A naphthalene diimide G-quadruplex ligand inhibits cell growth and down-regulates BCL-2 expression in an imatinib-resistant gastrointestinal cancer cell line

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Abstract

Gastro-intestinal tumours (GISTs) are driven by aberrant expression of the c-KIT oncoprotein. They can be effectively treated by the kinase inhibitor imatinib, which locks the c-KIT kinase domain into an inactive conformation. However resistance to imatinib, driven by active-site mutations, is a recurrent clinical challenge, which has been only partly met by the subsequent development of second and third-generation c-KIT inhibitors.

It is reported here that a tetra-substituted naphthalene diimide derivative, which is a micromolar inhibitor of cell growth in a wild-type patient-derived GIST cell line, has a sub-micromolar activity in two distinct patient-derived imatinib-resistant cell lines. The compound has been previously shown to down-regulate expression of the c-KIT protein in a wild-type GIST cell line. It does not affect c-KIT protein expression in a resistant cell line to the same extent, whereas it profoundly down-regulates the expression of the anti-apoptopic protein BCL-2. It is proposed that the mechanism of action involves targeting quadruplex nucleic acids, and in particular those in the *BCL-2* gene and its RNA transcript. The BCL-2 protein is up-regulated in the GIST-resistant cell line, and is strongly down-regulated after treatment. The compound strongly stabilises a range of G-quadruplexes including a DNA one from the *BCL-2* promoter and an RNA quadruplex from its 5'-UTR region. A reporter assay construct incorporating the 5'-UTR quadruplex sequence demonstrates down-regulation of *BCL-2* expression.

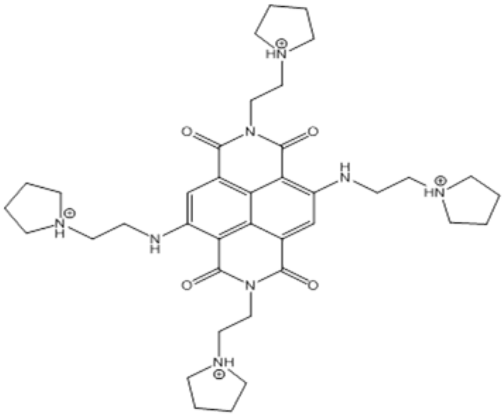
1. Introduction

Gastrointestinal stromal tumours (GIST) are dependent in large part for tumour initiation and their continuing growth on the aberrant expression of the c-KIT growth factor receptor, which contains a tyrosine kinase domain.[1-4] This kinase can be down-regulated by the CML (chronic myelogenous leukaemia) kinase inhibitor drug imatinib (Glivec®), which maintains c-KIT in an inactive state by binding to the ATP active site. Imatinib has received clinical approval for the treatment of GIST, where it has a major effect in terms of shrinking tumour size and thus resulting in increased patient life-span.[5-7] However a proportion of GIST patients are inherently resistant to imatinib and for a substantial majority of others, mutations in the c-KIT active site, for example in exon 9, eventually result in the onset of secondary clinical resistance and tumour regrowth.[4,8] A number of second-generation c-KIT inhibitors have been developed[8], with the multi-targeted kinase inhibitors Sunitinib[9,10] and more recently Regorafenib[11,12] having received regulatory approval. However Sunitinib treatment frequently results in further secondary mutations. It is also ineffective against mutations in exon 17. Regorafenib is active in tumours that are resistant to both imatinib and Sunitinib, although it can cause severe liver toxicity in a substantial number of patients.

We have previously reported[13] a distinctive approach to directly targeting the c-*KIT* gene which has exploited our earlier findings that the c-*KIT* promoter contains two putative quadruplex-forming sequences.[14,15] These, c-*KIT1* and c-*KIT2*, can form G-quadruplex higher-order structures, at least *in vitro*.[16-20] Such intramolecular quadruplexes are formed from G-rich repeat nucleotide sequences folded into four-stranded structures, stabilized through Hoogsteen hydrogen bonding of G-quartet motifs together with potassium or sodium counter-ions coordinated between the quartets.[21] A number of studies have now shown that the *c-KIT* quadruplexes can be stabilised by appropriate small molecules, resulting in the inhibition of c-*KIT* transcription.[13,22-24] In particular we have used a tetra-substituted naphthalene diimide derivative[13,25] (Compound **1**: Figure 1) for this purpose. This compound produces potent cell growth arrest in several c-KIT expressing cell lines, as well as in the patient-derived GIST cell line GIST882.[13] By contrast, this cell line did not show sensitivity to the established quadruplex-binding compounds BRACO-19[26] and TMPyP4.[27] We now report that compound **1** also potently

inhibits growth in two imatinib-resistant patient-derived GIST cell lines, and we present evidence supportive of distinctive quadruplex-mediated mechanisms of action.

Figure 1



Over-expression of the BCL-2 protein has also been associated with worsening prognosis following imatinib treatment in GIST patients[28] and the BCL-2 inhibitor ABT-737 can induce apoptosis in GIST cell lines.[29] BCL-2 (the B-cell lymphoma gene-2) encodes a 25kDa membrane protein that is involved in a complex system of signalling that controls apoptosis. The over-expression of *BCL*-2 prevents apoptosis and can contribute to metastasis in certain cancer types and is often associated with drug resistance. BCL-2 protein expression down-regulation can be used to increase the effectiveness of anti-cancer drugs and has been validated as a therapeutic target.[30] *BCL*-2 over-expression was first identified in follicular lymphomas linked to the chromosomal translocation t(14;18) where the gene is positioned adjacent to the IG heavy chain locus on chromosome 14.[31]

The human *BCL*-2 gene has been shown to contain a number of putative quadruplex forming sequences. Two in the promoter region,[32-35] and one in a 5'-UTR region of the mRNA,[36,37] 42-nucleotides upstream of the translation start site, have been previously reported to form quadruplex arrangements, at least *in vitro*. A number of quadruplex-binding small molecules have been reported to bind to one or other of the BCL-2 promoter quadruplexes and thereby down-regulate BCL-2 expression.[38-42] To our knowledge, there are no reports to date of translational down regulation of BCL-2 via the BCL-2 5'-UTR RNA quadruplex. However a small number of other RNA quadruplexes have been successfully targeted, notably the 5'-UTR N-RAS[43] and k-RAS ones,[44] as well as an intronic RNA quadruplex transcribed by the *C9orf72* gene.[45]

Our aim in this study has been to investigate the effects of the specific quadruplex stabilizing small molecule compound **1**, on c-KIT and BCL-2 protein expression levels in both the wild-type

GIST882 and an imatinib-resistant GIST cell line. The clinically-approved drugs for GIST are kinase inhibitors. An aim of this study then has been to examine whether drug resistance in GIST cells can be overcome at the gene rather than the protein level.

2. Results

The extent of quadruplex stabilisation by compound **1**, BRACO-19 and imatinib was estimated using a high-throughput FRET (Fluorescence Resonance Energy Transfer) method, for a group of quadruplexes in 60 mM KCl or NaCl-cacodylate solution.

Table 1. Changes in melting temperature (ΔT_m), in °C, for five quadruplex-forming sequences and a representative duplex sequence, with 0.5 μM of compound **1**, imatinib and the quadruplex ligand BRACO-19, obtained using a FRET procedure. All measurements were made in triplicate and the mean s.d. is ± 0.1 °C. Solutions were in 60 mM KCl-cacodylate buffer, apart from those with the BCL-2 RNA quadruplex, which were in 60 mM NaCl-cacodylate buffer.

	F21T	**c-KIT1**	**c-KIT2**	**Promoter BCL-2**	**5'-UTR BCL-2**	**Duplex**
Compound **1**	29.7	11.2	29.0	27.6	10.7	3.5
Imatinib	0.2	0.2	0.2	0.1	0.1	0.1
BRACO-19	21.6	6.5	18.5	17.9	n/d	7.2

The FRET results (Table 1) show that imatinib does not significantly stabilise any of the quadruplexes or duplex

DNA, whereas compound **1** in particular, is an outstandingly potent stabiliser of quadruplex structures, and superior to BRACO-19 for all the quadruplexes examined in this study. It is notable that compound **1** also appears at first sight to show selectivity for the promoter DNA quadruplex of the BCL-2 gene over the 5'-UTR RNA quadruplex. However the melting experiments have been performed in different ionic conditions, 60 mM in K^+ for the DNA quadruplexes and 60 mM in Na^+ for the RNA one, since the melting temperature for the highly stable RNA BCL-2 quadruplex is 91 ºC in 60 mM K^+ solution, compared to 64.5 ºC in 60 mM Na^+ solution, so a clear conclusion cannot be made on the basis of this data alone.

The cell growth inhibition studies (Table 2) show that imatinib is active in the non-resistant c-KIT cell line GIST882 and the two non-GIST tumour lines HT-29 and HCG-27, whereas it is less active in the MCF-7 breast carcinoma line and the two patient-derived imatinib-resistant lines GIST48 and GIST62. By contrast, compound **1**, which is readily taken up into cells, is active across all lines, and in the two resistant lines results in an IC_{50} value of < 1.0 µM. BRACO-19 does not show significant activity in any of the GIST lines.

	GIST882	**GIST48**	**GIST62**	**HT-29**	**HGC-27**	**MCF-7**
Compound **1**	1.6	0.5	0.4	0.04	0.1	0.01
Imatinib	1.7	19.8	>25	0.06	2.4	11.3
BRACO-19	>25	>25	>25	2.7	2.3	2.2

Table 2. Short-term 96 hr anti-proliferative activity for the three compounds in a panel of six cancer cell lines, shown as IC_{50} values (in µM). Standard deviations from >3 individual determinations are in the range ±0.05-0.2 µM.

The previous study on the mode of action of compound **1** in GIST882 cells indicated that two quadruplex-linked mechanisms were involved in (i) inhibition of telomerase action and (ii) down-regulation of c-*KIT* expression at the transcriptional level. Both of these have now been examined in the GIST48 imatinib-resistant cell line. Compound **1** does not show any significant effect on the ability of telomerase to add telomeric repeat units to the 3´ ends of telomeres in GIST48 (Figure 2a), at least at the concentrations used here. This is in contrast to its high potency against telomerase in the wild-type GIST882 cell line. The compound also shows a reduced effect on c-KIT protein expression levels (Figure 2b) with a 17% drop in protein levels after 24 hrs and 37% after one week compared to β-actin controls. By contrast, in the GIST882 cell line, at a

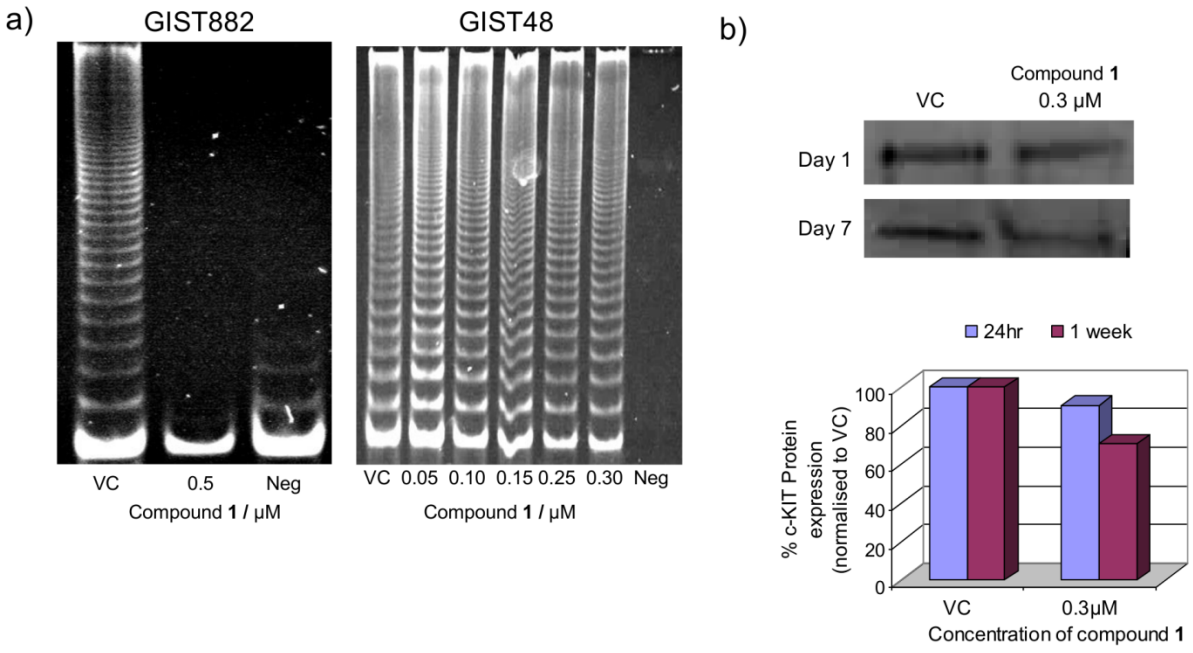


Figure 2

sub-cytotoxic concentration of 1 µM, c-KIT protein expression was reduced by 90% in 24 hrs, relative to a GAPDH control.[13] The expression levels of the anti-apoptopic protein BCL-2 have been compared in the GIST882 and GIST48 cell lines (Figure 3a), showing significant up-regulation in the latter line and evidence of only low-level expression in GIST882. A time-dependent study of the effects of a sub-cytotoxic concentration of compound **1** in the GIST48 line (Figures 3b, 3c), shows a ca 85% reduction in BCL-2 protein levels after one week of exposure, relative to β-actin controls.

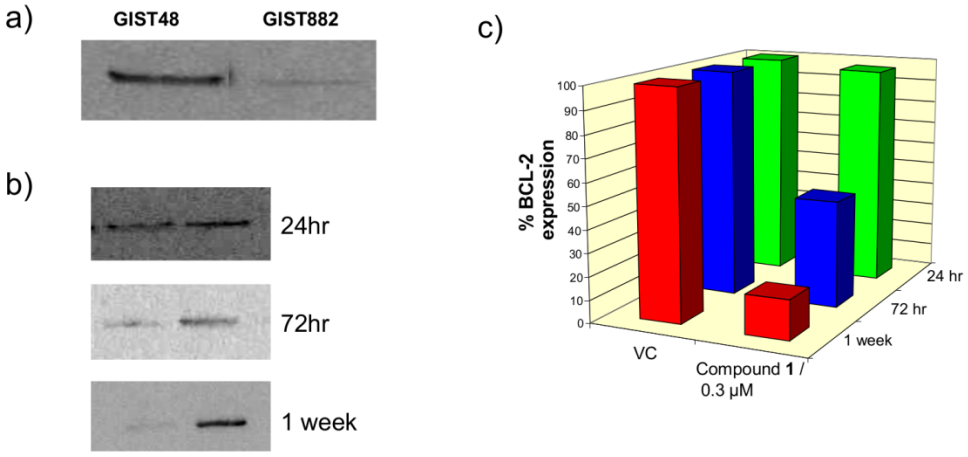


Figure 3

In view of the identification of a quadruplex-forming sequence in the 5'-UTR region of the BCL-2 mRNA, upstream of the translation start site, we have investigated the role that this site might play in regulating BCL-2 translation in the presence of compound **1**. A dual luciferase reporter assay has been established in which the 67nt sequence directly upstream of the BCL-2 translation start site has been inserted upstream of

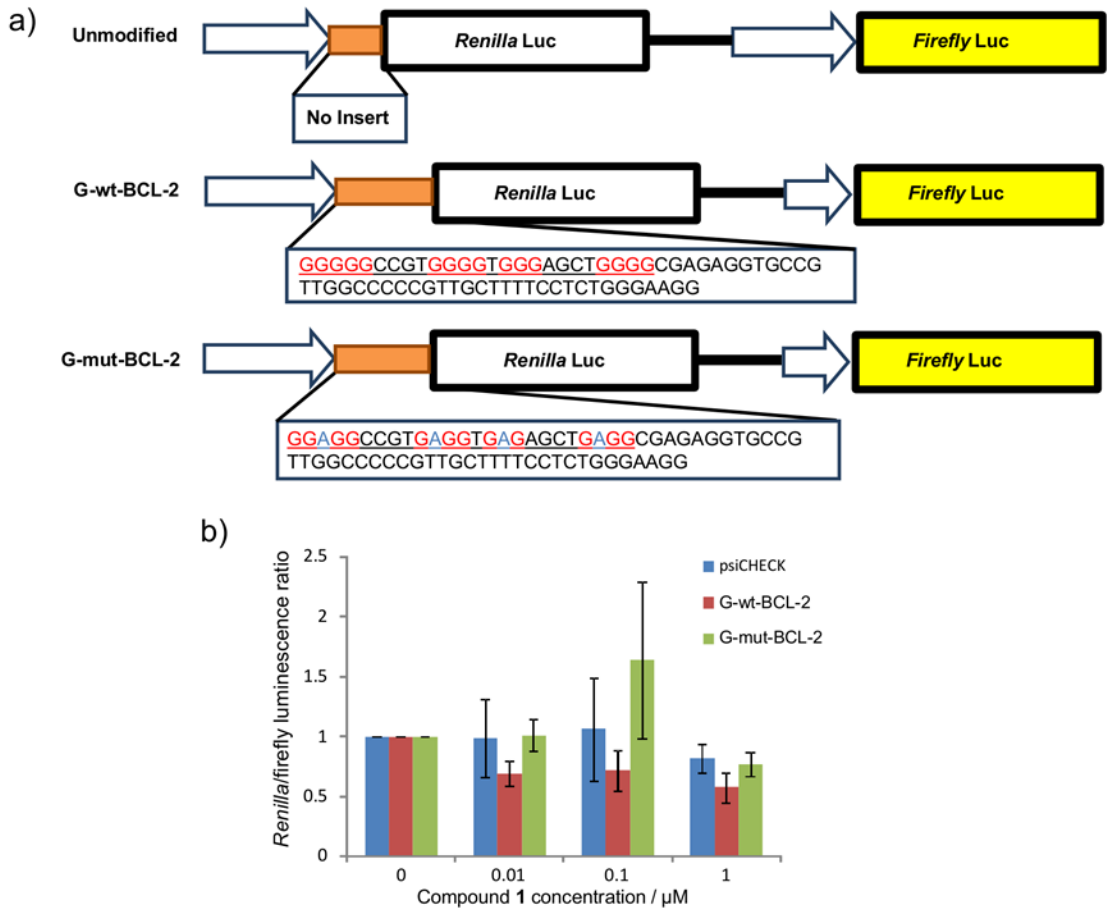


Figure 4

Renilla and firefly luciferase genes. A control reporter construct was also designed in which all G-rich tracts were modified with a single G-to-A substitution. The reporter assay constructs are shown in Figure 4a. The translation activities (as measured by the *Renilla*/firefly luminescence ratio) of these plasmids were initially assessed *in vitro* using a rabbit reticulocyte lysate system. These experiments showed that the insertion of the 67nt sequence from the BCL6 5' UTR (G-wt-BCL-2) significantly reduced protein expression (as measured by the *Renilla*/firefly luminescence ratio) compared to the mutated construct (G-mut-BCL-2) and the empty vector control (Figure S1). These findings are in line with previously reported results.[36] The plasmids were then transfected into GIST882 cells and the *Renilla* and firefly luciferase luminescence activities were recorded as a measure of protein expression. Addition of compound **1** showed an approximately dose-dependent inhibition of expression response with the wild-type BCL-2 5'-UTR sequence G-wt-BCL-2 compared to the psiCHECK sequence (containing no insert) and

the mutated BCL-2 5'-UTR plasmid G-mut-BCL-2 (Figure 4b), with a 30% reduction in translation at a ligand concentration of 10 μM, with no reduction for the controls.

3. Discussion

This study has shown that the naphthalene diimide derivative **1** is a potent inhibitor of cell growth in both wild-type and imatinib-resistant GIST patient-derived cell lines (Table 2). This property is not common to all quadruplex-binding small molecules, as shown by the selectivity of the acridine compound BRACO-19, which is active in a number of laboratory cancer cell lines (as well as *in vivo* against tumour xenografts[46]). The fact that both compounds stabilise a range of quadruplexes *in vitro* (Table 1) can only be taken as correlative evidence that quadruplex targeting is involved in their mechanism of action. The exceptionally high degree of stabilisation shown by compound **1** with the c-*KIT2* promoter quadruplex is in accord with the observed down-regulation of the high level of c-KIT expression in the wild-type GIST882 cell line[13]; the resistant line GIST48 has a lower level[47] and the compound does not have a large effect on this gene. On the other hand, BCL-2 is shown here to be expressed at high levels in GIST48 cells and may play a role in chemo-resistance, in this cell line at least. BCL-2 expression is reduced to a very low level by compound **1** and we suggest that this effect is likely to be a major contributor to the overall potency of compound **1** in imatinib-resistant cells.

The concept of BCL-2 as a target in resistant GIST is in accord with several previous studies. Amplification of BCL-2 has been previously reported in GIST disease progression in the clinic[48] and BCL-2 protein expression has been found to be elevated in iminatib GIST-resistant cells.[49] Also the BCL-2 protein small-molecule inhibitor ABT-737 shows activity in GIST lines.[29]

The observation that BCL-2 translation efficiency is reduced by compound **1** (in a reporter system) suggests that this compound is acting at the RNA (i.e. translation) level. However, in the absence of quantitative mRNA data, it is possible that compound **1** also acts at the DNA (i.e. transcriptional) level. We cannot also exclude the possibility that compound **1** has an effect on other targets and pathways in addition to the BCL-2-BAX apoptosis pathway. Indeed our recent study[50] on a trisubstituted naphthalene diimide in pancreatic cancer cell lines, using whole-genome RNA-seq methodology, revealed 47 major targets, almost all of which are quadruplex-containing. Also we cannot at this stage exclude other intronic targets in the BCL-2 gene, which

contain putative quadruplex sequences (see Figure S2 in the Supplementary Information), from being also targeted by compound **1**. In the context of GIST, the ability of a small molecule to down-regulate BCL-2, a major contributor to drug-induced resistance at the gene level (thereby bypassing the mutational consequences of protein targeting) is by itself of potential therapeutic benefit in this disease. Although we cannot at present comment directly on the suitability of compound **1** as a potential therapeutic agent in GIST, several members of the broad class of highly substituted naphthalene diimide compounds have shown promising *in vivo* activity in models for human cancers, indicating that their pharmacological properties are compatible with acceptable norms for drug-like properties.[39,48]

4. Methods

4.1 *General*

The quadruplex-binding naphthalene diimide derivative compound **1** was synthesized and purified in-house by reverse-phase hplc following published procedures[25] and was used as the 99% pure free base. It was dissolved in 100% DMSO to make a 10mM stock solution and aliquots were kept at -20°C. Stock solutions of compound **1** as free base and imatinib mesylate (Glivec®), purchased from ACC Corporation, were also prepared in filtered, sterilized distilled water and 100% DMSO respectively and stored as above. A 1mM working solution of each compound was prepared freshly on the day of the experiment in filter sterilized distilled water. The established quadruplex-binding ligand, the acridine compound BRACO-19 (synthesised and purified in-house) was also used in this study. A 1mM working solution of each compound was freshly prepared in filter-sterilised distilled water with 1% HCl, on the day of an experiment.

4.2 *Cell culture*

MCF-7 (human breast adenocarcinoma), HGC-27 (human gastric carcinoma) and HT-29 (human colorectal adenocarcinoma) cells were purchased from ATCC. MCF-7 and HT-29 cells were both maintained in DMEM. HGC-27 was maintained in MEM. Both DMEM and MEM were supplemented with 10% FBS. MCF7 cells were cultured at 37°C containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS)

Three patient-derived GIST cell lines were made available by Dr JA Fletcher. GIST 882 is a primary GIST line with a gain-of-function K642E mutation in c-KIT exon 13 and is not resistant to imatinib. GIST48 is an Imatinib-resistant line from a patient with a primary homozygous V560D exon 11 missense mutation and a heterozygous secondary D820A exon 17 kinase activation loop mutation, following treatment. GIST62 is an imatinib-resistant line from a patient who did not respond to imatinib. GIST882 cells were maintained in RPMI 1640 supplemented with 15% fetal bovine serum. GIST48 and GIST62 cells were routinely cultured and maintained in F10 media supplemented with fetal bovine serum and growth supplements.

4.2.1 *Short term cytotoxicity assay*

Cellular growth inhibition was measured using the sulforhodamine B (SRB) assay in 96 well plates as described previously.[13,48] 50% inhibitory concentrations (IC_{50}) were determined by taking the mean absorbance at 540 nm for each drug concentration expressed as a percentage of the absorbance of untreated control wells. All measurements were made >3 times

4.3 *In vivo telomerase activity*

Telomerase activity following treatment with compound **1** was determined using the TRAP-LIG modified telomere repeat amplification protocol. 1000 ng of protein from GIST48 cells treated with compound **1** for one week was incubated with the TS forward primer (0.1 μg of 5′-d(AATCCGTCGAGCAGAGTT)-3′) at 30 °C for 10 min to allow the initial elongation to take place. Elongated products were purified using the QIA quick nucleotide purification kit (Qiagen) according to the manufacturer's instructions and the samples were freeze-dried. Re-dissolved PCR products were subjected to amplification in master mix containing ACX reverse primer (1 μM, 5′-d(GCGCGG[CTTACC]$_3$CTA ACC)-3′), TS forward primer (0.1 μg, 5′-d(AATCCGTCGAGCAGAGTT)-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.

4.4 *Expression of c-KIT and BCL-2 proteins*

GIST48 cells were treated with compound **1** at sub-cytotoxic concentrations and samples were taken at different time points. Total protein was extracted with RIPA lysis buffer (Santa Cruz) at 4 °C and protein concentration was quantified with the BCA Protein Assay kit (Pierce). For the expression analysis of c-KIT, 500 μg of total protein was immune-precipitated with c-KIT primary antibody and Protein-G beads overnight at 4°C. Following this, the immune-precipitates were washed and protein samples were boiled at 95 °C for 5 min. Samples were separated on SDS-PAGE and probed with primary and HRP-secondary antibodies (Santa Cruz). For the expression analysis of BCL-2 and β-actin (as a control), 200 μg and 50 μg of total protein was separated on SDS-PAGE respectively and probed with appropriate primary and secondary antibodies (Santa Cruz). 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 protein band and are shown as normalised to β-actin.

4.5 *FRET studies*

FRET DNA melting assays with compound **1,** imatinib and BRACO-19 were performed using a fluorescence resonance energy transfer (FRET) assay. The labelled oligonucleotides had the fluorophores FAM (6-carboxyfluorescein) as donor and fluorophore TAMRA (6-carboxytetramethyl-rhodamine) attached as acceptor. All labelled sequences were purchased from Eurogentec Ltd., U.K. and used without any further purification beyond the manufacturer's hplc procedures. The sequences used were:

Human telomeric G4 sequence (F21T):

5'-FAM-d(GGG[TTAGGG]$_3$)-TAMRA-3'

Duplex sequence:

5'-FAM-d(TATAGCTATA-[(-CH_2-CH_2-O-)$_6$])-TATAGCTATA)-TAMRA-3'

BCL-2 5'-UTR G4 sequence

5'-FAM-r(GGGGGCCGUGGGGUGGGAGCUGGGG)-TAMRA-3'

BCL-2 promoter G4 sequence

5'-FAM-d(GGGCCAGGGAGCGGGGCGGAGGGGGCGGTCGGG)-TAMRA-3'

The DNA quadruplex FRET probe sequences were diluted from stock to the correct concentration (400 nM) in a 50 mM KCl + 10 mM potassium cacodylate buffer (pH 7.4) and then annealed by heating to 95 °C for 10 min, followed by cooling to RT in a heating block (3 - 3.5 hr). An identical procedure was adopted for the RNA BCL-2 sequence, which used a 50 mM NaCl + 10 mM sodium cacodylate buffer, pH 7.4. Drug stock solutions (at 2 x concentration) were prepared using the appropriate cacodylate buffer (pH 7.4). 96-well plates (MJ Research, Waltham, MA) were prepared by aliquoting 50 µL of annealed DNA into each well, followed by 50 µL of the compound solutions. Measurements were made on a DNA Opticon Engine (MJ Research) with excitation at 450–495 nm and detection at 515–545 nm. Fluorescence readings were taken at intervals of 0.5 °C in the range 30–100 °C, with a constant temperature being maintained for 30 sec prior to each reading to ensure a stable value. Final analysis of the data was carried out using a script written in the Origin 7.0 (OriginLab Corp., Northampton, MA) package and its advanced curve-fitting function for derivation of ΔT_m values.

4.6 *Construction of plasmids*

To construct the plasmids G-wt-BCL-2 and G-mut-BCL-2 two synthetic DNA duplexes encoding a 67 nt sequence from the 5'-UTR of the wild-type BCL-2 sequence and a mutated BCL-2 sequence were inserted into the *NheI* and *Not1* restriction sites of the psiCHECK-2 plasmid (Promega) upstream of the *Renilla* luciferase start codon. To build the DNA inserts the oligonucleotides shown below were used in a series of three PCR reactions. The reverse primer (Reverse-1) was designed to clone the entire *hRluc* gene from a psiCHECK-2 vector, including a *Not1* restriction enzyme site at the 3’ terminus, and includes an overlap on the 5' terminus for

the first PCR reaction with the Forward-1 primer. The primers used to generate the G-wt-BCL-2 and G-mut-BCL-2 inserts were as follows:

Forward-1:

5'-d(CCCGTTGCTTTTCCTCTGGGAAGGATGGCTTCCAAGGTGTACGAC)-3'

Forward-2a G-wt:

5'-d(CTGGGGCGAGAGGTGCCGTTGGCCCCCGTTGCTTTTCCTCTGG)-3'

Forward-3a G-wt:

5'-d(NNNGCTAGCTGGGGGCCGTGGGGTGGGAGCTGGGGCGAGAGGTG)-3'

Forward-2b G-mut:

5'-d(GAGCTGAGGCGAGAGGTGCCGTTGGCCCCCGTTGCTTTTCCTCTGG)-3'

Forward-3b G-mut:

5'-d(NNNGCTAGCTGGAGGCCGTGAGGTGAGAGCTGAGGCGAGAGG)-3'

Reverse-1: 5'-d(NNNGCGGCCGCTTACTGCTCGTTCTTCAGCAC)-3'

The PCR reactions resulted in the following two DNA constructs where the start codon is in italics and the G-rich motif is underlined:

G-wt-BCL-2:

5'-d(CT<u>GGGGGCCGTGGGGTGGGAGCTGGGG</u>CGAGAGGTGCCGTTGGCCCCCG

TTGCTTTTCCTCTGGGAAGG*ATG*)-3’

G-mut-BCL-2:

5'-d(CT<u>GGAGGCCGTGAGGTGAGAGCTGAGG</u>CGAGAGGTGCCGTTGG*C*CCCCGTT

GCTTTTCCTCTGGGAAGG*ATG*)-3’

Both the wild-type construct (G-wt-BCL-2) and the mutated construct (G-mut-BCL-2) contain the 42 bases located between the G-rich PQF sequence and the translation start site found within the wild-type *BCL*-2 5'-UTR region (in addition to the 25 nt G-rich sequence). The two inserts above were digested with *Nhe1-hf* and *Not1-hf* (NEB), treated with polynucleotide kinase and ligated into the *Nhe1-hf* and *Not1-hf* restriction enzyme sites located in the psiCHECK-2 plasmid. Competent *E. coli* cells (DH5α) were then transformed with the ligated plasmids (by heat shock), and positive colonies checked first by restriction enzyme analysis, and

confirmed by sequencing. Plasmids were amplified in *E. coli* liquid culture and purified on standard anion-exchange spin columns (Promega).

4.7 *In vitro dual luciferase reporter assay*

G-wt-BCL-2, G-mut-BCL-2 and unmodified psiCHECK-2 plasmids were linearized by digestion with *BamHI* restriction endonuclease and purified by agarose gel electrophoresis. Approximately 1 µg of each linear plasmid template was then added to a 20 µL *in vitro* transcription reaction (Ambion) to generate mRNA transcripts. mRNA transcripts were purified using centrifugal columns (Ambion), with 2 µg of linear mRNA from each plasmid then used per 50 µL rabbit reticulocyte lysate *in vitro* translation reaction (Promega). These reactions were incubated at 30 ºC for 90 minutes followed by assessment of luminescence activity following the manufacturer's instructions (Promega) using a luminometer.

4.8 *Transient transfection and dual luciferase reporter assay in cells*

GIST882 cells (1.0×10^5) were seeded into 96-well plates with 100 µl/well of RPMI1640 medium plus 15% fetal calf serum and 2 mM glutamine. Transfection was carried out in 96-well plates using Lipofect- AMINE 2000 (Invitrogen) according to the manufacturer's instructions, using modified and unmodified psiCHECK-2 plasmids. 333 ng of plasmid DNA in 25 µL DMEM without serum, along with 0.4 µL of LipofectamineTM 2000 in 25 µL of DMEM were added sequentially into each well. Dilutions were incubated at room temperature for 5 min, then mixed together gently and incubated for 20 min. Thirty-six hours after transfection cells were re-suspended in PBS passive lysis buffer (Promega). Cells in each well were lysed with 20 µL of lysis buffer. The lysates were assayed by using the Dual-Luciferase® Reporter Assay System (Promega). The average value and standard deviation of triply prepared samples was calculated using the Microsoft Excel software. The firefly and *Renilla* luciferase activities were measured using the Dual-luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions with a luminometer

4.9 *Transient transfection with compound **1***

Compound **1** was used to quantify, in a concentration dependent manner, any possible stabilization *in vitro* of the 5'UTR BCL-2 putative quadruplex forming sequence. The ligand, dissolved in 100% DMSO, was added *in vitro* in concentrations ranging from 0.01 to 1 μM/well. Each sample of ligand addition to a cell sample was prepared in triplicate. The concentrations and the addition of compound **1** for plasmid psiCHECK-2, G-wt-BCL-2 and G-mut-BCL-2 were the same for the transfection.

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Figure legends

Figure 1. The structure of compound **1** (N,N'-Bis(2-(pyrrolidin-1-yl)ethylamino)-2,6-bis(2-(pyrrolidin-1-yl)ethylamino)-1,4,5,8-naphthalenetetracarboxylic acid diimide)

Figure 2a. Polyacrylamide gels showing telomerase activity in cells treated with compound **1** for one week. The LHS shows GIST882 treated cells, with lane 1 showing the vehicle control (VC), lane 2; 0.5 μM of compound **1**, lane 3 shows the negative control. The RHS shows GIST48 treated cells, with lane 1 showing the vehicle control (VC), and lanes 2-6 showing the results of treatment with increasing concentrations of compound; lane 7 shows the negative control.

Figure 2b. Immunoblotting of total c-KIT protein in GIST48 cells. The upper panel shows total c-KIT after treatment with compound **1** for 24 hrs and the lower panel shows c-KIT after 1 week treatment. In each case the left-hand blot shows the β-actin control.

Figure 2c. Quantitation of these changes in c-KIT expression, normalised against untreated control β-actin expression.

Figure 3a. Immunoblotting of total BCL-2 protein in GIST48 and GIST882 cells.

Figure 3b. Immunoblotting of total BCL-2 protein in GIST48 cells. The top panel shows total BCL-2 after treatment with compound **1** for 24 hrs, the central panel for 72 hrs and the lower panel shows BCL-2 after one week of treatment. In each case the right-hand blot shows the β-actin control.

Figure 3c. Quantification of the total BCL-2 protein in GIST48 cells, shown as a dose-response plot following compound **1** treatment at a 0.3 μM concentration.

Figure 4a. Schematic showing the three dual-luciferase plasmid constructs used here, with respectively, null, mutated (**G-mut-BCL-2)** and wild-type (**G-wt-BCL-2)** 5'-UTR quadruplex sequences inserted upstream of the *Renilla* luciferase gene. White arrows indicate viral promoter sites.

Figure 4b. Plot showing changes in protein expression at three different concentrations of compound **1**, as measured by the ratio of *Renilla*/firefly luminescence, following plasmid transfection into GIST882 cells. Standard deviations following >3 determinations are shown as error bars.