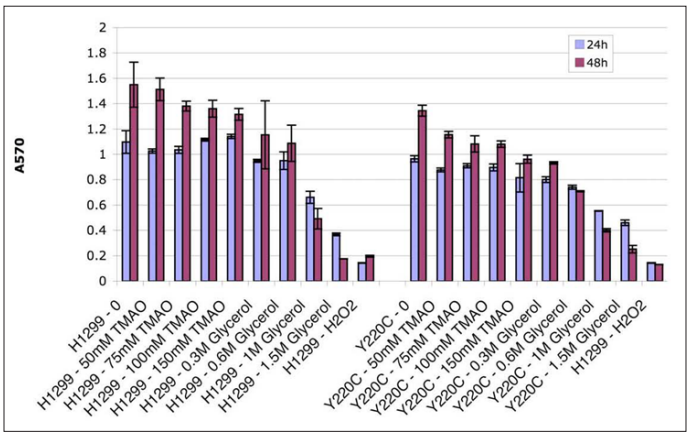

Figure 5: Effects of TMAO and glycerol on cell viability. H1299 cells that lack p53 (H1299) and H1299 cells that express the p53-Y220C mutant (Y220C) were grown for 24h or 48h in the presence of the indicated concentrations of TMAO, glycerol or 5mM H_2O_2. Cell viability was measured using the CellTiter 96® non-radioactive cell proliferation assay kit from Promega (cell assays done in collaboration with Dr. Fiona Townsley)

Conclusions

TMAO and glycerol stabilize proteins by generic mechanisms. They will affect the stability of wild type and point mutants of p53 identically because the change of one side chain will have minimal effects on the biophysical properties of the folded native state. The low concentrations of TMAO and glycerol used previously to rescue a ts mutant of p53 in cells have little effect on either the thermal or kinetic stability of p53 *in vitro* [15]. In our hands, the concentrations used did not rescue a typical ts mutant in the H1299 cancer cell line. The concentrations of TMAO and glycerol required to raise the stability of p53 significantly are in the toxic range. The idea of using a general, non-specific osmolyte to stabilize p53 would not seem a feasible strategy as the stability of most proteins in the cell would be increased and so the proteostasis of the cell would be disturbed.

Materials and Methods

Proteins

Experiments were performed on the core domain wild-type p53 or *T*-p53C-Y220C and *T*-p53C-F270L which have the mutations Y220C or F270L in a stabilized framework of human p53 that contains 4 mutations [4, 21].

Mutagenesis and Protein Purification

Mutagenesis, gene expression, and protein purification were performed as described in ref, for wild-type p53 or with a modification for *T*-p53C-Y220C or *T*-p53C-F270L, for which a construct comprising residues 94–312 was used [22]. After the final purification step (gel filtration), the mutant proteins were concentrated to 6–7 mg/mL, flash-frozen, and stored in liquid nitrogen.

Thermal Denaturation Studies

Thermal unfolding of p53 was followed by monitoring the fluorescence of the dye Sypro Orange that has a large increase in fluorescence on binding to the denatured state of proteins. The measurements were made in 25 mM phosphate, 150 mM NaCl, 5 mM DTT, pH 7.2, with 10 µM protein concentration using a Rotor-gene 6000 qPCR machine (Corbett Life Science) at a heating rate of 72 °C/h.

Stock solutions of TMAO and glycerol were prepared in water. Sample volumes of 20 µL containing p53 with the Sypro Orange dye (1:1000 v:v) and different diluted concentrations of TMAO or glycerol were heated from 30 to 60 ˚C. The melting temperature, Tm, was calculated by determining the maximum of the first derivative of the thermal denaturation curve using the supplied software. We verified independently that the measured values of Tm were the same as those determined from differential scanning calorimetry at similar heating rates [5].

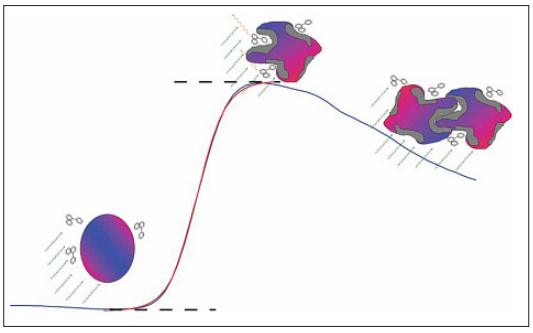

Figure 6: Shows a typical fluorescence intensity versus temperature spectrum for the unfolding of protein in the presence of SYPRO orange.

The molecular structure of the dye is symbolized as a three-ring aromatic molecule. In the presence of a globular protein (oval shape at the baseline of the curve), a basic fluorescence intensity is excited by light of 492 nm (depicted schematically by green curved arrows). Through unfolding of the protein, hydrophobic patches (in gray) become exposed, and strong fluorescent light of 610 nm (depicted by orange curved arrows) is emitted by the dye molecules bound to them. Following the peak in the intensity, a gradual decrease is observed, which is mainly explained by protein being removed from solution owing to precipitation and aggregation.

Kinetics of Denaturation

The protein was buffer exchanged on ice to a final concentration that varied between 1–20 µM and immediately transferred to 37 °C. The unfolding kinetics was followed from the emission of tryptophan at 340 nm on excitation at 280 nm, using a Cary Eclipse fluorescence spectrophotometer controlled by the supplied Cary software [23]. Reactions were followed for 10,000 s. Data were fitted to a single exponential (eqn 1) $F_{340} = A - B \exp(-_{kobs}t)) + C$ (1), where A is the starting value, B is the amplitude of the fluorescence curve, kobs is the observed unfolding rate constant, and C is the lower limiting value for F_{340}. The half-lives at the corresponding temperatures were calculated using $t_{1/2} = \ln 2/k_{obs}$.

Cell Assays

H1299 cells were grown and maintained in Roswell Park Memorial Institute (RPMI) medium (Invitrogen) supplemented with 10% foetal calf serum. The H1299 cell line expressing the p53-Y220C mutant was maintained by the addition of 600 µg/mL G418. TMAO and glycerol were diluted to the appropriate concentration in 50 µL of RPMI medium in a 96 well plate. 50 µL of cell suspension ($3x10^5$cells/ml) were added and the plates were incubated at 37 °C for 24 or 48 h. Cell viability was measured using a CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. A570 values were measured on a Spectra Max 190 plate reader (Molecular Devices). All conditions were tested in triplicate.

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