CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2007; **25**: 109–114.
Published online 19 July 2006 in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1027/cbf.1351

Inhibition of eukaryotic translation initiation factor 5A (eIF5A) hypusination impairs melanoma growth

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The eukaryotic translation initiation factor 5A (eIF5A) undergoes a specific post-translational modification called hypusination. This modification is required for the functionality of this protein. The compound N1-guanyl-1,7-diaminoheptane (GC7) is a potent and selective inhibitor of deoxyhypusine synthase, which catalyses the first step of eIF5A hypusination process. In the present study, the effects of GC7 on cell death were investigated using two cell lines: melan-a murine melanocytes and Tm5 murine melanoma. *In vitro* treatment with GC7 increased by 3-fold the number of cells presenting DNA fragmentation in Tm5 cells. Exposure to GC7 also decreased viability to both cell lines. This study also describes, for the first time, the *in vivo* antitumour effect of GC7, as indicated by impaired melanoma growth in C57BL/6 mice. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS — tumour growth; cell proliferation; melanoma; cytotoxicity; eIF5A; eIF-5A; hypusine; GC7

INTRODUCTION

Several proteins are involved in cell cycle progression and control of cell proliferation. Among them, the eukaryotic translation initiation factor 5A (eIF5A), whose precise function is not fully understood, has been shown to participate in protein translation, and consequently, its depletion or blockage severely impairs proliferation of yeast[1] and mammalian cells.[2] The protein eIF5A undergoes a specific and unique post-translational modification called hypusination. This process occurs when a lysine residue is converted into hypusine. Hypusination is carried out in two steps: (i) a 4-butylamine moiety of spermidine is transferred to the ε-amino group of a specific lysine residue in the eIF5A molecule (Lys^{50} in human eIF5A), by the action of deoxyhypusine synthase (DHS), giving rise to the deoxyhypusil residue; (ii) the deoxyhypusil residue carbon 2 is hydroxylated by desoxyhypusil hydroxylase (DHH) to form the hypusine residue [N-ε-(4amino-2 hydroxybutyl)lysine].[3] Even before the characterisation of eIF5A, cell proliferation was correlated with the levels of hypusine.[4,5] Hypusine synthesis is higher at the logarithmic phase than in the non-exponential phase during growth of various mammalian cell lines.[4,5] There is also evidence that a high hypusine synthesis follows the activation of a lymphocyte.[6]

Previous reports have shown that mimosine, a plant amino acid, inhibits the second step of the hypusination process, probably due to its action as a chelator of Fe^{2+} present in the DHH enzyme.[7] The hydroxylation

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Contract/grant sponsor: São Paulo State Research Foundation (FAPESP); contract/grant number: 00/11397-9.

Received 15 February 2006
Revised 22 May 2006
Accepted 8 June 2006

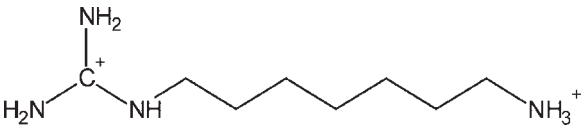

Figure 1. Chemical structure of the eIF5A hypusination inhibitor N1-guanyl-1,7-diaminoheptane (GC7)

blockage of the deoxyhypusil residue by action of mimosine was shown to impair cell proliferation.[8,9] The effectiveness of mimosine to block cell proliferation led to the investigation of its antineoplastic effect in mice. Mimosine was able to suppress lung tumour growth, probably by inducing apoptosis of the cells.[10] Although the inhibition of eIF5A by mimosine seems to be a potential new target for antineoplastic therapies, mimosine's mechanism of action by Fe^{2+} chelation may also lead to inhibition of metal-dependent enzymes others than DHH, and therefore result in unpredictable and uncontrolled side effects. To overcome this problem, other compounds, such as deoxyspergualin,[11] ciclopirox[12] and N1-guanyl-1,7-diaminoheptane, known as GC7[2,13,14] have been tested concerning their properties of inhibiting eIF5A hypusination. Probably due to its polyamine-like structure (Figure 1), the compound GC7 has been described as a DHS inhibitor of high affinity and selectivity.[13,15] The antiproliferative effects of this compound have been observed *in vitro* on different cell lines such as HUVEC, NIH-3T3, CHO-K1, H9 and HeLa.[14,16] Inhibition of eIF5A hypusination by GC7 has been revealed as a promising, not yet studied, strategy to impair tumour growth.

In the present study, the toxic effect of GC7 on melan-a, a murine melanocyte cell line, and on Tm5, a murine melanoma cell line derived from melan-a,[17] was investigated. *In vitro* treatment with GC7 induced more pronounced DNA fragmentation in Tm5 cells, and similarly decreased the viability of both cell lines. Evidence was also obtained that GC7 inhibits Tm5 melanoma growth in C57BL/6 mice. To our knowledge this is the first report of an *in vivo* effect of GC7; wherein inhibition of eIF5A hypusination by this compound led to a sound impairment on tumour growth.

MATERIALS AND METHODS

Cell culture

The melan-a murine melanocyte lineage and Tm5 murine melanoma cell line[17] were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cells were grown in RPMI-1640 (Roswell Park Memorial Institute medium), pH 6.9, supplemented with 5% of foetal calf serum (FCS) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) as antibiotics. RPMI-1640 medium and FCS were purchased from Invitrogen™ (Carlsbad, CA). Melan-a cell line was cultured in the presence of 200 nM 12-*o*-tetradecanoylphorbol-13-acetate (PMA, Tocris, Ellisville, MO).

Synthesis of N1-guanyl-1,7-diaminoheptane (GC7)

GC7 was synthesised as previously described.[18] Briefly, a solution of 3.95 g of 1,7-diaminoheptane and 2.85 g of *o*-methylurea sulphate in 105 ml water was stirred for 16 h at room temperature. The product mixture was filtered and the soluble fraction was frozen and lyophilised. The product was resuspended in 20 ml of methanol and concentrated to a viscous oil. The crude product was crystallised after being dissolved in 80 ml of methanol and incubated for 24 h at 4°C. The product of synthesis was analysed by an infrared absorbance assay in a FTIR-8300 equipment (SHIMADZU Corporation, Japan) using a GC7 sample as reference. Stock solutions of GC7 were prepared in phosphate buffered saline (PBS), which were further diluted as required before use. PBS was used as control in all assays.

Flow cytometry

Flow cytometric analysis of GC7-treated cells was performed using a FACSCalibur™ cytometer (Becton Dickinson, San Juan, CA) as described before,[19] with few modifications. Briefly, for synchronisation, cells were arrested in G_0/G_1 phase by culturing in medium without serum for 24 h, followed by the addition of fresh serum to stimulate growth in the presence of GC7 40 μM. After 48 h, cells were washed, fixed with ice-cold ethanol (70% final concentration), resuspended in 0.5 ml solution containing 50 μg/ml propidium iodide and 200 μg/ml DNAse-free RNAse in PBS, and incubated for 30 min at 37°C. Ten thousand events were evaluated per assay. The hypodiploid cells were determined using the CellQUEST™ software (Becton Dickinson, San Juan, CA).

Cytotoxicity assay

Cells were plated (5×10^3 cells/well) in a 96-well plate 24 h prior to the experiment. Cells were analysed

48 h after the addition of GC7 by the MTT assay.[20,21] Briefly, after incubation with GC7, 20 μl of a MTT solution (5 mg/ml) were added and cells were incubated for another period of 2 h. After washing twice with PBS, 100 μl of isopropanol were added and cell viability was determined by absorbance measurement at 570 nm.

Effect of GC7 on tumour growth in vivo

Two protocols were used to analyse the *in vivo* antitumour effect of GC7. In both protocols the methodologies for tumour induction and monitoring were the same, where tumour growth was monitored by volume measurements. In ''protocol 1'' administration of GC7 started on the day after tumour cells were implanted and followed daily in the subsequent days (until tumours reached ca. 1000 mm^3). In ''protocol 2'' administration of GC7 was initiated after tumours had reached 4 mm^3 and then proceeded as described in protocol 1.

Briefly, groups of C57BL/6 female mice (5–6 animals, 6–7-weeks old, 25–30 g) were subcutaneously injected with Tm5 cells (2×10^5) into the dorsal-side of the mice. On the next day (protocol 1), or when tumours reached 4 mm^3 of volume (protocol 2), administration of GC7 (900 μg/Kg) or the vehicle (PBS) was initiated. Daily subcutaneous injection of GC7 was performed into the dorsal-side of the animals at 1cm from the local of cell injection (protocols 1 and 2). When tumours reached ca. 1000 mm^3 animals were euthanased and the tumours were collected and weighed. C57BL/6 mice were kept in the Center for Development of Experimental Models for Medicine and Biology (CEDEME, UNIFESP, SP, Brazil) under the International Guiding Principles for Biomedical Research Involving Animals (CIOMS), Genebra (1985).

Statistical analysis

Comparisons between groups were performed by unpaired t test analysis, and differences were considered significant when $p < 0.05$.

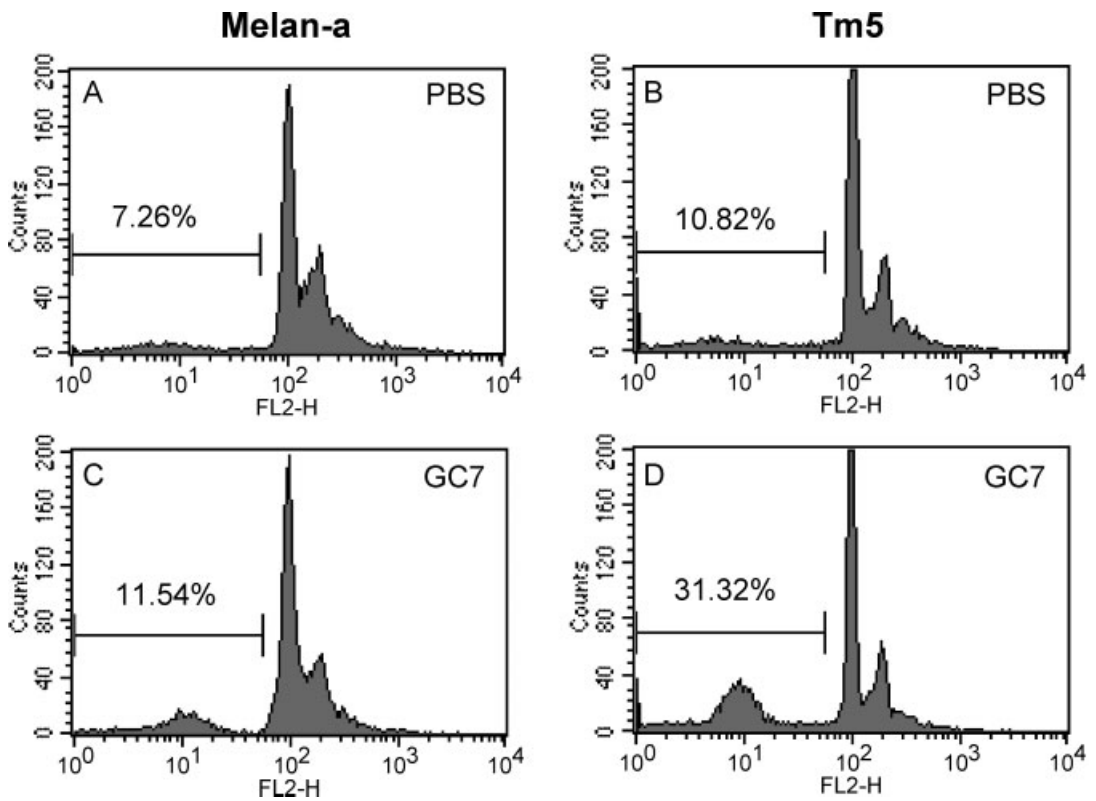

Figure 2. Flow cytometry of melan-a (panels A and C) and Tm5 cells (panels B and D) treated with 40 μM of GC7 or PBS for 48 h. The values are the mean ± SEM of two independent experiments with 10.000 events each

RESULTS

GC7 induces DNA fragmentation of Tm5 melanoma cells

DNA fragmentation analysis of Tm5 cells was performed by flow cytometry after treatment with GC7 (40 μM) or vehicle (PBS). The treatment with GC7 for a 48 h period showed a 3-fold (± 0.35) increase in the percentage of hypodiploid Tm5 cells compared with PBS-treated cells (Figure 2). Interestingly, Tm5 cells also displayed a higher degree of DNA fragmentation compared to melan-a cells treated with GC7. Two other tumour cell lines (Jurkat, a human leukemia T lymphocyte cell line) and THP-1 (a human monocytic leukemia cell line) were also analysed (data not shown). GC7 treatment for 72 h increased the fraction of hypodiploid cells by 1.9-fold (±0.06) in Jurkat cells (at 40 μM) and 2.8-fold (±0.26) in THP-1 cells (at 30 μM). To confirm the GC7 toxicity in Tm5 cells, lower GC7 concentrations were tested on cell viability assays.

GC7 decreases melan-a and Tm5 cells viability

The cell viability analysed in response to GC7 treatment was expressed as percentage of cell survival using the MTT assay. The GC7 treatment elicited a clear dose-response inhibition of both melan-a and Tm5 cells viability (Figure 3). The apparent IC_{50} ranged from 10 to 20 μM and a maximum of 28% ± 3.7 (melan-a) and 36% ± 3.1 (Tm5) of viability was detected at 40 μM. The effects of GC7 on DNA fragmentation and viability of Tm5 cells prompted us to carry out experiments on melanoma growth in C57BL/6 mice.

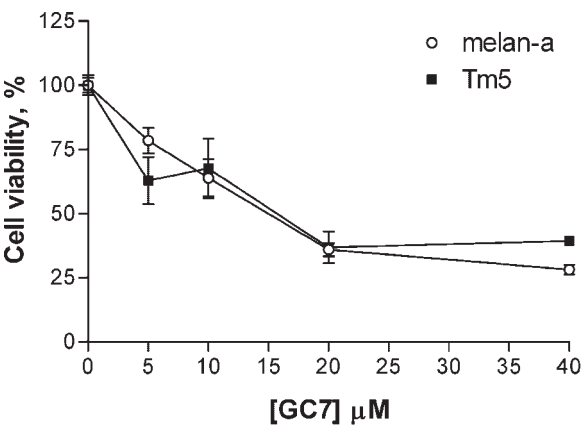

Figure 3. Melan-a and Tm5 cells viability as analysed by MTT assay after treatment with various concentrations of GC7 for 48 h. PBS treatment was used as control. The values represent the average of two independent experiments carried out in duplicate

GC7 inhibits melanoma growth in vivo

The treatment with GC7 during 18 days after Tm5 cells implantation was able to significantly reduce the melanoma growth (Figure 4); as indicated by monitoring the tumour volume during the period of treatment (Figure 4a). A significant ($p = 0.0321$) decrease in tumour weight was obtained at the18th day when the animals were euthanased (Figure 4b). When GC7 started to be administrated to established tumours of ca. 4 mm^3, the effect on tumour volume reduction was not significant, although a clear tendency could be observed (Figure 5).

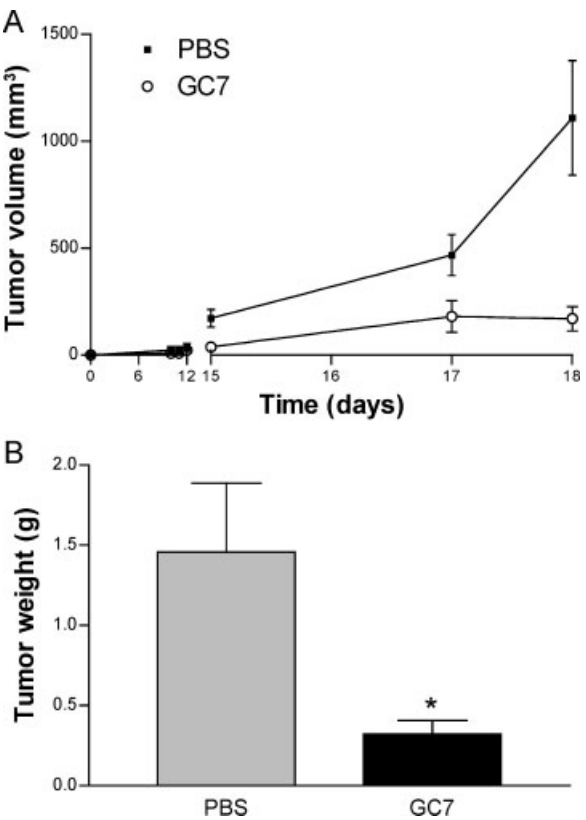

Figure 4. Effect of GC7 on melanoma growth in C57BL/6 mice. (A) Tumour volume was monitored during 18 days of the GC7 treatment (900 μg/Kg). (B) Tumour weight was determined at the 18th day, after death of the animals ($^{*}p = 0.0321$). Results are presented as mean ± SEM of two independent experiments of six animals per group

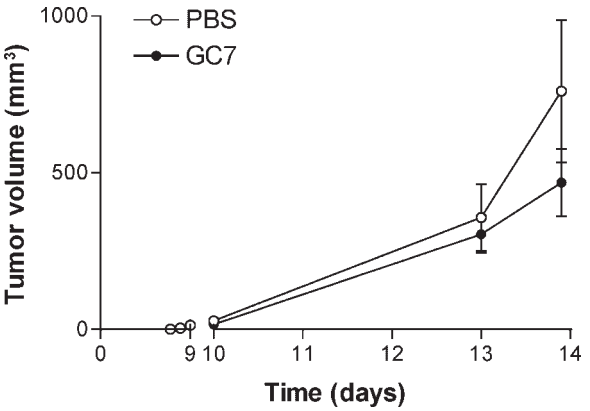

Figure 5. Effect of GC7 on melanoma growth in C57BL/6 mice. GC7 treatment (900 μg/Kg) started when established tumours of ca. 4 mm^3 of volume were detected. Treatment was followed for 14 days (p = non significant). Results are presented as mean ± SEM of two independent experiments of five animals per group

DISCUSSION

Despite the fact that eIF5A function is not fully elucidated yet; it has broadly been related to translation processes. A study on protein synthesis revealed a reduction of 30% in translation rates after eIF5A depletion.[1] A recent study clearly associated eIF5A with actively translating 80S ribosomes and polyribosomes,[22] confirming its role in translation. *In vitro* studies show that the inhibition of hypusination by N1-guanyl-1,7-diaminoheptane (GC7) impairs proliferation of various cell lines,[14,16] possibly due to an impairment in translation control. In the present study, the effect of GC7 on melan-a murine melanocytes and on the melan-a-derived Tm5 melanoma cells is reported. A previous study has shown that GC7 induces a protective effect against apoptosis induced by serum-starvation in HUVEC cells.[14] However, other authors have found a synergistic effect of GC7 and Interferon-α on apoptosis in epidermoid oropharyngeal KB and lung H1355 cancer cells.[23] In our study, GC7 treatment caused a significant increase in the proportion of Tm5 cells with fragmented DNA, compared to non-treated Tm5 cells and to treated melan-a cells (Figure 2). In parallel, GC7 also caused a marked increase in DNA fragmentation of other tumour cell lines (Jurkat and THP-1, not shown). It is possible that the eIF5A requirement for cell proliferation depends on serum availability, but the mechanisms involved in the pro- and anti-apoptotic effects of GC7 remain to be further examined. A dose-response pattern of reduction in cell viability was observed in both melan-a and Tm5 cell lines (Figure 3). This result confirms the toxic effect of GC7 on different cell lines, as reported before.[14,16] It is interesting to note that at 40 μM GC7, cytotoxicity was slightly more pronounced in melan-a cells than in Tm5 (Figure 3), although at this concentration DNA fragmentation was increased in Tm5 cells (Figure 2). We believe that these apparently conflicting data may be due to differences in the proliferation pattern of both cell lines, as well as in the mechanisms of GC7-induced apoptosis. The results obtained with Tm5 cells prompted us to evaluate the efficacy of GC7 in causing an *in vivo* blockade of melanoma tumour growth or mass reduction of established tumours. A clear inhibition of tumour growth was found (Figure 4), but despite of an observed tendency, GC7 was not capable of inducing a significant volume reduction in already established tumours (Figure 5). Whether GC7 may act efficiently in tumour mass reduction when used in higher concentrations remains to be analysed in future investigations, as well as its possible side-effects against other cells, such as stem cells and blood cells precursors. Nevertheless, we believe that the potent action of GC7 in blocking tumour growth could represent an interesting key-point in control of early tumours and microscopic metastasis (adjuvant treatment).

This is the first evidence that inhibition of eIF5A hypusination presents an antineoplastic effect *in vivo*. In addition, our results highlight GC7 as a potential new drug to be used in new anti-cancer therapies.

ACKNOWLEDGEMENTS

The authors thank Dr. Hans E. Johansson, from the Children's Hospital Oakland Research Institute, CA, USA, for the GC7 sample used as reference standard.

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