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The G-quadruplex-stabilising agent RHPS4 induces telomeric dysfunction and enhances radiosensitivity in glioblastoma cells

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ABSTRACT

G-quadruplex (G4) interacting agents are a class of ligands that can bind to and stabilise secondary structures located in genomic G-rich regions such as telomeres. Stabilisation of G4 leads to telomere architecture disruption with a consequent detrimental effect on cell proliferation, which makes these agents good candidates for chemotherapeutic purposes. RHPS4 is one of the most effective and well-studied G4 ligands with a very high specificity for telomeric G4.

In this work, we tested the *in vitro* efficacy of RHPS4 in astrocytoma cell lines, and we evaluated whether RHPS4 can act as a radiosensitising agent by destabilising telomeres.

In the first part of the study, the response to RHPS4 was investigated in four human astrocytoma cell lines (U251MG, U87MG, T67 and T70) and in two normal primary fibroblast strains (AG01522 and MRC5). Cell growth reduction, histone H2AX phosphorylation and telomere-induced dysfunctional foci (TIF) formation were markedly higher in astrocytoma cells than in normal fibroblasts, despite the absence of telomere shortening. In the second part of the study, the combined effect of submicromolar concentrations of RHPS4 and X-rays was assessed in the U251MG glioblastoma radioresistant cell line. Long-term growth curves, cell cycle analysis and cell survival experiments, clearly showed the synergistic effect of the combined treatment. Interestingly the effect was greater in cells bearing a higher number of dysfunctional telomeres. DNA double-strand breaks rejoining after irradiation revealed delayed repair kinetics in cells pre-treated with the drug and a synergistic increase in chromosome-type exchanges and telomeric fusions.

These findings provide the first evidence that exposure to RHPS4 radiosensitizes astrocytoma cells, suggesting the potential for future therapeutic applications.

1. Introduction

Telomeres are protein–DNA complexes that are located at the physical ends of linear eukaryotic chromosomes, and they confer protection against the action of exonucleases and ligases [1,2]. For this reason, telomeres are known to play a major role in the maintenance of genomic stability by preventing inappropriate chromosome end-to-end fusion [3]. Telomere attrition or telomeric dysfunction resulting from the loss of function of shelterin complex proteins [4] lead to cell cycle arrest, senescence, and apoptosis [5]. This makes telomeres and mechanisms involved in the maintenance of their length promising targets for the development of selective molecules for cancer therapy [6–10].

In particular, in the past decade, considerable attention has been focused on telomerase, because the activation of this enzyme

Abbreviations: G4, G quadruplex; TIF, telomere induced dysfunctional foci; GBM, glioblastoma multiforme; ALT, alternative lengthening of telomeres; hTERT, human telomerase catalytic subunit; PCC, premature chromosome condensation; cPDL, cumulative population doubling level; SER, sensitisation enhancement ratio; TRF1, telomere repeat binding factor 1; TRF2, telomere repeat binding factor 2; POT1, protection of telomeres 1; DSB, double strand break; CSC, cancer stem cells.

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is crucial in the maintenance of telomeres in most cancer cells [11]. The interest in the use of telomerase inhibitors for therapeutic approaches has decreased due the observation that significant effects on tumour growth were obtained only after long-term drug administration (*i.e.*, months of treatment) and only when the telomeres reached a critical length [12,13].

However, the telomeric structure itself is an appealing target for telomere-binding compounds in short-term treatments. In this respect, G4-ligands, a class of molecules that are able to interact with physiologically occurring G4 (G quadruplex) structures formed by the G-rich overhang of telomeric DNA [14–16] have recently received considerable attention. Telomestatin [17], Braco-19 [18] and RHPS4 [19] are just a few examples of this growing class of G4-stabilising molecules proven for their capability to reduce cell growth by rendering telomeres dysfunctional in *in vitro* and *in vivo* cancer models [18,20–24]. Furthermore, an additional advantage of G4-ligands is their effect on telomerase-positive cancer cells by destabilising telomere architecture and inhibiting an optimal access of the enzyme at the target site [25] and the telomerase-negative alternative lengthening of telomere (ALT) positive cells [19,26,27].

The pentacyclic acridine compound RHPS4 (3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate) is considered one of the most effective and selective G4-stabilising molecules. In particular, RHPS4 causes telomere deprotection in short-term exposure, leading to telomeric fusions, anaphase bridges and cell proliferation blockage [19,21,28]. In long-term exposure, it was found to induce telomerase inhibition and the down-regulation of the human telomerase catalytic subunit (*hTERT)* gene, telomere erosion, arrest at the G2/M transition and suppression of cell proliferation in cancer cells [19]. Interestingly RHPS4 seems to act preferentially on relatively short telomeres, a condition frequently encountered in cancer cells [19,21,29], although telomere-length independence in brain tumour cells has been recently reported [30]. Overall, RHPS4 has proven very effective in inducing *in vitro* and *in vivo* cytotoxicity in a wide panel of tumour cells, including melanoma [21], prostate [23,31], uterus carcinoma [23], non-small lung [31], malignant childhood and adult brain cancer cells [30] and in xenograft tumours [23,31].

An enhanced clinical benefit of the combined treatment of certain anticancer agents with RHPS4 has been demonstrated in published studies. These observations are consistent with the loss of the protective capping status of telomeres mediated by RHPS4, thus leading to a greater susceptibility of cells with shorter telomeres towards different classes of chemical anti-cancer agents [29,31].

Regarding radiotherapy, it should be noted that drugs that could specifically sensitise tumours to ionising radiations, and in particular radioresistant tumours such as glioblastomas (GBM), would greatly enhance our ability to deliver curative doses while avoiding off-target effects. In general, an increased sensitivity to reactive oxygen species [32], the radiomimetic agent bleomycin [33] and ionising radiations [6,7,34] has been shown in cells with dysfunctional telomeres and/or short telomeres. Therefore, telomere dysfunction has been proposed as new a factor in the sensitivity to ionising radiation treatment [35,36]. Furthermore, alterations in telomere maintenance have been shown to interfere with the proper repair of radiation-induced DNA double-strand breaks [37–39].

Here, we assessed the ability of the RHPS4 G4-quadruplex ligand to destabilise telomeres in a panel of astrocytoma (III/IV World Health Organisation (WHO) grade) cell lines. Furthermore, the role of RHPS4 in sensitising cells to ionising radiations has been studied in combined treatments. We focused our attention on astrocytoma cells and in particular on GBM cells, because this tumour shows extreme intrinsic radioresistance and represents the most common primary brain tumour in humans [40]. Since glioblastoma patients are treated by neurosurgery in combination with radiotherapy, increasing the sensitivity of tumour cells to ionising radiation by targeting telomeres could represent an innovative strategy to improve radiation therapy outcome in these patients.

2. Materials and methods

2.1. Cell lines and culture conditions

Unless otherwise indicated, media and supplements for cell culture were purchased from Euroclone (Euroclone, Pero, MI, Italy) and the plasticware was purchased from Corning (Corning Life Sciences, NY, USA). U251MG (Astrocytoma IV WHO grade) and U87MG (Astrocytoma IV WHO grade) cell lines, kindly provided by Prof. D. Bettega (Depth of Physics, University of Milan and INFN and) were originally purchased from Banca Biologica and Cell Factory (Banca Biologica and Cell Factory, Genoa, Italy). T67 [41] and T70 [42] human astrocytoma cells (WHO grade III and IV, respectively) were kindly provided by Prof. G. Lauro (Depth of Sciences, University Roma Tre). AG01522 (PD 18–25) and MRC5 normal human primary fibroblasts (PD 20–25) were purchased from Coriell Institute (Coriell Institute, Camden, NJ, USA). All the astrocytoma cell lines were proven to be telomerase positive based on the results from TRAP-assay (not shown).

U251MG and U87MG were routinely maintained in minimum essential medium with Earle's balanced salt solution (MEM/EBSS) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium Pyruvate, 1% non-essential aminoacids, 100 units/mL penicillin and 100 μg/mL streptomycin. T67, T70 and MRC5 were cultured in Dulbecco's modified MEM supplemented with 10% FBS, 2 mM L-glutammine, 100 units/mL penicillin and 100 μg/mL streptomycin. AG01522 normal human primary fibroblasts (Coriell Institute) (PD 18-25) were maintained in EMEM/EBSS with 15% FBS, 2 mM L-glutammine, 1% non-essential aminoacids, 100 units/mL penicillin and 100 μg/mL streptomycin. All the aforementioned cell lines were maintained at 37 °C in a 5% CO_2 95% air atmosphere.

2.2. Chemical compound and treatments

The 10 mM stock solution of pentacyclic acridine, 3,11-difluoro-6,8,13-trimethyl-8Hquino[4,3,2-kl]acridinium methosulfate (RHPS4) was prepared in dimethyl sulfoxide (DMSO). The drug was always added to the cells 24 h after plating. An appropriate volume of DMSO was employed as the negative control. Drug dilutions were freshly prepared periodically before each set of experiments.

2.3. Proliferation assessment

To assess the proliferation capability of cells exposed to increasing concentration of RHPS4, 10^5 cells were seeded in triplicate in 60-mm Petri plates and cell counts, using the Scepter handheld automated cell counter (EMD Millipore Corporation, Billerica, MA, USA) were determined daily, from day 1 to day 5 of culture. Experiments were conducted in triplicate.

2.4. TIF co-immunostaining

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked in PBS/BSA1%. Samples were then co-immunostained over night at 4 °C, using a rabbit telomeric protein TRF1 antibody (Santa Cruz Biotechnology, CA, USA) in combination with a mouse γH2AX antibody (Millipore) or a mouse 53BP1 antibody (Millipore). After washes in PBS/BSA1% samples were incubated with the secondary antibodies (anti-mouse Alexa 546

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