Synthesis and Biological Evaluation of Hybrid Acridine-HSP90 Ligand Conjugates as Telomerase Inhibitors

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ARTICLE

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The synthesis and biological evaluation of a series of bifunctional acridine-HSP90 inhibitor ligands as telomerase inhibitors is herein described. Four hybrid acridine-HSP90 inhibitor conjugates were prepared using a Click-Chemistry approach, and subsequently shown to display comparable results to the established telomerase inhibitor BRACO-19 in the TRAP-LIG telomerase assay. The conjugates also demonstrated significant cytotoxicity against a number of cancer cell lines, in the sub-μM range.

Introduction

The expression of the ribonucleoprotein telomerase is up-regulated in ~85-90% of human cancers, and is considered essential for the propagation of the immortal phenotype and to the survival and proliferation of cancer cells.[1] Telomerase plays a crucial role in maintaining telomere length in tumour cells, thus preventing replicative senescence,[2] and has also been implicated in telomere capping, which is thought to protect from cell cycle arrest and senescence through DNA damage-signalling pathways.[3] Mutations in the catalytic domain hTERT can themselves lead to cancer progression in a number of human cancers.[4]

For these reasons, telomerase has emerged as a promising therapeutic target for novel anticancer therapy and several strategies have been formulated for down-regulating telomerase function, on the basis of understanding the function of, particular, the hTERT and hTR RNA domains of the enzyme complex.[5] Such strategies include inhibition of the catalytic active site of the enzyme[6] and oligonucleotide competition with the 3' end of telomeric DNA for the template site on the RNA subunit (hTR) of telomerase.[7] Both approaches have shown to result in telomerase inhibition, telomere shortening. *In vivo* antitumor activity has been observed with the modified oligonucleotide imetelstat, which is currently in clinical trials.[8] An alternative approach[9] involves the induction and stabilization of higher-order quadruplex structures formed by the guanine-rich telomeric DNA primer, which is single-stranded for the terminal 100–200 bases at the extreme 3' end of telomeric DNA in human chromosomes.[10] This is based on the requirement to maintain a single-stranded conformation when the terminal bases are recognised by the complementary sequence on the RNA template of the hTR sub-unit of telomerase, the essential first step prior to the synthesis by hTERT of further telomeric DNA repeats. G-quadruplex DNA structures formed within the telomeric repeat sequence have been shown to inhibit telomerase activity, and ligands that stabilise these complexes are effective inhibitors of telomerase and exert growth inhibitory effects on tumour cells *in vitro* and *in vivo*.[9,11]

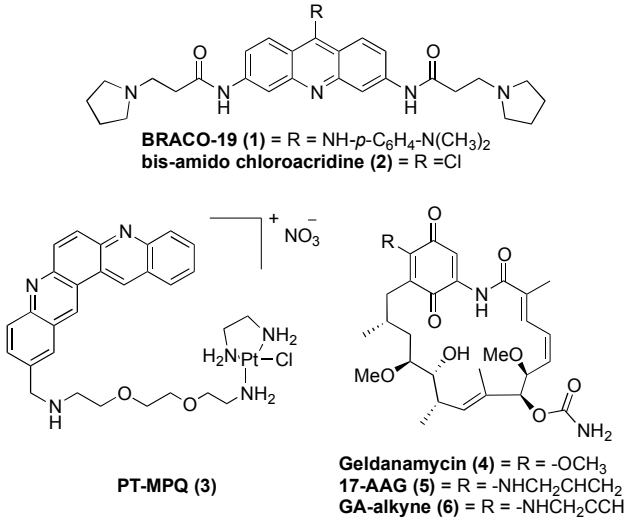

Figure 1: Structures of compounds discussed here

A large number of promising G-quadruplex interacting ligands have now been reported,[11] with BRACO-19 (**1**),[12a] RHPS4[12b] and the natural product telomestatin[12c] amongst the most-studied. G-quadruplex binding ligands have also been used in combination with established anticancer agents including, amongst others, cisplatin,[13a] paclitaxel[13b] and camptothecins.[13c] An advance on separate compounds being

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used in combination has been the development of a G4 quinacridine ligand-cisplatin conjugate, Pt-MPQ (**3**).[14] The bifunctional mode of this complex represents an intriguing example of ligand design and suggests new possibilities for achieving complementary biological activity in this area.

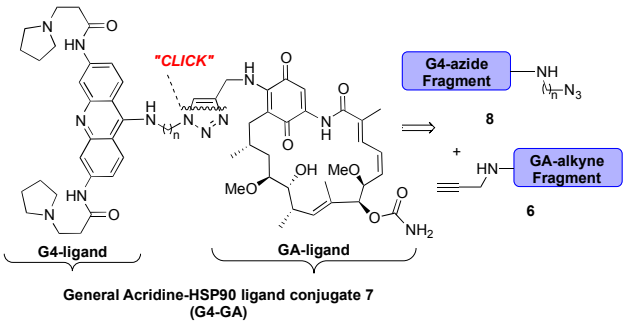
The heat shock protein 90 (HSP90) and the co-chaperone p23 have been shown to be required for the assembly and activation of telomerase in human cells.[15] Villa *et al.* subsequently reported using a TRAP assay, that the highly cytotoxic HSP90 inhibitors geldanamycin (Figure 1: GA, **4**) and 17-allylamino-17-demethoxygeldanamycin (17-AAG, **5**) inhibited telomerase catalytic activity in JR8 human melanoma cells.[16] Given the likely distinct and possibly complementary mechanisms for telomerase inhibition involving G-quadruplex- and HSP90-binding compounds, coupled with the reported successful demonstration of combination in this field,[14] we have conceived a bifunctional ligand. This is based on a G-quadruplex binder-geldanamycin conjugate as a potential telomerase inhibitor and anti-proliferative agent. Several promising GA-conjugates have already been reported with herceptin,[17] carbohydrates,[18] testosterone[19] and anti-HER2 MAb[20] amongst others,[21] providing broad support for the concept. We report here on the synthetic strategy and on preliminary biological evaluation of representative conjugates.

Results and discussion

In the design of our hybrid ligand series of the type-**7** (Scheme 1), we chose to incorporate features of the acridine based compound BRACO-19 (**1**) as the G-quadruplex (G4) binding and telomerase inhibition component, coupled through a 1,4-triazole-based linker to the geldanamycin-derived HSP90 interacting unit. In principle, the G4-binding acridine component should be sufficiently distanced from the quinone natural product derivative to allow efficient binding. On the other hand, it is known from structural data that the ansamycin ring of geldanamycin inserts into the binding pocket of the HSP90 protein, with the quinone ring exposed,[22] thus providing a convenient position for conjugating to the G4-binding acridine unit.

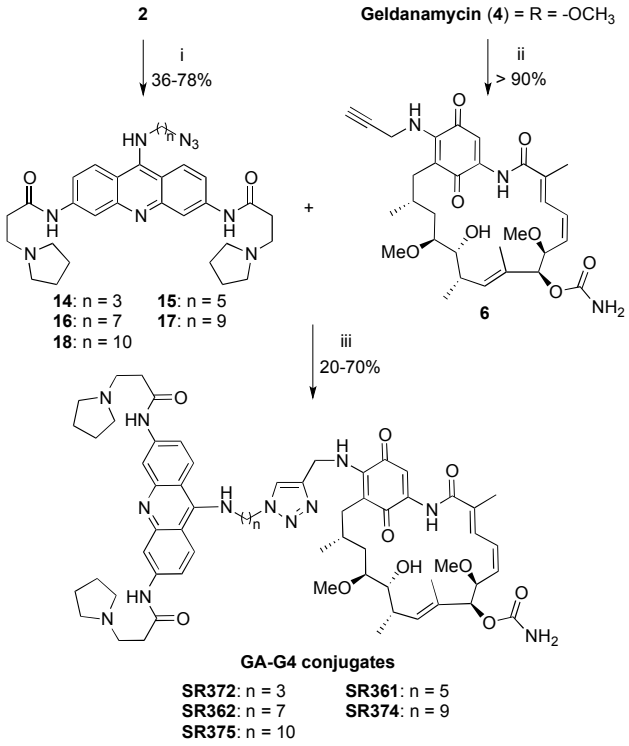
Retrosynthetically, the bifunctional ligand type-**7** could be achieved using the CuAAC 'click' reaction of the corresponding G4-azide type fragment (**8**) with the alkyne-GA derivative (**6**) (Scheme 1). We envisaged that this approach would allow the synthesis, for biological screening, of a selection of hybrid ligands of varying linker lengths.

The synthesis began with the preparation of the G4-azide bearing fragments (**8**), which began from the readily accessible starting material bis-amido chloroacridine **2**.[23] The compound **2** underwent efficient nucleophilic coupling with the amino azides (**9**-**13**)[24] to realise the fragments **14**-**18** in excellent yield. The preparation of the GA-alkyne (**6**) was achieved by reacting geldanamycin (**4**) with commercially available propargyl amine in DMF at room temperature, giving complete conversion in 16 hours. Optimal results were achieved when 1.2 equivalents of propargylamine was added slowly to the



Scheme 1: Retrosynthetic approach to the acridine-HSP90 ligand conjugates

reaction mixture followed by a further 0.6 equivalents after 5 hours. Stirring for an additional 15 hours gave reproducible and isolated yields of > 90%.[25] With the complementary GA-alkyne and G4-azido "click" fragments in hand, the final CuAAC reaction was performed using 1:1 *t*-butanol:water (20 vols), $CuSO_4.5H_2O$ (5 mol%) catalyst and sodium ascorbate (0.2 equiv). The reaction mixture was stirred at room temperature for 16 hours resulting in the successful formation of the GA-G4 conjugates (**SR361**, **SR362**, **SR374** and **SR375**, Scheme 2).[26]



Scheme 2 Synthesis of acridine-HSP90 ligand conjugates. Reagents and conditions: i) Amino azide linkers (**9**-**13**), $CHCl_3$, reflux, 16 h; ii) Propargylamine, DMF, rt, 16 h; iii) Sodium ascorbate, cat. $CuSO_4.5H_2O$, *t*-BuOH:H_2O (1:1), rt.

Unfortuantely, the coupling of the fragments **14** and **6** to give the target **SR372** was unsuccessful under the same reaction conditions and was not investigated further. The ability of the four conjugates to inhibit cell growth in a panel of cancer cell

lines was evaluated using the SRB assay (96 hr exposure). Data on the parent compound BRACO-19 (**1**) was also obtained (Figure 1). The 17-AAG (**5**) was selected as a suitable control for the HSP90 component, since it is 10-fold more soluble in DMSO (10 mg/mL) than geldanamycin (**4**) is (though neither are significantly soluble in aqueous media) and so was more tractable for biological studies. Both compounds have been reported to have telomerase inhibitory activity in the TRAP assay.[16]

Table 1. Cell growth proliferation data for the four conjugates, BRACO-19 (**1**) and 17-AAG (**5**) in a panel of three cancer and one normal human fibroblast cell lines, expressed as IC_{50} values, in μM. ESDS average ± 0.05 μM

Cell line	SR374	SR375	SR361	SR362	1	5
MCF7	1.6	0.4	2.9	<0.1	2.2	0.01
A549	0.3	0.1	0.5	0.1	2.4	0.01
GIST48	1.5	1.6	2.8	2.3	>25	0.02
WI38	7.4	4.5	10.3	0.7	10.7	0.02

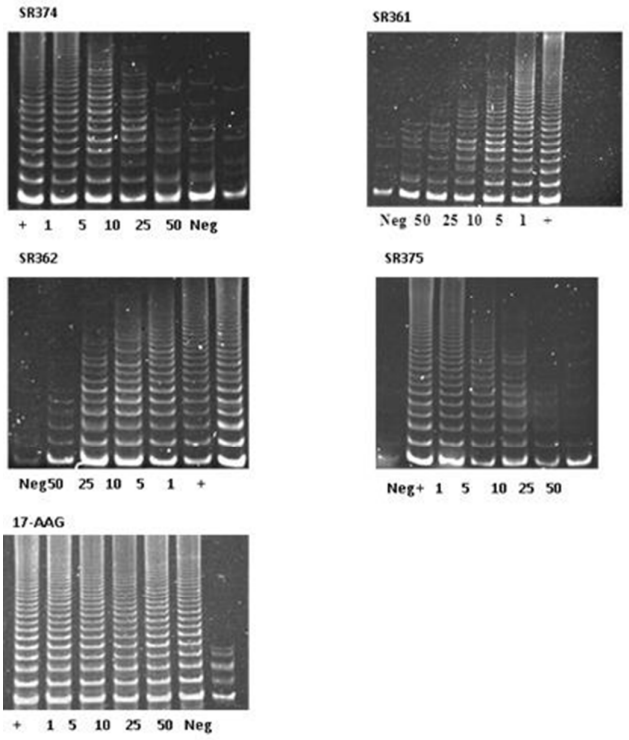
The results (Table 1) show that all four adducts are potent inhibitors of cell growth as measured by their IC_{50} values (the concentration required to produce 50% growth inhibition), with some conjugates showing sub-μM activity in several cell lines, notably the non-small cell lung cancer line A549. Three out of four show at least some selectivity for, in particular the A549 line over the normal non-cancer fibroblast WI38 line, with for example compound **SR375** showing 45-fold selectivity. 17-AAG (**5**) is highly potent in all the cell lines examined and shows no selectivity between them and the WI38 line. The conjugate behaviour is also distinct from that of the acridine compound BRACO-19 (**1**). This is especially apparent in responses to the Imatinib-resistant gastrointestinal cancer (GIST) cell line GIST48, which is unresponsive to BRACO-19 (**1**) but is sensitive to the conjugates. Data on just four conjugates is insufficient to derive a full set of structure-activity data. However Table 1 does suggest that linker length is important for anti-proliferative activity and that the compound **SR361**, with the shortest linker with $-(CH_2)_n-$ (n=5), is the least active. The two most active compounds, **SR374**, **SR375**, have the longest linkers with n = 9, and 10 respectively.

Table 2. Quantitation of the ability of compounds to inhibit telomerase catalytic activity, expressed as EC_{50} values in μM (concentration required to inhibit activity by 50%). ESDS average ± 2 μM

Ligand	SR374	SR375	SR361	SR362	1	5
EC_{50}	9.6	11.5	8.6	19	7.9	>50

Table 2 and Figure 2 show that the conjugates are all able to inhibit telomerase catalytic action, at EC_{50} values similar to that of BRACO-19 (**1**). By contrast, 17-AAG (**5**) itself is inactive in our hands, using the TRAP-LIG assay.[27] This result is at variance with the earlier report[16], which used the standard TRAP assay. We and others[28] have shown that the TRAP assay procedure when used with small molecules, is liable to contamination by compound in the PCR step unless precautions are taken to remove it, as in the TRAP-LIG[27] and direct assays.[28]

Figure 2. Polyacrylamide gels showing telomerase activity, one gel for each conjugate and one for 17-AAG (**5**), each with a negative control lane (with no telomerase or ligand) and a positive control lane, with telomerase only. Only the gels with the conjugates show dose-dependent diminution of the ladders of products, typical of telomerase inhibition.



Conclusions

Overall the data is strongly supportive of the HSP90 inhibitor conjugates being (i) stable in cells and (ii) being active as conjugates, since they differ from BRACO-19 (**1**) in being significantly more active across the cancer cell lines in our panel, and differ from 17-AAG (**5**) itself in being inhibitors of telomerase. Further studies will be needed to determine details of their mode of action.

Experimental

Human cancer cell lines MCF7 (Breast), A549 (Lung), and WI38 (Normal human lung fibroblast) were all purchased from American Type Culture Collection (ATCC). Cell lines MCF7, ALT and WI38 were maintained in Minimum Essential Medium supplemented with L-glutamine (2 mM, GIBCO 25030, Invitrogen, UK), essential amino acids (1%, GIBCO 11140, Invitrogen, UK) and foetal bovine serum (10%, S1810, Biosera,

UK). A549 cells were sub-cultured in DMEM as described above with the addition of non-essential amino acids. Cell lines were routinely passaged at ~70-80% confluency and maintained at 5% CO_2, 37 ^{o}C. GIST48 cells originate from gastro-intestinal cancers that had developed resistance to the drug Imatinib following initial clinical response (cells supplied by Dr JA Fletcher, Brigham and Women's Hospital, Boston).

Sulforhodamine B Assay

Short-term cell growth inhibition of the drugs was evaluated using a sulforhodamine B-based assay. Cells were seeded, at predetermined optimal densities, in 96-well plates and incubated overnight to aid cell attachment. Following overnight incubation, drugs were serially diluted in appropriate media and were directly added to cells. The cells were incubated for a further 96 hrs before removing the medium and fixing cells with TCA (10%, Sigma-Aldrich, UK) for 30 min at 4 °C. After removal of the TCA, the cells were washed with deionised water five times and dried at 60 °C for 1 hr. The cells were then incubated with sulforhodamine B (0.4% dissolved in 1 % acetic acid, Acros Organics, UK) for 15 min at RT. Excess unbound SRB was removed by washing wells with 1% acetic and plates were dried at 60 °C for 1 hr. Finally, Tris-base (10 mM, Acros Organics, UK) solution was added to each well, and the plates were gently shaken for 5 min at RT. The absorbance at 540 nm was measured with a plate reader (Spectrostar Omega, BMG Labtech, Germany). The data were normalized against the absorbance of control, untreated experiment and IC_{50} values were obtained as the concentration required for a reduction in absorbance intensity of 50%.

Immunoblotting

Known amount of protein (20-50 µg) from cellular samples were loaded onto a 4-12% Biorad precast gel and proteins were separated and then transferred onto a Nitrocellulose membrane (Invitrogen). The membranes were probed with primary antibodies against and β-actin (Santa Cruz Biotechnology). Following blocking in 5% milk, membranes were incubated overnight with primary antibodies (all purchased from Abcam) at various pre-determined optimal dilutions. Membranes were then probed with complementary secondary antibodies for 1hr at room temperature before visualisation with the horseradish peroxidase luminescent visualisation system (National Diagnostics).

Telomerase activity

This was determined using the TRAP-LIG assay[27], a modified telomere repeat amplification protocol that ensures that there is no carry-over of ligand into the second PCR step of the assay. Briefly, 1000 ng of protein from untreated and treated samples were incubated with TS forward primer (0.1 µg of 5'-AAT CCG TCG AGC AGA GTT-3') at 30^{o}C for 30min to allow the initial elongation to take place. Elongated products were purified using QIA quick nucleotide purification kit (Qiagen) according to the manufacturer's instructions. The eluted samples were freeze-dried and re-dissolved in PCR grade water. Redissolved PCR products were subjected to amplification in master mix containing ACX reverse primer (1 µM, 5'-GCG CGG $[CTTACC]_3$ CTA ACC-3'), TS forward primer (0.1 µg, 5'-AAT CCG TCG AGC AGA GTT-3'), TRAP buffer, BSA (5 µg), 0.5 mM dNTPs, and 2 U of TAQ polymerase (RedHot, ABgene, Surrey, U.K.) for 35 cycles of 94 °C for 30 s, at 61 °C for 1 min, and at 72 °C for 1 min. Samples were separated on a 12% PAGE and visualized with SYBR green (Aldrich) staining. Gels were quantified using a gel scanner and gene tool software (Sygene, Cambridge, U.K.). Intensity data were obtained by scanning and integrating the total intensity of each PCR product ladder in the denaturing gels. Background readings were corrected against a negative control and telomerase activity was expressed as a percentage of activity relating to activity in untreated sample.

Acknowledgements

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