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TCTP as a therapeutic target in melanoma treatment

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Background: Translationally controlled tumour protein (TCTP) is an antiapoptotic protein highly conserved through phylogeny. Translationally controlled tumour protein overexpression was detected in several tumour types. Silencing TCTP was shown to induce tumour reversion. There is a reciprocal repression between TCTP and P53. Sertraline interacts with TCTP and decreases its cellular levels.

Methods: We evaluate the role of TCTP in melanoma using sertraline and siRNA. Cell viability, migration, and clonogenicity were assessed in human and murine melanoma cells *in vitro*. Sertraline was evaluated in a murine melanoma model and was compared with dacarbazine, a major chemotherapeutic agent used in melanoma treatment.

Results: Inhibition of TCTP levels decreases melanoma cell viability, migration, clonogenicity, and *in vivo* tumour growth. Human melanoma cells treated with sertraline show diminished migration properties and capacity to form colonies. Sertraline was effective in inhibiting tumour growth in a murine melanoma model; its effect was stronger when compared with dacarbazine.

Conclusions: Altogether, these results indicate that sertraline could be effective against melanoma and TCTP can be a target for melanoma therapy.

Translationally controlled tumour protein (TCTP) initially named Q23, P21, and P23, according to its molecular mass, is involved in regulating fundamental cellular processes and is overexpressed in several tumour types (Chung *et al*, 2000; Amson *et al*, 2013). Since its characterisation, TCTP has been related to tumourigenesis and cancer progression (Kozioł and Gurdon, 2012). Numerous studies show that TCTP level in tumour is higher than that in the corresponding normal tissues, including prostate, renal, breast, and lung cancers (Amson *et al*, 2013; Acunzo *et al*, 2014; Ambrosio *et al*, 2015; Rocca *et al*, 2015). These observations point to TCTP's critical role in tumourigenesis and highlight its putative role as a therapeutic target in several cancers. Silencing TCTP was shown to induce tumour reversion, a process overriding the malignant process at the molecular level (Tuynder *et al*, 2002, 2004; Telerman and Amson, 2009). The decrease of TCTP levels were related to

inhibition of tumour growth and the loss of tumour features (high levels cell proliferation and migration) (Amson *et al*, 2013; Acunzo *et al*, 2014). Sertraline was first investigated in the context of TCTP and tumours because of the similarity of its structure to antihistaminic compounds; because TCTP encodes for a histamine-releasing factor, the hypothesis that inhibitors of the histaminic pathway could be effective against tumour cells was evaluated. Several articles had already shown that sertraline inhibits tumour growth *in vivo* (Tuynder *et al*, 2004). There are epidemiologic studies showing a protective effect and a decreased risk of tumour development (in breast, colorectal, lung cancers) among users of high doses of selective serotonin reuptake inhibitors, including sertraline (Xu *et al*, 2006; Coogan *et al*, 2009; Toh *et al*, 2009; Wernli *et al*, 2009). There is a negative feedback loop between TCTP and P53. Translationally controlled

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tumour protein promotes P53 degradation, inhibits MDM2 autoubiquitination, promotes MDM2-mediated ubiquitination, and degradation of P53. Additionally, P53 directly represses TCTP transcription (Amson *et al.*, 2012). In this context sertraline binds directly to TCTP (Amson *et al.*, 2013). The effects of decreasing TCTP in melanoma were analysed using both sertraline and siRNA. *In vivo* analysis was performed using a C57BL/6 mice model and compared with the alkylating agent dacarbazine (DTIC). Although DTIC is a long-established and standard treatment for metastatic melanoma, its efficiency is low (Pretto and Neri, 2013). The results reported here provide a basis for the evaluation of TCTP targeting in melanoma and suggests sertraline as a potential drug.

MATERIALS AND METHODS

Cell culture and animals. Human melanoma cell lines and murine melanoma cells (B16-F1 and B16-F10) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Murine cells were cultured in DMEM media and human cells were maintained in RPMI 1640, both supplemented with 10% (v/v⁻¹) fetal bovine serum (FBS) (Cultilab, Campinas, Brazil) and 40 mg ml⁻¹ gentamicin, in humidified 5% CO₂-95% air at 37 °C. C57BL/6 mice (female, 8–12 weeks old) were provided from the Central Animal House of the Pontifical Catholic University of Paraná, Brazil and received a standard laboratory diet (Purina). All procedures used in this study were approved by the Institutional Ethics Committee of the Federal University of Paraná (no. 730).

Small interfering RNA. The siRNAs of tpt1/TCTP were synthesised by Ambion (Life Technologies, Carlsbad, CA, USA). Sense siRNA, (5'-AGCACAUCUUGCUAAUUUTT-3'); antisense siRNA, (5'-AAAUUAGCAAGGAUGUGCUTA-3'). All procedures were performed under RNase-free conditions, using RNase-free water. Approximately 10⁵ B16-F10 cells were transfected with a final concentration of 50 nM of siRNA duplexes using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). After 24, 48, and 72 h transfection, cells were collected and used for cell viability, migration, and proliferation assays, RT-PCR analysis and western blot analysis. The siRNAs used herein were carefully evaluated concerning the main characteristics associated with highly active siRNAs: moderate-to-low (33,3%) guanine-cytosine content, lack of internal secondary structure within the siRNA (high-ΔG unfavoured secondary structures), and low stability of binding interactions at the 5' terminus of the guide siRNA strand, a uridine residue at position 10 of the sense strand, lack of immunostimulatory sequences within the siRNA, and lack of secondary structure of the target site (Reynolds *et al.*, 2004). BLASTn search to potential siRNAs and Smith-Waterman dynamic programming sequence alignment algorithm were performed: BLASTn analysis showed the confidence parameter (*E*-value) to the right annealing between the siRNA and the target sequence is 250-fold higher than the second predicted hypothetical sequence; furthermore, the TCTP is the only known protein sequence that has 100% identity.

Quantitative real-time PCR. Total RNA was extracted from tissues or cells using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentrations were measured with a NanoVue Plus spectrophotometer (GE Healthcare) and 10 pg total RNA was used. Real-time PCR was performed using the Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems, Foster City, CA, USA). Primers for TCTP (sense 5'-GAGCTGTTCTCCGACATCTACAAG-3'; antisense 5'-AGTCACACCATCTTCACGGTAGTC-3'), P53 (sense 5'-GTCACAGCATGACGGAGG-3'; anti-sense 5'-TCTTCCAGATGCTCGGGATAC-3') and GAPDH (sense 5'-

AACGACCCCTTCATTGACCTC-3'; antisense 5'-TCCACCACCCTGTTGCTGTAG-3') were based on validated sequences from Primer Bank (Spandidos *et al.*, 2010). The relative quantification value for each target gene was compared with the endogenous control (GAPDH), which was expressed as 2^{-(Ct - Cc)} (Ct and Cc are the mean threshold cycle differences after normalising to GAPDH).

Protein extract and western blot. After treatment for 24, 48, and 72 h with sertraline or siRNA, cultured cell pellets were homogenised and lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulphonyl fluoride, 2 μg ml⁻¹ aprotinin, 2 μg ml⁻¹ leupeptin, 1 mM dithiothreitol, 1% Triton X-100, and 20% glycerol). The lysates were separated by centrifugation at 20 000 g for 30 min at 4 °C; the supernatants were collected and aliquots were made. All protein concentrations were determined using the MicroBCA Assay (Thermo Scientific, Waltham, MA, USA). An aliquot (50 μg protein per lane for cellular extract and 100 μg protein per lane for tumour extract) of the cell per tissue lysate was separated by 15% SDS-PAGE gel, and then the proteins were transferred onto a nitrocellulose membrane. The primary antibodies against TCTP, GAPDH, and P53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used according to the manufacturer's instruction. The membranes were further incubated with HRP-linked anti-rabbit IgG and HRP-linked anti-mouse IgG (1:5000). The protein-antibody complexes were detected by using the chemiluminescent substrate according to the manufacturer's instructions and the emitted light captured on X-ray film or using Amersham Imager 600 (Little Chalfont, UK). The intensity of each band was analysed using 'histogram analyses' in the ImageJ Analysis Software (Schneider *et al.*, 2012) to confirm reductions or increases (data not shown).

Cell viability assays. Human melanoma (MeWo and A2058) cells and murine melanoma (B16-F10) (5 × 10³ cells per well) cells were plated for 16 h on 96-well plates and then grown in medium containing FBS. The medium was then replaced by a serum-free one. After 16 h, this was replaced with medium containing 10% of FBS plus sertraline at different concentrations (0.01, 0.1, and 1 μM) in pentaplicate. Controls consisted of the respective medium alone and in the presence of 550 μM DMSO, which was the sertraline solvent. After 24, 48, and 72 h, the viability of cells in each well was determined using Cell Titer-Glo luminescent assay reagent, following the manufacturer's instructions (Promega, Madison, WI, USA). Luminescence was quantified using Tecan Infinite X-100 (Männedorf, Switzerland). Experiments were performed in pentaplicate and repeated two times. Alternatively, murine melanoma B16-F1 and B16-F10 cells were plated (5 × 10⁴ cells) in 24-well plates and transfected using tpt1/TCTP siRNA (50 nM) and negative siRNA control. After 24, 48, and 72 h, cells were stained with Trypan blue and counted in a haemocytometer. Trypan blue experiments were performed in pentaplicate and repeated three times.

Cell migration assays. Human melanoma cell lines' migration was measured in a two-dimensional 'scratch' assay. To repress proliferation cells were treated with mitomycin C (10 μg ml⁻¹) for 2 h, before the cell monolayer was scratched with a pipette tip. Cells were then incubated with sertraline (0.01, 0.1, and 1 μM) for 24 h. Scratches were monitored using the Olympus CellR Live Cell Imaging System (Tokyo, Japan) with an IX81 motorised inverted microscope (Tokyo, Japan) and a Hamamatsu camera (Tokyo, Japan), fitted with a climate chamber. Images were acquired using the Olympus excellence RT software (Olympus, Hamburg, Germany). Relative migratory capacity was determined by calculating the percentage of the cell-free area. Experiments were performed in pentaplicate and repeated twice. Alternatively, migration assays were performed using uncoated cell culture

inserts with 8 μm pores (Corning Life Sciences, Corning, NY, USA) according to the manufacturer's instructions. Briefly, B16-F10 cells were transfected with siRNA to tpt1/TCTP and with a negative control for 24, 48, and 72 h. Cells were harvested and resuspended in serum-free medium at a density of 5×10^4 cells per well. B16-F1, B16-F10, B16-F10-tpt1 siRNA, and B16-F10 control siRNA were seeded into the upper chamber. Lower chambers were filled with medium containing 10% FBS as a chemoattractant. After 6 h, cells were fixed and permeabilised with methanol and stained with 0.5% Crystal violet/20% methanol. The non-migrating cells on the upper surface of the filter were removed by cotton swab. The number of migratory cells was measured by counting at $\times 100$ magnifications using a microscope. Experiments were performed in triplicate and repeated three times.

Clonogenic assay (anchorage-independent cell transformation assay). Soft agar method was used to evaluate colony formation capacity of melanoma cells. Human and murine melanoma cells (5×10^3 cells per well) were treated with sertraline (0.1, 1, and 5 μM) in media containing 1.5 ml of 0.5% agar (DMEM or RPMI 1640) and supplemented with 10% (v/v) of FBS, and 40 mg ml⁻¹ gentamicin. Cultures dishes were maintained at 37 °C in a 5% CO₂ incubator for 15 days. Cell colonies were stained with 0.005% Crystal violet, and counted

at light microscope. Experiments were performed in duplicate and repeated three times.

Cell proliferation assays. Murine melanoma cells (10^4 cells per well) were plated for 16 h on 96-well plates in medium containing FBS. The medium was then replaced by a serum-free one. After 16 h, serum-free medium was replaced with medium containing 10% of FBS and cells were transfected with siRNA to tpt1/TCTP and with a negative control (control siRNA). After 24, 48, and 72 h, the number of cells in each well was determined using Violet crystal method (Borges *et al.*, 2013). Experiments were carried out in pentaplicate and repeated three times.

In vivo tumour growth. The C57BL/6 mice were subcutaneously injected with B16-F10 cells (5×10^5 cells per animal), and a solid tumour developed at the injection site. Intraperitoneal treatments started 5 days after injection of the cells. Mice were treated with a daily dose of sertraline (10 mg kg⁻¹, in 100 μl). The control groups received 100 μl of DTIC solution (60 mg kg⁻¹) and the respective vehicle under the experimental conditions described for the treated groups: water for DTIC and/or aqueous solution containing DMSO (2.4 mM) for sertraline. All mice were kept under observation for 17 days (12 days of treatment) and then killed using the combination (1:1) of xilazine cloridrate/ketamine

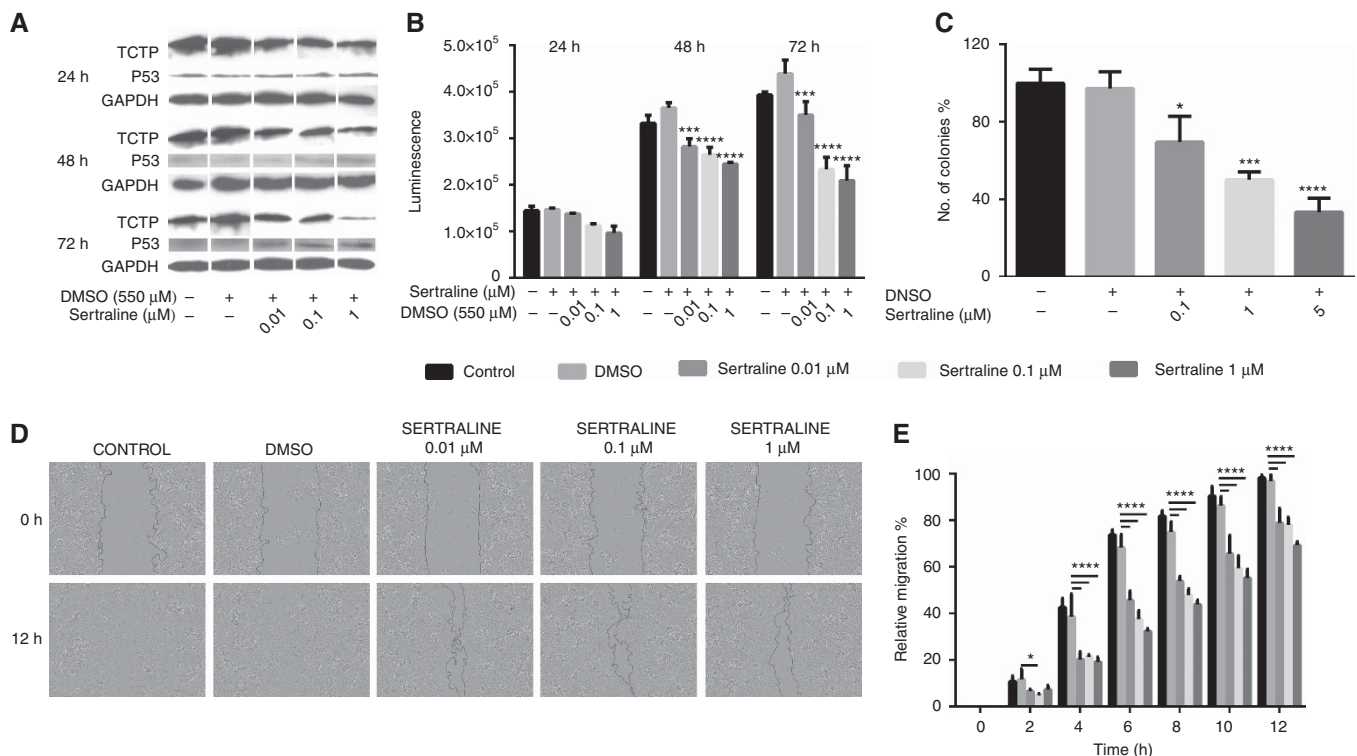


Figure 1. (A) Western blot analysis of TCTP, P53, and GAPDH protein levels in MeWo cells treated with sertraline (0.01, 0.1, and 1 μM) and with a negative control (dimethyl sulphoxide (DMSO)) (24, 48, and 72 h). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control for each sample. (B) Cell viability was determined using Cell Titer-Glo assay (Promega). MeWo cells were plated in 96-well plates (5×10^3 cells per well) and treated with sertraline (0.01, 0.1, and 1 μM), negative control (DMSO) or only medium (control) in soft agar. After 24, 48, and 72 h, Cell Titer-Glo reagent was added in the medium and luminescence was measured by Tecan (Tecan Infinite M200, Männedorf, Switzerland). Data were analysed by comparing the lines treated with sertraline and the negative control (DMSO) using two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. Two independent experiments were performed ($n = 5$) ($***P < 0.001$, and $****P < 0.0001$). (C) Clonogenic assay. A total of 5×10^3 cells were incubated with sertraline (0.1, 1, and 5 μM), negative control (DMSO) or only medium (control) in soft agar. After 15 days, cells were stained with Crystal violet and the number of colonies was counted. Three independent experiments were performed ($n = 2$) ($*P < 0.1$, $***P < 0.001$ and $****P < 0.0001$). Data represent mean \pm s.d. of three independent experiments. (D) Representative images from 'scratch' migration assay. A total of 5×10^4 cells were plated in 96-well plates, treated with mitomycin C and then the cell monolayer was scratched and incubated in the presence of sertraline (0.01, 0.1, and 1 μM), DMSO or only medium (control) in soft agar. Scratches were monitored hourly for 12 h. (E) Relative migratory capacity was determined by calculating the percentage of the cell-free area using the ImageJ Analysis Software. Data represent mean \pm s.d. of three independent experiments ($*P < 0.1$ and $****P < 0.001$).

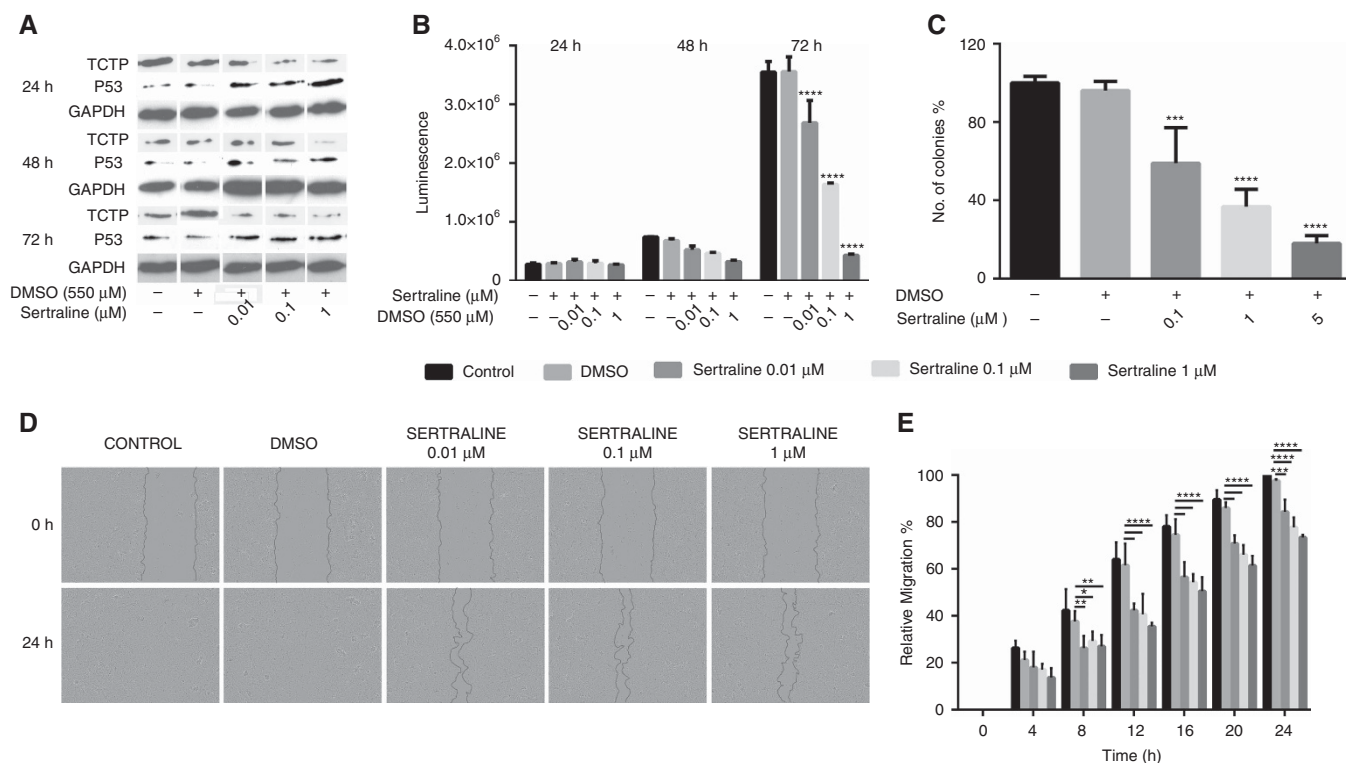


Figure 2. Effect of sertraline in A2058 human melanoma cell line. **(A)** Western blot analysis of TCTP, P53, and GAPDH proteins in A2058 cell line. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control for each sample. **(B)** Cell viability was determined using Cell Titer-Glo (Promega). A2058 cells were plated in 96-well plates (5×10^3 cells per well) and treated with sertraline (0.01, 0.1, and 1 μM), a negative control (dimethyl sulphoxide (DMSO)) or only medium (control) in soft agar. After 24, 48, and 72 h, Cell Titer-Glo reagent was added in the medium and luminescence was measured by Tecan (Tecan Infinite M200). Data were analysed by comparing the lines treated with sertraline and the negative control (DMSO) using two-way analysis of variance (ANOVA) with Tukey's *post hoc* test. Two independent experiments were performed ($n=5$) ($*P<0.1$ and $****P<0.0001$). **(C)** Clonogenicity assay. A total of 5×10^3 cells were incubated with sertraline (0.1, 1, and 5 μM), negative control (DMSO) or only medium (control) in soft agar. After 15 days, cells were stained with Crystal violet and the number of colonies was counted. Three independent experiments were performed ($n=2$) ($***P<0.001$ and $****P<0.0001$). Data represent mean \pm s.d. of three independent experiments. **(D)** Representative images from 'scratch' migration assay. A total of 5×10^4 cells were plated in 96-well plates, treated with mitomycin C and then the cell monolayer was scratched and incubated in the presence of sertraline (0.01, 0.1, and 1 μM), DMSO or only medium (control) in soft agar. Scratches were monitored hourly for 24 h. **(E)** Relative migratory capacity was determined by calculating the percentage of the cell-free area using Image J program. Data represent mean \pm s.d. of three independent experiments ($*P<0.1$, $**P<0.01$, $***P<0.001$, and $****P<0.001$).

cloridrate (10%) in 50 μl . Tumours were excised, photographed and their weights were determined. Tumour tissues were collected for total protein extraction and histochemical analysis. Experiments were performed using groups of five animals per condition and repeated two times. All procedures involving animals were performed in accordance with Brazilian Federal Laws, and with the Ethical Subcommittee on Research Animal Care Agreement number 730 of the Federal University of Paraná. The protocols applied in carrying out the investigations require a high standard of ethics and are in agreement with the Ethical Principals established by Experimental Animal Brazilian Council (COBEA), and with the requirement of the 'Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care)'.

Immunohistochemical studies. Immunohistochemical analyses of TCTP expression were performed on Tissue-Tek OCT compound-fixed tumour sections obtained from B16-F10 tumours from the *in vivo* experiments. Tumour glass slides were blocked with PBS containing 1% BSA and 0.025% Triton X-100 for 20 min. Samples were incubated with primary antibodies against TCTP (Santa Cruz Biotechnology), Ki67 (Spring Bioscience, Pleasanton, CA, USA), caspase-3 (Biocare Medical, Pacheco, CA, USA), and P53 (Santa Cruz Biotechnology) in 1% BSA/PBS overnight at 4 $^{\circ}\text{C}$, and then washed and incubated with fluorescent secondary antibody (Invitrogen) for 1 h at room temperature. Samples were

washed, dried and mounted in medium containing DAPI (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and imaged on Nikon A1RSiMP (Nikon, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using ANOVA with Tukey's *post hoc* test for average comparisons using the GraphPad Prism 6 program. Statistical significance was established at $P<0.1$.

RESULTS

Effects of sertraline on human melanoma cell lines. Sertraline treatment was analysed in the context of the human melanoma cell lines MeWo and A2058, and were further assessed for protein expression levels and biological effects. Figure 1A shows TCTP and P53 levels after incubation with sertraline. A decrease in TCTP protein level and an increase in P53 were observed. Sertraline decreased the viability of MeWo melanoma cells in a time- and concentration-dependent manner (Figure 1B). In the soft agar assay, the number of colonies formed in the presence of sertraline was reduced by $\sim 50\%$ (1 μM) and by 65% (5 μM) when compared with the control (DMSO) (Figure 1C). The effects of sertraline on the reduction of the migration phenotype were also highly significant (Figure 1D). A scratch assay was performed in the

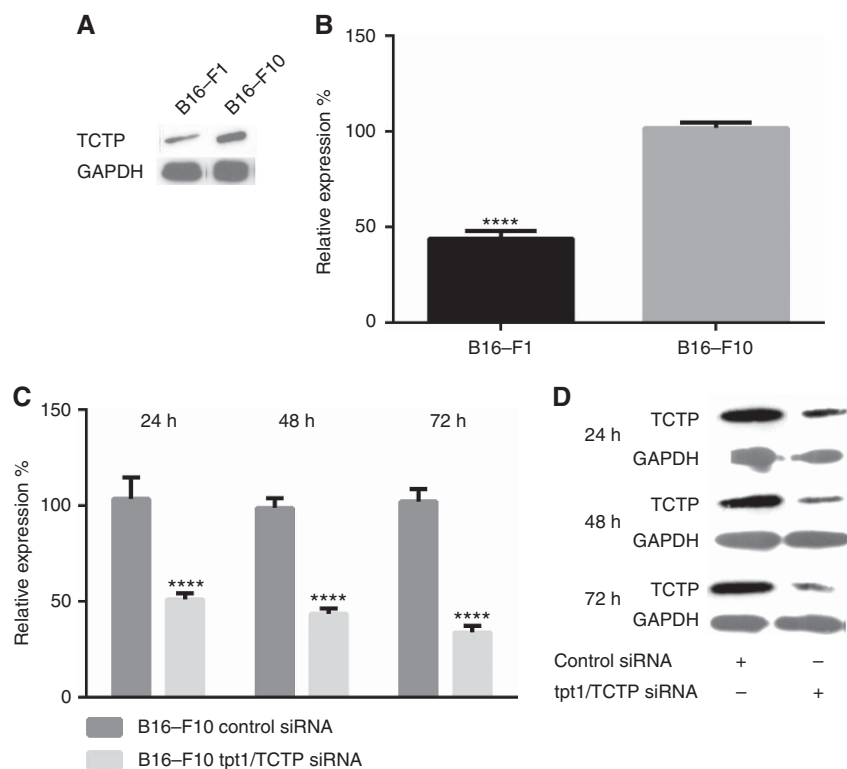


Figure 3. Expression of TCTP protein in B16-F1 and B16-F10 cells. **(A)** Western blot analysis of TCTP and GAPDH proteins in B16-F1 and B16-F10 cell lines. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control for each sample. **(B)** Quantitative real-time PCR of TCTP mRNA levels in B16-F1 and B16-F10 melanoma cells. For quantification, we used the $\Delta\Delta C_t$ method with GAPDH as an endogenous control for each sample ($****P < 0.001$, compared with control) in soft agar. Data represent mean \pm s.d. of three independent experiments. **(C)** Silencing of tpt1/TCTP by siRNA in B16-F10 cells. Percentage of relative expression of TCTP after transfection with 50 nM of siRNA for tpt1/TCTP or negative control (control siRNA). Analysis of TCTP levels using RT-PCR in B16-F10 cell line at 24, 48, and 72 h after transfection. For quantification, we used the $\Delta\Delta C_t$ method using GAPDH as an endogenous standard for each sample. For graphics, $\Delta\Delta C_t$ was normalised to the controls. Data were analysed by comparing the lines transfected with tpt1/TCTP siRNA and the negative control using *t*-test. Two independent experiments were performed ($n = 5$) ($****P < 0.0001$). **(D)** Western blot analysis of TCTP and GAPDH protein levels in B16-F10 cells were determined after transfection with 50 nM of tpt1/TCTP siRNA and negative control (24, 48, and 72 h). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control for each sample.

presence of different concentrations of sertraline and cell migration was analysed each hour for 12 h (Figure 1D graph). Even low doses (0.01 and 0.1 μM) could significantly inhibit the melanoma cells migration.

The same set of experiments was performed on the A2058 human melanoma cell line that was also very sensitive to sertraline treatment (Figure 2). Translationally controlled tumour protein levels were decreased and P53 levels were increased after sertraline treatment (Figure 2A). Cell viability was significantly decreased after sertraline treatment (Figure 2B). Sertraline also caused a marked decrease in the number of colonies formed in the soft agar (Figure 2C). The higher concentration (5 μM) inhibited by $\sim 80\%$ the formation of colonies. Figure 2D shows that melanoma cell migration was also strongly affected by sertraline treatment, in all tested concentrations (0.01, 0.1, and 1 μM). Taken together, these results show that, in MeWo and A2058 cells, sertraline decreases TCTP and inhibits cell viability, colony formation and cell migration.

High TCTP levels in B16 (B16-F1, B16-F10) murine melanoma cell lines. B16 melanoma cell lines are very interesting models for tumour biology studies. Compared with B16-F1, which has low metastatic potential, B16-F10 cell line displays a higher metastatic capacity (Fidler, 1973; Morris *et al.*, 2015). Translationally controlled tumour protein levels are markedly higher in B16-F10 cells (Figure 3A). A q-PCR analysis revealed that expression of TCTP mRNA in B16-F10 cells was 2.3-fold higher than that in

B16-F1 cells (Figure 3B). A knockdown (siRNA) of TCTP was performed and the transfectants were analysed by qRT-PCR after 24, 48, and 72 h (Figure 3C). A decrease of 50–70% in the amount of TCTP mRNA was observed and confirmed by the protein levels detected in the western blot assays (Figure 3D).

Effects of TCTP inhibition in B16-F10 cell viability, proliferation, and migration. The inhibition of TCTP by siRNA in the B16 cells slightly affected the viability and proliferation of these cells. A decrease of 15% and 25% in viability was observed after, respectively, 24 and 72 h of transfection (Figure 4A). The proliferation was reduced by 20% and 40% (Figure 4B). When the migration of these cells was evaluated by transwell, a substantial decrease in the migration potential of the TCTP-silenced cells was observed (Figure 4C). The number of cells that reached the other side of the well membrane was 53% and 67% smaller when TCTP was inhibited after 48 h and 72 h of transfection. The migration of TCTP knockdown cells was lower than the one observed for B16-F1 cells (Figure 4C).

In vitro effects of sertraline on B16-F10 cells. The effect of sertraline on the downregulation of TCTP was initially assessed by western blot analysis. B16-F10 melanoma cells were treated with sertraline at different concentrations (0.01, 0.1, and 1 μM). Translationally controlled tumour protein levels were evaluated after 24, 48, and 72 h (Figure 5A). The observed decrease of TCTP levels by sertraline is time and concentration dependent. Sertraline

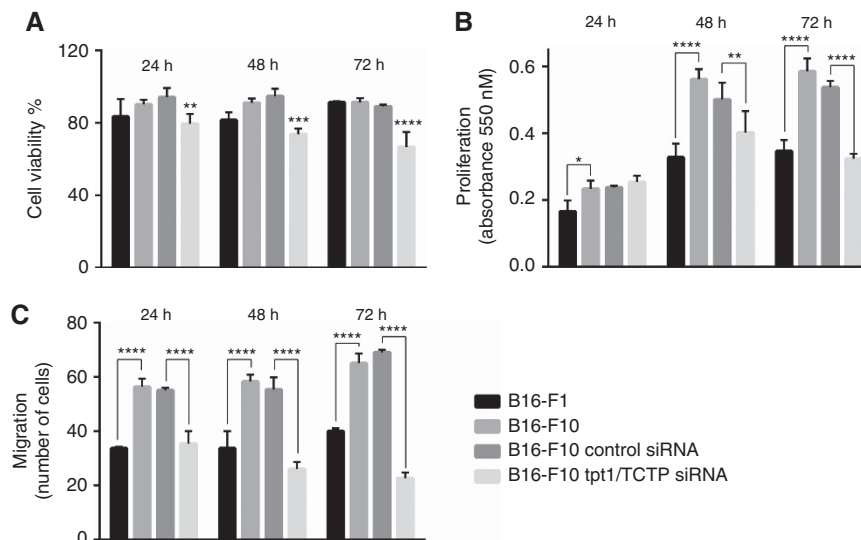


Figure 4. Silencing TCTP by siRNA in B16-F10 melanoma cells: viability, proliferation, and migration. **(A)** Cell viability was determined using Trypan blue exclusion assay. B16-F1, B16-F10, and B16-F10 cells transfected using tpt1/TCTP siRNA (50 nM) and negative control (control siRNA) were plated (5×10^4 cells) in 24-well plates. After 24, 48, and 72 h, cells were stained with Trypan blue and counted in a haemocytometer. Data were statistically analysed by comparing the viability of the cell lines transfected with tpt1/TCTP siRNA and the negative control using one-way ANOVA with Tukey's *post hoc* test. Two independent experiments were performed ($n=5$) (** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$). **(B)** Cell proliferation by Crystal violet staining. B16-F1, B16-F10, and B16-F10 cells transfected using tpt1/TCTP siRNA (50 nM) and negative control (10^4 cells per well in 96-well plates). At 24, 48, and 72 h after transfection, cells were fixed and stained, and the absorbance was measured at 550 nm. Data were statistically analysed by comparing the lines transfected with tpt1/TCTP siRNA and the negative control using two-way ANOVA with Tukey's *post hoc* test. Two independent experiments were performed ($n=5$) (* $P<0.1$, ** $P<0.01$, **** $P<0.0001$). **(C)** Migration assay by Transwell. The migratory potential of the B16 cell lines was determined by assays on $8 \mu\text{M}$ transwell culture chambers (Corning). A total of 5×10^4 cells (B16-F1, B16-F10, and B16-F10 transfected using 50 nM tpt1/TCTP siRNA or control siRNA) with serum-free medium were plated on the top. Media with 10% of fetal bovine serum were used as a chemoattractant in the bottom. After incubation, the cells were fixed, stained and counted. Data were analysed using one-way ANOVA using Tukey's *post hoc* test. Three independent experiments were performed ($n=3$) (**** $P<0.0001$).

at the concentration of $1 \mu\text{M}$ markedly reduces the intracellular TCTP levels. Even the lower dose ($0.01 \mu\text{M}$) was able to significantly decrease the amount of TCTP after 72 h of treatment. Figure 5B shows the results of TCTP relative expression by qRT-PCR in B16-F10 cells treated with sertraline. These results show that TCTP mRNA levels were diminished by sertraline in a time- and concentration-dependent manner. Viability was assessed by measuring the metabolic active cells after 24, 48, and 72 h of treatment (Figure 5C). Even low concentrations of sertraline triggered a significant decrease of the viability after 48 and 72 h. The capacity of murine melanoma cell to form colonies in a semisolid medium was evaluated in the presence of sertraline (Figure 5D). Sertraline reduced the number of colonies to 76% ($0.1 \mu\text{M}$), 48% ($1 \mu\text{M}$), and 32% ($5 \mu\text{M}$), when compared with the control. Interestingly, $5 \mu\text{M}$ of sertraline made B16-F10 cells even less clonogenic than B16-F1 cells.

In vivo effects of sertraline treatment on C57BL/6/B16-F10 mice model. The *in vivo* antitumour activity of sertraline was evaluated using C57BL/6 mice in which subcutaneous B16-F10 melanoma cells were inoculated (Figure 6). At 5 days after the cells were injected in the mice, sertraline/DTIC daily intraperitoneal treatment was started. At this point, a very small but palpable tumour could be observed. Animals were treated for 12 days with DTIC (60 mg kg^{-1}) and/or sertraline (10 mg kg^{-1}). Control animals were treated with the drug solvent (DMSO and/or water). At the end of the experiment, tumours were excised and their weights were determined (Figures 6A and B). Sertraline (10 mg kg^{-1}) inhibited tumour growth by 84.4%. When animals were treated with both sertraline and DTIC, tumour growth was reduced to 88%. A stronger antitumour effect of sertraline could be observed when compared with DTIC singly treatment (47% tumour growth inhibition). However, when the suppression of tumour growth of

DTIC and sertraline is compared with sertraline alone, there is no increase in the antitumour activity of sertraline. Translationally controlled tumour protein content in these mice tumours was assessed by western blot analysis and a marked decrease could be observed. As expected, P53 levels were increased in tumours from animals treated with sertraline (Figure 6C). Collected tumours were also evaluated regarding the amount of TCTP, Ki67, caspase-3, and P53 by immunohistochemistry (Figure 6D). In line with the results observed by western blot analysis, tumours from animals treated with sertraline presented lower levels of TCTP and higher levels of P53 protein. Collectively, these results indicate that B16-F10 cells show a decrease in TCTP and malignant status, both *in vitro* and *in vivo* when treated with sertraline.

DISCUSSION

Melanoma is the deadliest of skin cancers, caused by the transformation of melanocytes that accumulate genetic alterations, leading to abnormal proliferation and dissemination (Bastian, 2014). The incidence of melanoma is rising all over the world and an effective treatment for advanced cases is still to be determined. Despite improvements, metastatic melanoma (stage IV) is still associated with a poor prognosis and a median survival of 6–12 months (Schadendorf *et al*, 2015).

Melanoma tumours and cell lines overexpress TCTP (Tuynder *et al*, 2004; Baylot *et al*, 2012; Sade *et al*, 2012). Translationally controlled tumour protein is involved in fundamental cellular processes, such as cell cycle, proliferation, and apoptosis (Amson *et al*, 2013; Thebault *et al*, 2016).

In tumour reversion, a cellular reprogramming, with a marked decrease in malignancy, more than 300 genes are involved

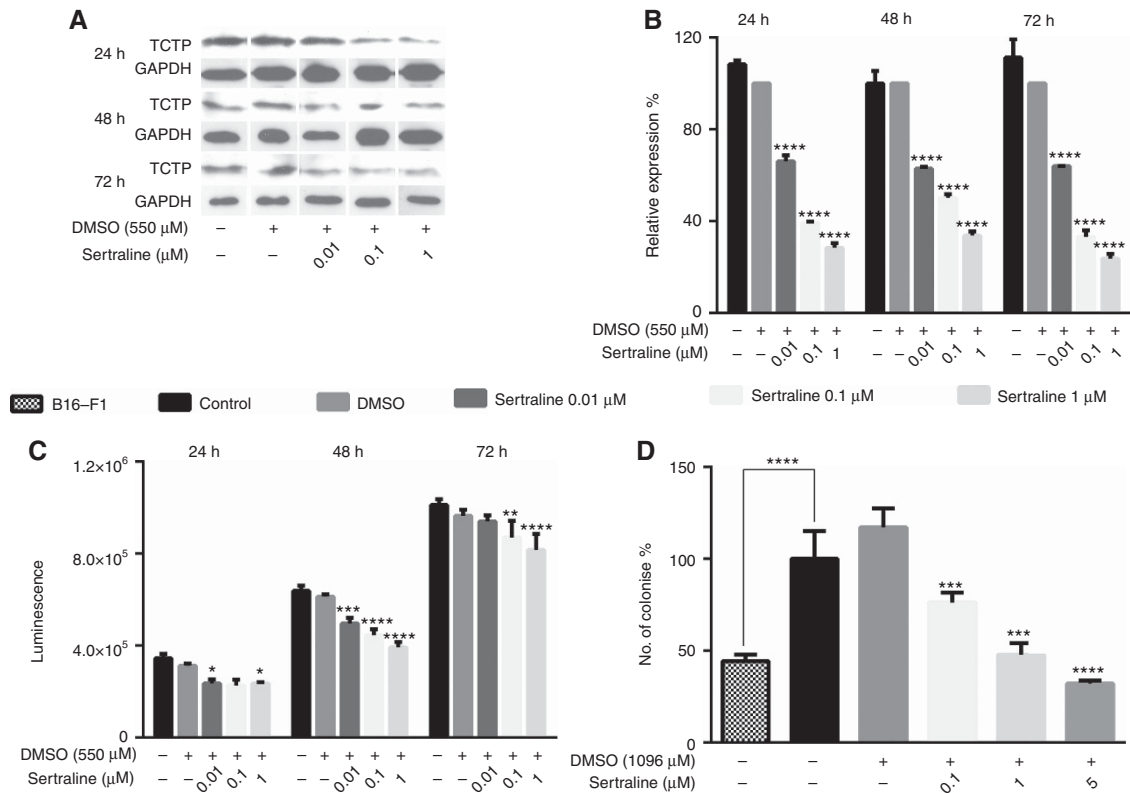


Figure 5. Effect of sertraline on TCTP expression levels: evaluation of cell viability and colony formation. **(A)** Western blot analysis of TCTP and GAPDH protein levels in B16-F10 cells treated with sertraline (0.01, 0.1, and 1 μM) and a negative control (24, 48, and 72 h). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control for each sample. **(B)** Analysis of TCTP relative expression using qRT-PCR in B16-F10 cell line after treatment with sertraline (0.01, 0.1, and 1 μM at 24, 48, and 72 h), a negative control (dimethyl sulphoxide (DMSO)) and only medium in soft agar. The $\Delta\Delta\text{Ct}$ method was used for quantification of the TCTP mRNA levels, using GAPDH as an endogenous standard for each sample. For graphics, $\Delta\Delta\text{Ct}$ was normalised to the controls. Data were analysed by comparing the lines treated with sertraline and the negative control (DMSO) using one-way ANOVA test with Tukey's *post hoc* test. Two independent experiments were performed ($n = 5$) (**** $P < 0.0001$). **(C)** Cell viability was determined using Cell Titer-Glo (Promega). B16-F10 cells were plated in 96-well plates (10^4 cells per well) and treated with sertraline (0.01, 0.1, and 1 μM), DMSO (negative control) and only medium in soft agar. After 24, 48, and 72 h, Cell Titer-Glo reagent was added in the medium and luminescence was measured by Tecan (Tecan Infinite M200). Data were analysed by comparing the lines treated with sertraline and the negative control (DMSO) using two-way ANOVA test with Tukey's *post hoc* test. Two independent experiments were performed ($n = 5$) (* $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$). **(D)** Clonogenic assay. A total of 5×10^3 B16-F10 cells were incubated with sertraline (0.1, 1, and 5 μM), DMSO (negative control) and only medium in soft agar. After 15 days, cells were stained with Crystal violet and colonies were counted. Four independent experiments were performed ($n = 2$) (*** $P < 0.001$, **** $P < 0.0001$).

including TCTP (Tuynder *et al*, 2002). Accumulating data on TCTP and tumour reversion suggest that such a reprogramming could be clinically interesting and a therapeutic target for cancer treatment, overcoming regular drawbacks of standard procedures as resistance by clone selection (Tuynder *et al*, 2002, 2004; Telerman and Amson, 2009; Amson *et al*, 2013; Powers and Pollack, 2016).

Relevance of TCTP for tumour reversion in melanoma was shown by Tuynder *et al* (2004) in WM-266-4, WM-115, SK-MEL-28 and Hs852T cell lines, and human tumours. Using an oncolytic virus (H1 parvovirus), revertant cells were isolated from these melanoma cells. These revertant clones presented decreased TCTP levels and reduced tumorigenicity *in vivo*. Translationally controlled tumour protein was also implicated to the development of chemoresistance in melanoma (Sinha *et al*, 2000). MeWo cells induced to resistance by drugs presented higher TCTP levels when compared with their sensitive-counterparts. Mewo cell lines chemoresistant to cisplatin, vindesin, etoposide or fotemustine presented marked TCTP overexpression when compared with the parental cell line MeWo.

Both MeWo and A2058 cells showed reduced levels of TCTP when treated with sertraline. This decrease in TCTP was

accompanied by an increase in P53 levels (Figures 1 and 2). The decrease in the migration phenotype and also in the capacity of forming colonies caused by sertraline was striking in both human cell lines.

We took B16 melanoma a model to study TCTP. From B16 cells, different phenotypic cell lines were established: B16-F10, which is highly malignant and metastatic, and B16-F1, less proliferative with a lower metastatic capacity (Fidler, 1973; Morris *et al*, 2015). When TCTP levels were assessed in these cell lines, a marked difference was observed. The smaller content of TCTP protein in B16-F1 was consistent with low TCTP mRNA levels. In comparison with B16-F10 levels, B16-F1 presented half of the TCTP protein and mRNA content (Figure 3). The results suggest that knockdown of TCTP correlated with a decrease in malignant features of B16-F10 cells (Figure 4). These results are similar to those of WM266, in which decreased TCTP levels result in decreased tumour development (Tuynder *et al*, 2004). Most striking is the reduced migration capacity of F10 melanoma cells silenced for TCTP, which is even lower than B16-F1 (Figure 4C). Recently, the knockdown of TCTP was shown to reduce B16-F10 cells' capacity to form pulmonary metastasis *in vivo* (Bae *et al*, 2015). Sertraline (even at the lowest dose: 0.01 μM) was very

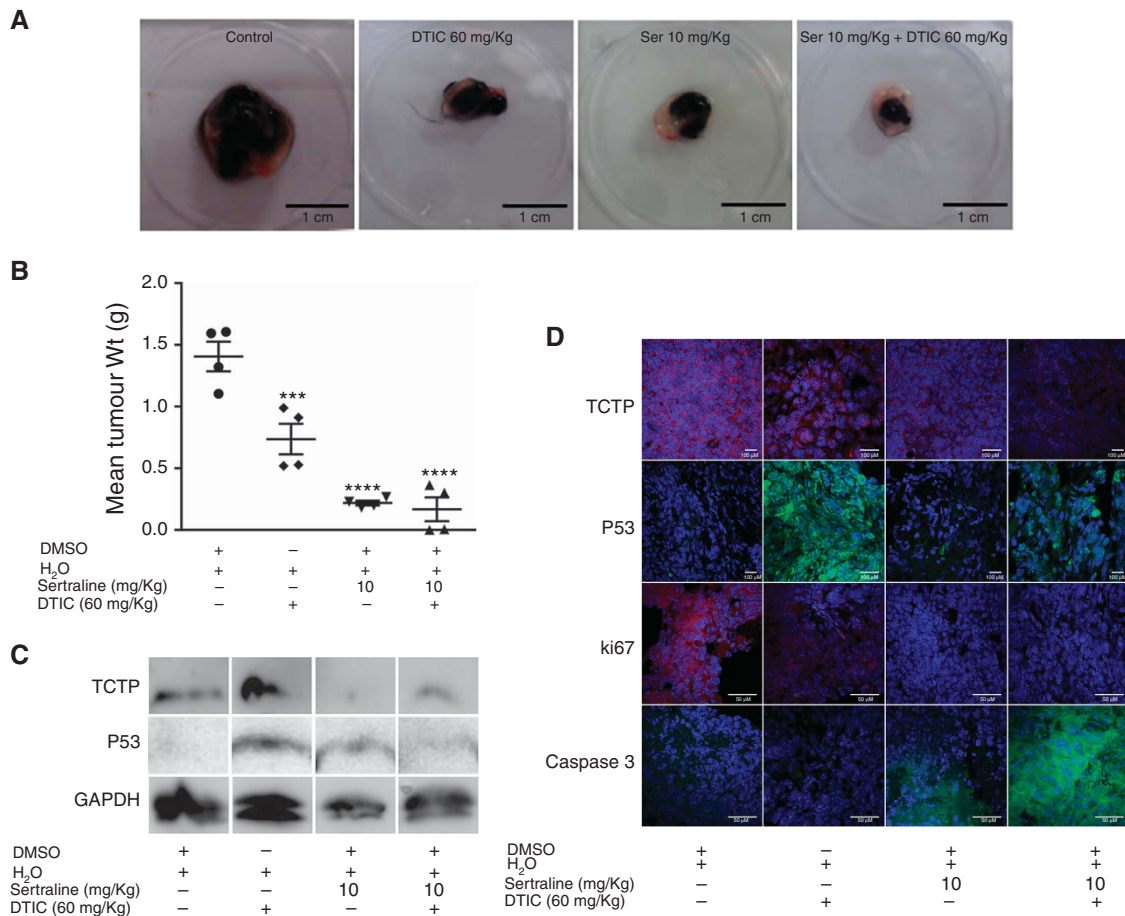


Figure 6. *In vivo* effect of sertraline on melanoma murine model. The C57BL/6 mice were subcutaneously injected with B16-F10 melanoma cells (5×10^5 cells). Daily intraperitoneal treatment with DTIC (60 mg kg^{-1}) and/or sertraline (10 mg kg^{-1}) was started on the fifth day after the cells were injected in the mice. Negative controls were treated with water and/or dimethyl sulphoxide (DMSO). **(A)** Representative images from tumours excised after 17 days. **(B)** Weights from tumours were determined and data were analysed by comparing the animal treated with DTIC, sertraline and sertraline/DTIC. The negative control was analysed using t-test. Three independent experiments were performed ($n = 5$) ($***P < 0.001$ and $****P < 0.0001$). **(C)** Western blot analysis of TCTP, P53, and GAPDH protein levels in tumour samples after treatment with DTIC and/or sertraline and negative control. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control for each sample. **(D)** Immunohistochemistry (IHC) of TCTP, Ki67, caspase-3, and P53 obtained from B16-F10 tumours. Tumour sections obtained from B16-F10 tumours from the DTIC- and/or sertraline-treated mice or -untreated (H_2O and/or DMSO). Sample sections were incubated with primary antibodies against TCTP (1 : 1000), Ki67 (1 : 1000), caspase-3 (1 : 1000), and P53 (1 : 1000). Protein expression was detected by immunohistochemistry using a secondary antibody conjugated to FITC (P53 and caspase-3) (1 : 250) or Alexa Fluor 594 (TCTP and Ki67) (1 : 250). Images were merged with a 4',6-diamidino-2-phenylindole (DAPI) channel. The scale bars are visualised on the right.

effective in the reducing TCTP levels (Figure 5). Sertraline was effective in reducing the capacity of melanoma cells to form colonies in soft agar. The results concerning the reduction of migration and clonogenic capacity are quite interesting in the context of melanoma as this tumour type is very metastatic, and difficult to treat when disseminated (Shain and Bastian, 2016).

Results of sertraline treatment using the *in vivo* model are very promising (Figure 6). Although new treatment strategies are being developed against melanoma (mainly targeting specific mutations and others based on immune therapy), DTIC is still the main chemotherapy drug. However, 10 mg kg^{-1} of sertraline triggered a marked inhibition of tumour growth, which was better than DTIC (60 mg kg^{-1}). We observed a decrease in TCTP and an increase in P53 levels in the tumours of the animals treated by sertraline (Figure 6C). Dacarbazine also triggered an increase in P53 levels, because of its action on DNA. These results point to a complex reprogramming of tumour cells by sertraline, suggesting its mechanism of action goes further than reactivation of P53. Sertraline also induced a decrease in Ki67 levels and an increase in caspase-3 levels (Figure 6D).

Translationally controlled tumour protein had already been described as a cell survival protein, modulating apoptosis (Amson *et al*, 2012; Acunzo *et al*, 2014; Thebault *et al*, 2016). Reciprocal repression of TCTP-P53 is particularly interesting in the context of melanoma because of its wild-type p53 status prevalence. Although p53 tumour suppressor gene is rarely mutated in melanoma, its functional attenuation is needed for tumour development. Reactivation of p53 was already pointed as an alternative therapeutic strategy for melanoma treatments, in combination with other strategies (Jochemsen, 2014; Lu *et al*, 2014).

The underlying mechanism of sertraline is based on its direct interaction with TCTP. Translationally controlled tumour protein's function on the autoubiquitination of MDM2 is hereby impaired, resulting in the increase of P53 levels (Amson *et al*, 2012).

Our results suggest that sertraline effects are related not only to P53 activation and induction apoptosis but also to a more complex pathway, which probably enable cells to suppress tumour features. When sertraline treatment of human melanoma cell lines was evaluated, we choose a p53 wild-type (MeWo) and a mutant p53

(A2058) cell line (TP53 web site: <http://p53.fr>) (Figures 5 and 6). Sertraline effects were quite similar in both cell lines; migration and clonogenicity were markedly reduced. Translationally controlled tumour protein levels decreased and P53 increased by sertraline, even in the p53 mutant cell line (A2058). As TCTP is a multifunctional protein with several partners, involved in crucial cell pathways, the decrease of TCTP levels probably influence other molecules than P53, leading to a complex alteration of cell behaviour and to tumour reversion. However, a specific and detailed mechanism remains elusive.

It is important to highlight TCTP as a target for melanoma therapy in a context of cellular reprogramming and tumour reversion, as the decrease of TCTP levels lead to the loss of tumour features such as migration, clonogenicity, strongly associated with metastatic disease and tumour growth *per se*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DISCLAIMER

As the corresponding author, I declare that all named authors have read the manuscript and have agreed to this manuscript (TH-2017-4183R) in its present form. These research data have not been and will not be submitted to another journal, in whole or in part. This manuscript reports previously unpublished work. All those named as authors have made important and sufficient contribution to the work.

ETHICAL CONSIDERATIONS

We have observed appropriate ethical guidelines and legislation in conducting the animal studies described in this Manuscript. All procedures involving animals were performed in accordance with Brazilian Federal Laws, in accordance with the Ethical Subcommittee on Research Animal Care Agreement number 730 of the Federal University of Paraná. The protocols applied in carrying out the investigations require a high standard of ethics and are in agreement with the Ethical Principals established by Experimental Animal Brazilian Council (COBEA), and with the requirement of the 'Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care)'.

REFERENCES

Acunzo J, Baylot V, So A, Rocchi P (2014) TCTP as therapeutic target in cancers. *Cancer Treat Rev* **40**(6): 760–769.

- Ambrosio MR, Rocca BJ, Barone A, Onorati M, Mundo L, Crivelli F, Di Nuovo F, De Falco G, del Vecchio MT, Tripodi SA, Tosi P (2015) Expression of translationally controlled tumor protein in human kidney and in renal cell carcinoma. *BioMed Res Int* **2015**: 730390.
- Amson R, Karp JE, Telerman A (2013) Lessons from tumor reversion for cancer treatment. *Curr Opin Oncol* **25**(1): 59–65.
- Amson R, Pece S, Lespagnol A, Vyas R, Mazzarol G, Tosoni D, Colaluca I, Viale G, Rodrigues-Ferreira S, Wynendaele J, Chaloin O, Hoebeke J, Marine JC, Di Fiore PP, Telerman A (2012) Reciprocal repression between P53 and TCTP. *Nat Med* **18**(1): 91–99.
- Bae SY, Kim HJ, Lee KJ, Lee K (2015) Translationally controlled tumor protein induces epithelial to mesenchymal transition and promotes cell migration, invasion and metastasis. *Scientific Rep* **5**: 8061.
- Bastian BC (2014) The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. *Annu Rev Pathol* **9**: 239–271.
- Baylot V, Katsogiannou M, Andrieu C, Taieb D, Acunzo J, Giusiano S, Fazli L, Gleave M, Garrido C, Rocchi P (2012) Targeting TCTP as a new therapeutic strategy in castration-resistant prostate cancer. *Mol Ther* **20**(12): 2244–2256.
- Borges BE, Teixeira VR, Appel MH, Steclan CA, Rigo F, Filipak Neto F, da Costa Ferreira AM, Chammas R, Zanata SM, Nakao LS (2013) *De novo* galectin-3 expression influences the response of melanoma cells to isatin-Schiff base copper (II) complex-induced oxidative stimulus. *Chemico-biol Interact* **206**(1): 37–46.
- Chung S, Kim M, Choi W, Chung J, Lee K (2000) Expression of translationally controlled tumor protein mRNA in human colon cancer. *Cancer Lett* **156**(2): 185–190.
- Coogan PF, Strom BL, Rosenberg L (2009) Antidepressant use and colorectal cancer risk. *Pharmacoepidemiol Drug Saf* **18**(11): 1111–1114.
- Fidler IJ (1973) Selection of successive tumour lines for metastasis. *Nat New Biol* **242**(118): 148–149.
- Jochemsen AG (2014) Reactivation of p53 as therapeutic intervention for malignant melanoma. *Curr Opin Oncol* **26**(1): 114–119.
- Kozio MJ, Gurdon JB (2012) TCTP in development and cancer. *Biochem Res Int* **2012**: 105203.
- Lu M, Miller P, Lu X (2014) Restoring the tumour suppressive function of p53 as a parallel strategy in melanoma therapy. *FEBS Lett* **588**(16): 2616–2621.
- Morris VL, Toseef T, Nazumudeen FB, Rivoira C, Spatafora C, Tringali C, Rotenberg SA (2015) Anti-tumor properties of *cis*-resveratrol methylated analogs in metastatic mouse melanoma cells. *Mol Cell Biochem* **402**(1–2): 83–91.
- Powers S, Pollack RE (2016) Inducing stable reversion to achieve cancer control. *Nature* **16**: 266–270.
- Pretto F, Neri D (2013) Pharmacotherapy of metastatic melanoma: emerging trends and opportunities for a cure. *Pharmacol Ther* **139**(3): 405–411.
- Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* **22**(3): 326–330.
- Rocca BJ, Ginori A, Barone A, Calandra C, Crivelli F, De Falco G, Gazaneo S, Tripodi S, Cevenini G, del Vecchio MT, Ambrosio MR, Tosi P (2015) Translationally controlled tumor protein in prostatic adenocarcinoma: correlation with tumor grading and treatment-related changes. *BioMed Res Int* **2015**: 985950.
- Sade YB, Boia-Ferreira M, Gremski LH, da Silveira RB, Gremski W, Senff-Ribeiro A, Chaim OM, Veiga SS (2012) Molecular cloning, heterologous expression and functional characterization of a novel translationally-controlled tumor protein (TCTP) family member from *Loxosceles intermedia* (brown spider) venom. *Int J Biochem Cell Biol* **44**(1): 170–177.
- Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, Herlyn M, Marchetti MA, McArthur G, Ribas A, Roesch A, Hauschild A (2015) Melanoma. *Nat Rev Dis Primers* **1**: 15003.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature methods* **9**(7): 671–675.
- Shain AH, Bastian BC (2016) From melanocytes to melanomas. *Nat Rev Cancer* **16**(6): 345–358.
- Sinha P, Kohl S, Fischer J, Hutter G, Kern M, Kottgen E, Diel M, Lage H, Schnolzer M, Schadendorf D (2000) Identification of novel proteins associated with the development of chemoresistance in malignant melanoma using two-dimensional electrophoresis. *Electrophoresis* **21**(14): 3048–3057.
- Spandidos A, Wang X, Wang H, Seed B (2010) Primer Bank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res* **38**: 792–799.

- Telerman A, Amson R (2009) The molecular programme of tumour reversion: the steps beyond malignant transformation. *Nat Rev Cancer* **9**(3): 206–216.
- Thebault S, Agez M, Chi X, Stojko J, Cura V, Telerman SB, Maillet L, Gautier F, Billas-Massobrio I, Birck C, Troffer-Charlier N, Karafin T, Honore J, Senff-Ribeiro A, Montessuit S, Johnson CM, Juin P, Cianferani S, Martinou JC, Andrews DW, Amson R, Telerman A, Cavarelli J (2016) TCTP contains a BH3-like domain, which instead of inhibiting, activates Bcl-xL. *Scientific Rep* **6**: 19725.
- Toh S, Mitchell AA, Louik C, Werler MM, Chambers CD, Hernandez-Diaz S (2009) Selective serotonin reuptake inhibitor use and risk of gestational hypertension. *Am J Psychiatry* **166**(3): 320–328.
- Tuynder M, Fiucci G, Prieur S, Lespagnol A, Geant A, Beaucourt S, Duflaut D, Besse S, Susini L, Cavarelli J, Moras D, Amson R, Telerman A (2004) Translationally controlled tumor protein is a target of tumor reversion. *Proc Natl Acad Sci USA* **101**(43): 15364–15369.
- Tuynder M, Susini L, Prieur S, Besse S, Fiucci G, Amson R, Telerman A (2002) Biological models and genes of tumor reversion: cellular reprogramming through tpt1/TCTP and SIAH-1. *Proc Natl Acad Sci USA* **99**(23): 14976–14981.
- Wernli KJ, Hampton JM, Trentham-Dietz A, Newcomb PA (2009) Antidepressant medication use and breast cancer risk. *Pharmacoeconom Drug Saf* **18**(4): 284–290.
- Xu W, Tamim H, Shapiro S, Stang MR, Collet JP (2006) Use of antidepressants and risk of colorectal cancer: a nested case-control study. *Lancet Oncol* **7**(4): 301–308.

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