Preclinical pharmacokinetics in tumors and normal tissues of the antigene PNA oligonucleotide MYCN-inhibitor BGA002

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KEYWORDS

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ABSTRACT

Although MYCN has been considered an undruggable target, MYCN alterations confer poor prognosis in many pediatric and adult cancers. The novel MYCN-specific inhibitor BGA002 is an antigene PNA oligonucleotide covalently bound to a nuclear-localization-signal peptide. In the present study, we characterized the pharmacokinetics of BGA002 ~~by the setting-up of a novel specific ELISA assay. After~~after single and repeated administration to mice~~,~~ using a novel specific ELISA assay. BGA002 concentrations in plasma showed linear pharmacokinetics, with dose proportional increase across the tested dose levels, and similar exposure between male and female, and between intravenous and subcutaneous route of administration. Repeated dosing resulted in no accumulation in plasma. Biodistribution up to seven days after single subcutaneous administration of [^{14}C]-radiolabeled BGA002, showed broad tissues and organ distribution (suggesting a potential capability to reach primary tumor and metastasis in several body sites), with high concentrations in kidney, liver, spleen, lymph nodes, adrenals, and bone marrow. Remarkably, we demonstrated that BGA002 concentrates in tumors after repeated systemic administrations in three mouse models with MYCN-amplification (neuroblastoma, rhabdomyosarcoma and small-cell-lung-cancer), leading to a significant reduction in tumor weight. Taking into account the available safety profile of BGA002, these data support further evaluation of BGA002 in patients with MYCN-positive tumors.

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INTRODUCTION

MYCN is a member of the MYC family of oncogenes, and is an oncogenic driver in many types of cancers[1]. Deregulation of MYCN occurs in both pediatric and adult cancers. MYCN-amplification (MNA) and/or overexpression have been found in pediatric cancers including neuroblastoma (NB), rhabdomyosarcoma (RMS), medulloblastoma, Wilms tumor and retinoblastoma. In particular, NB is one of the deadliest cancers that occur in early childhood and represents 7% of pediatric malignancies[1–3]. About 25% of patients with NB present MNA which is linked to a poor prognosis, metastasis, and advanced stage disease[1–3]. RMS is the most common pediatric soft tissue sarcoma, and a major cause of cancer death in children. MNA is present in about 25% of cases and MYCN overexpression occurs in 55%. It is associated with adverse prognosis and is a feature of the more aggressive alveolar subtype (ARMS)[4]. Similarly, MNA is also present in adult cancers, and occurs in small cell lung cancers (SCLC)[1] (15-20%)[5,6], in neuroendocrine prostate cancers (40%), in prostate adenocarcinomas (5%)[7], and in basal cell carcinomas (17.5%)[1], while overexpression of MYCN is present in a subset of T-cell acute lymphoblastic leukemias, glioblastoma multiforme and breast cancer[1]. Importantly, the amplification or overexpression of MYCN in the majority of these adult cancers is also associated with a poor prognosis[1]. ~~Normally~~

From an physiological perspective, MYCN expression is restricted during embryogenesis and has a very limited pattern of expression in normal cells after birth[8]. Given its crucial role in MYCN-positive tumors, one should consider this as a promising target[1]. However, N-Myc protein is deemed an undruggable target and drug discovery approaches aimed at blocking N-Myc protein have largely failed[9]. While indirect strategies have been proposed, none of these has proven to be effective in standard clinical practice [1,3,10].

We have previously demonstrated that an alternative approach consists in the specific gene expression inhibition at the level of chromosomal DNA through MYCN-specific antigene peptide nucleic acid (PNA) oligonucleotide covalently linked to a nuclear localization signal (NLS) peptide[2,4,11–14]. The antigene oligonucleotide approach (via persistent and specific block at the level of the target

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gene transcription) has shown advantages compared to the block of mRNA translation by antisense PNA oligonucleotide strategies[2,4,11–15]. In particular, BGA002 is a MYCN specific antigene PNA that inhibits MYCN gene expression, yielding a potent and specific anti-tumor activity both *in vitro* and *in vivo*[2,13,14]. PNAs are oligonucleotide~~-~~-mimetics, in which the anionic sugar-phosphate backbone of the nucleic acids is replaced with an achiral, uncharged polyamide backbone[16]. PNAs showed promising results in diagnostic or therapeutic applications as antisense or antigene drugs, due to their extraordinary stability against enzymatic degradation by proteases and nucleases and their ability to potently and specifically bind with high affinity their target complementary sequences in DNA or RNA[2,4,11,15]. PNAs have distinctive characteristics, which allow them to be used for antigene application because their neutral backbone avoids the electrostatic repulsion normally encountered between the negatively charged double strand DNA[2,4,11,17]. While PNAs are difficult to synthesize with high efficiency, adding biological function is a more reliable process~~, in~~. In fact, a straightforward approach to improve their pharmacological properties lies in the conjugation of PNA to short synthetic carrier peptides[18,19]. ~~Different type of peptides have been evaluated[2,4,11,18,20–25], with the aim to improve the~~Different type of peptides have been evaluated with the aim to improve limited solubility, low cellular uptake, poor biodistribution and rapid excretion of naked PNA oligonucleotides~~[26]~~[2,4,11,18,20–26], which has prevented their broad application as oligonucleotide therapeutics.

In an attempt to explore the pharmacokinetic-pharmacodynamic relationship of BGA002, and predict the exposure required for anti-tumor activity in humans, as well as establish the dosing requirements for a first-time-in-human (FTIH) study, we investigated the ~~pharmacokinetics~~pharmacokinetic (PK) and ~~pharmacodynamics~~pharmacodynamic (PD) profiles of BGA002 in pediatric and adult MYCN-positive tumor mouse models after single and repeated ~~systemic administration of BGA002~~doses [27–31]. The characterization of the pharmacokinetics in plasma was complemented by the evaluation of the biodistribution in healthy tissues, organs, and blood, taking into accounts the potential effect of route of administration, sex, and accumulation. In addition, to overcome the lack

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of a standardized methodology, here we report the development and application of a novel hybridization-based ELISA approach for the quantification of the MYCN-inhibitor BGA002 PNA-peptide in biofluids, as well as in murine tissues and tumors. We anticipate that, together with information on the safety profile of BGA002, these results provide the basis for the design of oligonucleotide-based cancer therapeutics in a prospective ~~first-time-in-human~~FTIH study. Characterization of the pharmacokinetics and pharmacodynamics of BGA002 may also give a benchmark for the development of other PNA-peptide inhibitors.

MATERIALS AND METHODS

Chemicals and dose preparation

BGA002 was produced by Biogenera ~~produced BGA002.~~SpA(Bologna, Italy). PNA-peptide was ~~either~~ available~~,~~ from stored ~~at -20°C, and~~quantities ready for use, or freshly produced by the chemistry department and delivered after purification and dilution ~~to the biology department for non-GLP studies or to the CROs (GLPLife Test, Wil Research, Aptuit and Selcia) for~~ for both GLP and non-GLP studies. A solution of BGA002 was quantified through spectrophotometer (λ_{260}=170300 L mol^{-1} cm^{-1}) and adjusted to reach 3 mg/mL, then stored at +°4C. Biogenera synthesized an acetylated form of BGA002. The radio labeled [^{14}C]-Ac-BGA002 (Batch No. 8249DCP006-6, radiochemical purity 99.8%) was ~~supplied~~ synthesized and supplied by Selcia ~~Limited~~Ltd (UK) as a solution in acetic acid: sodium acetate: HCl (75 mM: 25 mM: 3.6 mM) with 2.5% mannitol at a concentration of 1.16 MBq/mL (3.35 mg [^{14}C]-Ac-BGA002 /mL). The solution was stored in a fridge set to maintain a temperature of +4°C.

Synthesis of BGA002 compound

BGA002 is synthesized by standard ~~Solid Phase Peptide Synthesis~~solid phase peptide synthesis using an automated synthesizer (CEM ~~liberty blue~~Liberty Blue instrument with ~~Microwave~~microwave equipment).

Solid support is a ChemMatrix resin type and the strategy employed is Fmoc/Boc. Building blocks employed are protected aminoacids (Fmoc and Boc/Pbf) and peptide nucleic acid monomers (protected as Fmoc/Bhoc). Activation strategy employs 4eq of AA/PNA and HATU [445460_Sigma Aldrich]/diisopropilethylamine/2,6-lutidine in dimethylformamide (for AA) or N-methylpyrrolidone (for PNA). Loading of first aminoacid is performed in molar defect respect to resin loading with capping (by standard acetic anhydride solution) of the remaining active sites. In this way resin loading is lowered to ~0.2 mmol/g (to minimize intra-chain interactions during synthesis).

After loading of the first aminoacid, like in SPPS, the elongation of the oligomer continues by performing iterative synthetic routines for every building block added to the main chain, consisting of:

• Deprotection of Fmoc group, by reaction with excess 20% piperidine in DMF 2 times for 5min.

• Coupling (single for AA, double for PNA monomers) with the next building block, DIPEA/2,6-lutidine solution and HATU as activator at 90°C 170W for 15sec and 50W for 110sec for 1hour.

• Capping by standard Ac_2O/2,6-lutidine solution for 15min.

At the end of the coupling cycles the compound is then cleaved from the resin as Fmoc derivative by reaction with a 8:2 TFA: m-cresol solution and after 3 hour the desiderated crude will be obtained after precipitation in diethylether.

Fmoc-ON purification is required to remove guanidilated compounds. The crude is purified by RP-HPLC using a C18 column and standard eluents (water, acetonitrile, TFA 0.1%). Unfortunately, the synthesizer cannot perform a ~~preactivation~~pre-activation reaction between monomer, base and acti-

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vator because only one vessel is present in the instrument. The activator reacts faster with the terminal amine than the monomers and can produce the ~~Guanidilate~~guanidilate derivative that block the sequence. The purified Fmoc compound is dissolved in DMF and a solution of diluted piperidine is slowly added for the deprotection reaction. The corresponding crude material obtained is purified again using the same instrument and conditions, obtaining the BGA002 as a TFA salt.

Final steps of BGA002 production consist in counter-ion exchange (trifluoroacetate to chloride) and lyophilization. TFA counterion could be exchanged both by basic precipitation and aqueous HCl dissolution or by chromatography. Yield of the whole process is ~5%.

Labeling Ac-BGA002 with carbon-14

The labeling of [^{14}C]-Ac-BGA002 has been performed in accordance with Selcia Quality Program by the Radiochemistry group at Selcia Ltd.. 2g of resin bound PNA oligomer Fmoc protected was placed into SPPS reactor, the resin was rinsed with dimethylformamide then covered with dimethylformamide and left to swell for 2h. The remaining DMF was flushed away with nitrogen and the resin was exposed to the deprotection solution (20% of piperidine in DMF) then ~~shaked~~shook for 20min at room temperature for two times. A Kaiser ~~Test~~test was performed on a small amount of resin to confirm that deprotection of the amino functionality had occurred. [1-^{14}C]sodium acetate was azeotroped with toluene and suspended in DMF and lutidine and DIPEA were added. The resulting mixture was stirred at room temperature, under nitrogen until a clear solution was obtained (around 10 minutes). The resulting orange solution ~~were~~, containing the preformed mixture was then added to the SPPS reactor ~~containing the performed on a small amount of resin~~and showed that the sampling had not gone to completion. The solution was flushed away using nitrogen and the resin was rinsed with DMF. Another equivalent of [1-^{14}C]sodium acetate was activated using HATU, 2,6-lutidine, DIPEA in DMF. The activated mixture was added to SPPS reactor and shaken overnight at room temperature~~. A~~, unfortunately the Kaiser ~~Test performed on a small amount of resin~~ test showed that the coupling had not gone to completion therefore the mixture was given more time

to react and was stirred at room temperature for another 5 days. A Kaiser test has been performed on a small amount of resin ~~showed that~~at the end of each coupling ~~had not gone~~reaction to check the completion ~~but had progresses significantly~~of reaction itself. . Another equivalent of [1-^{14}C]-sodium acetate was activated using HATU 2,6-lutidinide, DIPEA in DMF and the resulting mixture was transferred into the SPPS reactors. The mixture was shaken overnight at room temperature. ~~A~~The Kaiser ~~Test~~test performed ~~on a small amount of resin~~ showed that the coupling had not gone to completion therefore the mixture was given more time to react and was stirred at room temperature for another 24h. ~~A Kaiser Test performed on a small amount of resin showed that the coupling had gone to completion.~~ The solution was flushed away using nitrogen and the resin was rinsed with DMF followed by DCM and diethyl ether. This rinsing sequence was repeated another 3 times. The resin was covered with the cleaving solution and the SPPS reactors were placed on the shaker for 3h. The reaction mixtures were discharged in falcon tubes containing ethyl ether. This caused a precipitate to form. The resin was washed with TFA and the rinsing were collected into a falcon tube as well. The tubes were stored at -15°C for 2 h then centrifuged at 4000 rpm for 15 min. The supernatant was removed and the resulting solid deposit was further washed with diethyl ether using the centrifuge. The falcon tubes were stored in fridge at -4°C overnight. The solid residues at the bottom of the falcon tubes were dried under a stream on nitrogen for 2h then over a phosphorus pentoxide in dessicator for another 2h. The crude is purified by RP-HPLC using a C18 column and standard eluents (water, acetonitrile, TFA 0.1%).

Animals: mice

NOD/SCID CB17 mice for PK analysis in tumor and liver and for efficacy studies were born in the animal facility of the Department of Veterinary Medical Science of the University of Bologna (Italy. All experiments were approved by the Scientific Ethical Committee of Bologna University according to protocol no. 7/73/2012 and authorization 564/2018-PR. Pharmacokinetics plasma analysis was performed in juvenile mice (CD1-Swiss, 5 weeks old) by Glp Life Test~~.~~ (Bologna, Italy).

Aptuit (Verona, Italy) and Farefarma (Novara, Italy) performed respectively ELISA analysis and pharmacokinetics analysis. Biodistribution analysis in mice (CD1-Swiss, 5 weeks of age) was performed by Charles River. (Tranent, Edinburg). Animals were ~~maintained~~handled according to ~~each CRO~~ internal standard operating procedures. PK and biodistribution studies in mice were performed under GLP compliance.

In-vivo PK/TK experiments in mouse plasma

~~The studies were conducted with the support of the following CRO:~~

- ~~In-vivo phase: Glp Life Test, Via Saliceto 3, 40010 Bentivoglio, Bologna, Italy~~
- ~~Bioanalysis: Aptuit Verona Srl, Via Alessandro Fleming, 4 - 37135 Verona, Italy~~
- ~~Pharmacokinetic Analysis: Farefarma Srl, Via Ferrari, 9 - 28045 Invorio, Italy~~

The animals used for the studies include Swiss mice (CD1) (5 weeks old at start of treatment) from the Charles River Laboratories Italy Srl with a health certificate according to Legislative Decree 24/20142.

The rationale for the dose selection of the single dose PK study in mice was based on feasibility studies performed ~~in~~by Biogenera ~~lab that~~, which showed ~~good level of quantification~~detectable concentrations at 15 mg/kg dose in tumor and liver, thus this dose and the 3-fold lower dose 5 mg/kg were selected to obtain data in plasma. ~~Rational~~The rationale for the dose ~~selection of~~selected for the repeated dose PK studies in mice was based on the ~~above-mentioned~~ results from the single dose PK study~~; since the two tested~~. Given that doses of 5 and 15 mg/kg ~~gave well separated kinetic curves~~resulted in distinct concentration vs. time profiles, the ~~following doses were selected for the repeated dose PK~~PK/TK study: included doses of 2.5, 7.5, and 25 mg/kg/day, under the assumption linearity across the dose range.

For the single dose study, 24 male and 24 female mice for each dose ~~of BGA002, 5 or 15 mg/kg,~~level and ~~each~~ route of administration, ~~single~~i.e., intravenous (bolus) injection (IV) ~~in~~through the caudal vein or ~~by a single~~ subcutaneous injection (SC) on the back~~,~~ were treated with

a volume of 100 µL solution containing BGA002 ~~at the desired concentration.; in~~. In addition, for each route of administration a control group ~~(control)~~ of 4 males and 4 females was treated with 100 µL of vehicle. ~~A~~In total ~~of,~~ 104 mice ~~were administered IV,~~received BGA intravenously and ~~a total of~~ 104 mice ~~were administered SC.~~received the drug subcutaneously. The animals were ~~given a single administration and subsequently were~~ sacrificed at 6 different time points after dose for blood collection (time points IV: 5-15-30-60-240-480 minutes; time points SC: 10-30-60-90-180-480 minutes).

For the repeated dose study, 36 female mice for each dose of BGA002~~, 2.5 mg/kg, 7.5 mg/kg or 25 mg/kg,~~ were treated by SC injection ~~on the back~~ with ~~BGA002 solutions at~~ a constant injection volume of 5 mL/kg/day~~.~~ BGA002. The volume of solution administered was calculated weekly using the last mean body weight for group. Eighteen animals per group were treated with a single injection while another eighteen animals per group were treated for 28 days with a once-a-day SC injection ~~(for a~~. In total ~~of,~~ 108 female mice ~~and~~were allocated to six different ~~groups of~~ treatment groups). Animals ~~in each group of treatment were then~~ sacrificed at six (6) different time-points after dose (10-30-60-90-180-480 minutes) for blood collection ~~(for a total of~~, with three animals per time point~~).~~.

In both studies, the blood was collected from the *vena cava* ~~before sacrifice of the animal that was anesthetized~~under anesthesia by inhalation of a mixture of CO_2/O_2 (70% and 30% respectively). A total of 0.3 mL of whole blood was drawn from each ~~of the 3 animals~~animal per time point and, due to technical ~~sample limitation~~limitations in juvenile animals (5 weeks old at start of treatment), the samples were pooled in a single tube containing ~~enough~~ lithium-heparin ~~to treat 1 mL of blood volume~~ (APTACA ~~company~~ mod 2400/1). The plasma collected from each tube was frozen at about -20°C ~~at least~~ overnight and shipped on dry ice ~~to the Test Site (Aptuit)~~ for ~~the analyses~~analysis.

Hybridization-based ELISA in plasma and organs (tumor/liver)

A hybridization-based ELISA assay has been developed and validated to measure the concentration of BGA002 present in plasma samples and in tumor/liver samples. This assay is an attractive bioanalytical tool owing to its ultra-sensitivity, micro scale sample volume, and ease of use with little or no sample cleanup, which make it a suitable tool to study the TK/PK profiles of therapeutic oligonucleotides to support preclinical and clinical development programs.

Neutravidin coated plates were incubated with a capture probe, biotin-labeled and partially ~~complements~~complementary to BGA002. After incubation and washing, plasma samples were added to the plate where the complex is immobilized by the neutravidin-biotin binding. After washing, a ~~detection probe,~~ digoxigenin-labeled ~~and~~detection probe complementary to the other part of BGA002 was added. Then a detection solution containing anti-digoxigenin-peroxidase (anti-DIG-POD) was added, making possible visualization by addition of tetramethylbenzidine (TMB~~) that~~), which produces a colored product. The amount of color correlates to the amount of BGA002 present. More in detail, plates were washed 3 times with 300 µL 5X SSCT buffer; 100 µL of 0.2 µM capture probe solution in 5X SSCT buffer is added and incubated at RT for 30 minutes. Plates were washed 3 times with 300 µL 2X SSCT buffer; 100 µL of plasma samples/CS/QC were added and incubated at 37°C for 60 minutes. Plates were washed 4 times with 300 µL 2X SSCT buffer; 100 µL of 0.2 µM of detection probe solution in 5X SSCT buffer was added and incubated at RT for 60 minutes. Plates were washed 3 times with 300 µL 2X SSCT buffer and 3 times with 300 µL of purified water; 100 µL of Anti-DIG-POD Fab fragments (1:8000) in PBST and incubated at RT for 60 minutes. Plates were washed 3 times with 300 µL 2X SSCT buffer; 100 µL of substrate solution (1-step Ultra TMB ELISA) and incubated at RT for a period of 15 minutes. Reaction was stopped by adding 100 µL of 0.5 M sulfuric acid. Reading is performed on Spectra Max 250 at 450 nm with reference at 750 nm.

Validation of hybridization-based ELISA

The hybridization-based ELISA described above has been validated in mouse tumor and liver for GLP-like use, and in mouse plasma and rabbit plasma for GLP use as ~~follow.~~follows:

Validation in mouse tumor and liver homogenate has been performed over the range 200 to 3200 ng/mL ~~of~~ BGA002 and ~~in mouse liver homogenate over the range~~ 100 to 3200 ng/mL ~~of~~ BGA002, respectively . Calibration standards were prepared from a set of working solutions at 10 concentrations of the test item plus blank in the appropriate matrix ~~which gave a concentration range of~~, yielding concentrations between 15.63 ~~to~~and 8000 ng/mL ~~of~~ BGA002 (Figure 1 b). Validation samples to establish the accuracy and precision of the method were prepared from test item aliquot ~~to give~~at nominal concentrations of ~~BGA002 of~~ 50, 100, 200, 400, 800, 1600 and 3200 ng/mL BGA002. The concentration for the dilution quality control (DQC) tested in the ~~linearity section~~linear range was 80000, 40000, 20000 and 10000 ng/mL.

Validation in mouse plasma has been performed over the range 20 to 200 ng/mL. Calibration standards were prepared from a set of working solutions at 8 concentrations of the test item plus blank in the appropriate matrix ~~which gave a concentration range of 3.91 to 500 ng/mL for BGA002 in mouse plasma. Validation samples to establish the accuracy and precision of the method were prepared from test item aliquot to give nominal concentrations of BGA002 in mouse plasma at 20, 50, 100, 150, 200 ng/mL. The plasma concentration for the dilution quality control (DQC) tested in the linearity section was 20000 ng/mL.~~

~~Validation in rabbit plasma has been performed over the range 20 to 200 ng/mL. Calibration standards were prepared from a set of working solutions at 8 concentrations of the test item plus blank which gave a concentration range of 3.91 to 500 ng/mL for BGA002 in rabbit plasma.~~, yielding concentrations between 3.91 to 500 ng/mL BGA002. Validation samples to establish the accuracy and precision of the method were prepared from test item aliquot ~~to give~~at nominal concentrations of ~~BGA002 in rabbit plasma at~~ 20, 50, 100, 150, 200 ng/mL BGA002. The plasma concentration for the dilution quality control (DQC) tested in the linear range was 20000 ng/mL.

~~For each validation, the~~The following parameters were calculated: LLOQ, ULOQ, precision (%CV) intra-assay, precision (%CV) inter-assay, accuracy (%bias), selectivity interference with the analyte from individual lots of plasma, specificity, stability (at room temperature, +4°C, -20°C), freeze-thaw stability, prozone effect.

Pharmacokinetic analysis

Pharmacokinetics analysis was performed using a noncompartmental approach in Phoenix WinNonlin 6.3 (Pharsight Corporation, USA). All concentration data points were weighted by the inverse square of the fitted value. The following parameters were evaluated: time to reach maximum concentration (T_{max}), ~~highest~~peak concentration (C_{max}), area under the concentration vs time curve (AUC), elimination half-~~time~~life ($t_{1/2\ elim}$), volume of distribution (V_d), total clearance (Cl_b), bioavailability (F%).

Peak concentration (C_{max}) and the time to reach C_{max} (T_{max}) after administration of the BGA002 were determined from the observed data. The area under curve (AUC) was calculated using the linear trapezoidal rule (calculation method linear trapezoidal with linear interpolation). The first order constant (λ_z) associated with the terminal (log-linear) portion of the curve was estimated by linear regression of the time vs. log concentration and the terminal half-life was calculated as $\ln(2)/\lambda_z$. The volume of distribution (V_d) was calculated based on the terminal phase as Dose/ λ_z ·~~AUCINF~~AUCinf_obs (where ~~AUCINF~~AUCinf_obs is the AUC from the time of dosing extrapolated to infinity). The total clearance (Cl_b) was calculated as Dose/~~AUCINF~~AUCinf_obs. The bioavailability (F) after subcutaneous administration was calculated as the ratio of the AUC after subcutaneous (AUCsc) and intravenous (AUCiv) administration as (AUCsc/AUCiv) multiplied by 100.

Biodistribution of BGA002 in mice (QWBA-Charles River/Selcia)

The [^{14}C]-Ac-BGA002 (Batch No. 8249DCP006-6, radiochemical purity 99.8%) was supplied by Selcia ~~Limited~~Ltd (UK) as a solution in acetic acid: sodium acetate: HCl (75 mM: 25 mM: 3.6 mM)

with 2.5% mannitol at a concentration of 1.16 MBq/mL (3.35 mg [^{14}C]-Ac-BGA002 /mL~~). The solution~~), and was stored ~~in a fridge set to maintain a temperature of~~at +4°C. CD-1 mice (28-35 days of age) were supplied by Charles River, UK and acclimatized to the experimental unit for 7 days prior to use ~~on~~in the study. The [^{14}C]-Ac-BGA002 formulation was administered by injection into the nape of the neck (single subcutaneous administration) of 6 male and 6 female mice at a target volume of 4.5 mL/kg, to achieve a nominal dose level of 15 mg/kg (target radioactive dose of 5 MBq/kg). One male and one female mouse were ~~humanely killed~~euthanized by CO_2 narcosis ~~at each of~~after 1, 4, 8, 24, 48 and 168 h post dose.

The carcass of each animal was then frozen by immersion in a mixture of solid CO_2 in hexane for ca 15 min~~. The frozen carcass was then~~ and embedded in a block of carboxymethylcellulose~~, which was frozen in the same way.~~.. After equilibration at -20°C, sagittal sections (30 µm thick) were taken through each animal using a whole body cryomicrotome (Leica Instruments GmbH). The sections were freeze dried prior to storage at ambient temperature. All samples prepared in scintillation fluid were subjected to liquid scintillation counting (LSC) for 5 min, together with representative blank samples~~,~~ using a ~~Liquid Scintillation Analyzer~~liquid scintillation analyzer with automatic quench correction by an external method. Where possible, samples were analyzed in duplicate and allowed to heat and light stabilize prior to analysis. Prior to calculation of each result, a background count rate was determined and subtracted from each sample count rate. For scintillation counting, a limit of reliable measurement (LRM) of 30 c.p.m. above background has been ~~instituted~~introduced in these laboratories. Where results have arisen from data below the LRM, the fact ~~is~~was noted. The radioactivity present in various organs and tissues in whole body sections was determined by QWBA using a Typhoon FLA7000 scanner and AIDA image analysis software (version 4.06, raytest isotopenmeβgerate GmbH, Germany). For analysis, representative whole-body sections were placed into close contact with phosphor screens and left for a period of 7 days. On each phosphor screen, a set of external standards was also exposed. These standards were prepared from blood spiked with a serial dilution of a [^{14}C]-labeled reference solution, which was dispensed into holes

drilled into a block of carboxymethylcellulose, frozen and then sectioned in the same way as the animal samples. After the phosphor screen was scanned, an image of the radioactivity in the sample was stored digitally. For quantitative analysis, six background areas were defined on each storage phosphor screen image. The software automatically calculated the mean background and subsequently subtracted this from all standards and tissues analyzed. A regression coefficient was derived by comparing the response of each standard with the nominal concentration over the range of radioactive concentrations used and forcing the response curve through the origin. The concentrations of the standards used were in the range of 0.05 to 258.48 µg equiv/g. The response curve ~~is~~was linear over these concentrations and ~~was~~ assumed to be linear to the limit of reliable determination. Each organ or tissue of interest was then identified and integrated, and the software automatically calculated the concentration (µg equiv/g) using the regression equation derived from the standards.

The limit of reliable measurement for each storage screen was calculated from the assessment of the mean background of the plate and defined as 3 times the standard deviation ~~of the mean above background.~~. At the specific activity used in this study, the limit of reliable measurement was in the range of 0.01 to 0.04 µg equiv/g.

Xenograft mouse models

Luminescent cells (Kelly-Luc, H69AR-Luc, RMZ-Luc) and xenograft ectopic mouse models (~~Neuroblastoma, Small Cell Lung Cancer, Rhabdomyosarcoma~~neuroblastoma, small cell lung cancer, rhabdomyosarcoma) were prepared according to previously published studies[2]. Kelly cell line was originally established from a 1 year old female patient with a stage IV NB with MYCN amplification ~~and it has been~~, purchased from DSMZ (DSMZ Cat# ACC-355, RRID:CVCL_2092). The ~~Alveolar Rhabdomyosarcoma~~alveolar rhabdomyosarcoma cell line RMZ was kindly provided by Prof. Lollini (Department of Experimental Pathology ~~at~~, University of Bologna), and is characterized by MYCN amplification[32]. The ~~Small Cell Lung Cancer~~small cell lung cancer H69AR cell line has been originally established from the pleural fluid of a 55 year old Caucasian male with small cell

carcinoma of the lung ~~and it has been~~, purchased from ATCC (CRL-11351). The H69AR cell line is a multiple drug resistant cell line as compared to the parental NCI-H69 cell line. The H69AR cell line presents MYCN gene amplification, and high expression of MYCN mRNA and protein[33,34].

Pharmacokinetics evaluation of BGA002 in tumor and liver of ~~xenograft~~ NB, RMS and SCLC xenograft mouse models

NB, SCLC and RMS mouse models were established as described above. In each mouse model, systemic treatment with BGA002 was administered subcutaneously and started when specific bio-luminescent value (in NB and SCLC mouse model), or tumor volume (in RMS mouse model) was reached. ~~When the starting point was reached, NB mouse model was~~ NB mice were treated with BGA002 daily at 10 mg/kg/day (tumor n=4; liver n=7) or vehicle (n=5) for 15 consecutively days, SCLC ~~was~~mice were treated with BGA002 at 15 mg/kg/day (tumor n=13; liver n=13) or vehicle (n=7) for 9 consecutive days, while RMS ~~was~~mice were treated with BGA002 at 15 mg/kg/day (tumor n=3; liver n=3) or vehicle (n=5) for 15 days. Tumors and livers were extracted immediately after animal euthanasia, weighted on a precision scale and homogenized with a probe sonicator in RIPA extraction buffer at concentration of 10 mg/mL. Concentrations of BGA002 in tumor and liver were evaluated by the hybridization-based ELISA assay as described above. Samples were stored ~~frozen~~ at -20°C.

Pharmacodynamics ~~evaluation~~ of BGA002 in RMS and SCLC mouse models

BGA002 or vehicle were administered subcutaneously to mice in the SCLC and RMS ~~mouse~~tumor models ~~were established as described above~~, respectively at doses of 15 mg/kg/day for (n=4)/vehicle (n=5) or 5 mg/kg/day (n=4)/ vehicle (n=3) for 14 consecutive days. In both cases, treatment ~~was administered subcutaneously and~~ started when ~~specific~~a predefined tumor volume (in RMS mouse model), or bioluminescent value (in SCLC mouse model) was reached. ~~RMS mouse model was treated with BGA002 at the dose of 15 mg/kg/day for (n=4) or vehicle (n=5) for 14 con-~~

~~secutive days, while SCLC mouse model was treated with BGA002 at 5 mg/kg/day (n=4), 15 mg/kg/day (n=3), or vehicle (n=14) for 14 consecutive days.~~ Twenty-four hours after the last injection, animals were euthanized and necropsy was performed.

Tumors were extracted and weighted on a precision scale and all organs collected for subsequent analysis and studies. Western blot Production of N-Myc protein was assessed in total protein extracts. Sample was removed from animal as quickly as possible after animal sacrifice and homogenized with a probe sonicator on ice in sample lysing solution added with a Halt protease inhibitor cocktail (Cat# 78429, Thermofisher) in a 1:100 rate. Sample lysing solution volume ~~must~~should be at least 200 ~~uL~~µL and at ~~maximum~~most 2 mL and adjusted to a concentration of 100 mg/mL~~:~~. Total protein extract was quantified with BCA method at NanoDrop ND-1000 spectrophotometer against a standard curve of BSA in sample lysing solution. Proteins were separated by one-dimensional precast polyacrylamide gel [Bolt Bis-Tris Plus Gels 10%], that provides a neutral pH environment with the aim to minimize protein modifications, under denaturing conditions. Samples containing proteins were denatured by incubation at 95°C for 10 min after the addition of 4X Sample buffer, 10X Reducing Agent and water to a final volume of 30 µL. In each lane were loaded 30 µg of protein and run at 200 V for about 15 min in electrophoresis buffer [Bolt MES SDS Running buffer] with the molecular weight marker SeeBlue Plus2 Pre-Stained Standard. They were transferred from gel to PVDF membrane [IBlot Transfer Stack] using the IBlot Gel Transfer Device, a dry system that transfers proteins in 7 minutes [all materials and reagent were purchased from Life Technologies]. The membrane was subjected to overnight incubation at 4°C with blocking solution (5% milk in PBS-Tween 0.1%). The day after it was incubated 1 hour with primary antibody, a mouse monoclonal anti-N-Myc antibody [SC-53993_Santa Cruz] diluted 1:200 in 3.5% BSA in PBS-Tween 0.1%. After three washes in PBS-Tween 0.1%, the membrane was incubated one hour with the corresponding secondary antibody, a sheep anti-mouse IgG-HRP [Amersham Biosciences] diluted 1:10,000 in 3.5% BSA in PBS-Tween 0.1%. The membrane was washed 3 times in PBS-Tween 0.1% for 5 min before detection. N-Myc chemiluminescent signal was measured adding the ECL

Select Western Blotting solution [GE Healthcare] at ChemiDoc-It [UVP] to the membrane. The signal quantification was carried out with Alliance software [Uvitec] and was performed following colloidal staining with Coomassie brilliant blue dyes. All N-Myc protein signals were normalized using the Coomassie brilliant blue signal for each lane.

Immunohistochemistry analysis was performed on formaldehyde fixed and paraffin embedded samples, cut on microtome. Antigen retrieval was performed with EDTA-NaOH pH 8. Antibody used were Anti-MYCN primary antibody (OP13 Calbiochem) and HRP conjugated secondary antibody (Dako). Staining was performed with the DAKO kit for HRP Colorimetric revelation using DAB and ~~Haematoxylin~~haematoxylin contrast.

Statistical analyses

All statistical analysis were performed using GraphPad Prism software (GraphPad Software Inc.) version 6. Data from distinct groups were compared using the Mann-Whitney test. ~~P~~Statistical significance level was set at $p < 0.05$ ~~was considered significant~~.

RESULTS

Validation of the hybridization-based ELISA approach for the quantification of the MYCN-inhibitor BGA002 PNA-peptide in biofluids and tissues. ~~For the evaluation of BGA002 in organ and tissue Biogenera has developed a new specific hybridization based ELISA assay to allow fast and precise quantification of BGA002 itself. This assay is an attractive bioanalytical tool owing to its ultra-sensitivity, micro-scale sample volume, and ease of use with little or no sample cleanup, which make it a suitable tool to study the TK/PK profiles of therapeutic oligonucleotides to support preclinical and clinical development programs. Hybridization based ELISA have been used or validated~~

Hybridization-based ELISA have been used for the determination of different kinds of ODN, including phosphorothioate ASO[35–37], mRNA[38], modified oligonucleotides including PNA and 2'-O-

MOE PS[39], siRNA and unmodified DNA ODN[35]. We set up the method based on the assay developed by Straarup et al[38] having a sandwich structure with ~~the~~ BGA002 ~~able to link~~that links together the capture and detection probe, specifically binding this two key elements (Figure 1 a). The general sensitivity of the assay is very high, LLOQ in plasma reaches the value of 20 ng/mL and of 200 ng/mL in tissue homogenate (Figure 1 b and Table 1). Also precision is very high, considering that the inter assay and intra assay precision are always under about 10%CV ~~and only the rabbit plasma assay shows values reaching 17.0 and 17.1%CV respectively~~ (Figure 1 b and Table 1). ~~Differently~~ On the other hand, accuracy shows ~~higher~~larger differences between ~~the validations performed and reaches its higher value on mouse liver~~ liver tissue and tumor, with bias ranging between -29.0 ~~to~~and 19.6 %bias ~~and its lower in the~~for mouse ~~tumor at~~ liver and between-15.7 ~~to~~and 2.4 %bias for mouse tumor (Figure 1 b and Table 1). For GLP study only, we also evaluated the stability of the assay. ~~In mouse plasma we found high~~System stability at ~~-~~-20°C (nominal~~), where~~) was high, with the system ~~maintains~~maintaining its characteristics up to 3 weeks. ~~Interestingly, in rabbit at the same condition, the system is more stable, maintaining its feasibility up to 5 weeks.~~ At room temperature the stability is at least 4 hours ~~in both cases~~. Also the freeze-thaw stability is the same and is settled for at least 3 cycles from -20°C (nominal) to room temperature. No prozone effect has been observed. Overall, our results demonstrated that this method is specific for the intended analyte, BGA002, and is able to quantify it in different complex matrices~~, including plasma of different animal species and organs. This confirms~~. These results confirm that the hybridization-based ELISA assay is an appropriate tool to obtain oligonucleotides TK/PK profiles in preclinical and clinical drug development.

Pharmacokinetics of BGA002 in mice.

The pharmacokinetic ~~profile after single and repeated administration~~profiles of BGA002 ~~to mice was evaluated with a new specific hybridization-based ELISA assay of plasma. The concentration~~

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~~vs. time profiles~~ were comparable after single dose and after 28 days of repeated SC administration, as demonstrated by both C_{max} and $AUC_{(0-t)}$ values. Absorption following SC administration was fast, with T_{max} being achieved after 10 min. Systemic exposure was similar between male and female, irrespective of the dose level, with ~~comparable exposure between intravenous (IV) and subcutaneous (SC) route of administrations (Figure 2 a, b, c, S1, S2 and S3).~~ similar exposure between IV and SC administration (Figure 2 a, b, c, S1, S2 and S3). In addition, exposure increased with increasing doses, despite the lack of evidence for dose proportionality ~~BGA002 was quantifiable up to eight hours in plasma (Figure 2 a, b and c, Supplementary Table S1 and S2), after which drug levels were below the limited of detection. Despite a certain grade of variability in the first 90 minutes between concentrations of BGA002 at specific timepoints after single or repeated administration (daily for 28 days), none of the doses showed accumulation after repeated dosing (Figure 2 d, Supplementary Table S3). This conclusion is based on AUC and C_{max} values which did not show notable (greater than 2 fold) differences in exposure. In addition, with both route of administration, the exposure increased with increasing of the dose, despite it was not possible to identify a dose-proportional effect~~[40] (Figure 2 e, Supplementary Table S3). BGA002 was quantifiable up to eight hours in plasma (Figure 2 a, b and c, Supplementary Table S1 and S2), after which drug levels were below the limited of detection. Despite interindividual variability, none of the doses showed accumulation after repeated dosing (Figure 2 d, Supplementary Table S3). The $AUC_{(0-t)}$ after single SC administration of 2.5, 7.5 and 25 mg/kg dose were 478.1, 1331.1 and 3433.6 h*ng/mL, respectively. ~~No accumulation was observed following repeated dosing, which resulted in~~ A similar exposure range ~~(~~was observed after repeated dosing, namely~~,~~ 428.8, 1208.7 and 2801.5 h*ng/mL after 2.5, 7.5 and 25 mg/kg dose, respectively) (Table 2). Likewise, C_{max} also increased in a dose-dependent manner ~~after SC administration~~ (198, 410, 1031 ng/mL after 2.5, 7.5 and 25 mg/kg dose, respectively). ~~Similar findings were observed between sexes (Table 2).~~

Volume of distribution (V_d) values suggest a high distribution ~~in~~into tissues~~. Nevertheless, our pharmacokinetics data overall show a tendency of the BGA002 to accumulate when administered~~

subcutaneously, with increasing doses (see V_d values). This effect of potential tissue uptake and accumulation is counterbalanced by an increase of the elimination process as indicated by the systemic body clearance (Cl_b) values, which are high and show a tendency to increase with higher doses. This phenomenon could explain the progressive reduction of the half-life observed at higher doses, even though plasma levels remain comparable after repeated dosing. The elimination half-life ($t_{1/2}$) was in fact generally short, with a tendency of becoming shorter at higher doses. We did not find significant differences between males and females, for both IV and SC administration, except for the dose 5 mg/kg administered SC where a notable (greater than 2-fold) difference was observed between males and females (Table 2). Nevertheless, we believe that the female value could be related to some sort of technical issue because is not in in line with the trend we described above. Systemic body clearance (Cl_b) estimates showed high clearance, even though results were not affected by induction or inhibition mechanisms (Table 2of systemic clearance (Cl_b). The median of relative bioavailability (F) for the subcutaneous route, independently from the dose was calculated as 98.41 in male and 83.67 in female (Table 2). PK in rabbits showed a higher (up to ten fold) exposure compared to mice (Supp Fig. S5). Volume of distribution was higher in mice after SC administration (35218.41 mL/kg) than in rabbits after IV infusion (3086.70 mL/kg) at the same dose of 2.5 mg/kg (this could indicate a higher distribution in tissue in mice than in rabbits) (supp Table S5). Clearance was higher in mice after SC administration (3261.74 mL/h/kg) than in rabbits after IV infusion (694.43 mL/h/kg) (this indicates a higher elimination rate in mice compared to rabbits) (supp Table S5).4% in male and 83.6% in female mice (Table 2). .

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BGA002 has a broad biodistribution in organs and tissues in mice

In order to examine the tissue biodistribution of BGA002 after single subcutaneous administration, radioactive BGA002 containing a [^{14}C]-acetylated at the C-terminus ([^{14}C]-Ac-BGA002) was injected into male and female CD-1 mice at a target dose of 15 mg/kg. Total radioactivity in selected tissues was investigated by quantitative whole-body autoradiography (QWBA) at several time-

points (1, 4, 8, 24, 48 and 168 h post dose) (Figure 3). The ~~concentrations of~~ total radioactivity in selected organs and tissues of male CD-1 mice are presented in Figure 3 a and b with a representative example of the autoradiography shown in Figure 3 c. The results indicate a wide distribution of BGA002 into several tissues and organs, with the kidney, liver, spleen, bone marrow, lymph nodes and adrenals (Figure 3 a and b) exposed to the highest levels of radioactivity. The results also confirmed fast clearance from blood, with the highest concentrations of BGA002 detected at the first sampling time point (i.e., 1 h post dose, 1.20 µg equiv/g) and less than half the ~~quantity~~levels at 4 h post dose, with further decrease in the subsequent time points. The majority of the tissues showed high concentration between 4-24 h post dose, but it was not possible to identify a clear T_{max}. In both sexes, diffusion and biodistribution from the dose injection site was very slow, with radioactivity still visible at the injection site and in the surrounding areas at 168 h post dose (last time point) (Figure 3 a and b). Radioactivity remained well above the limit of detection in most tissues at this time point. The elimination half-life could not be estimated due to the high level of radioactivity still present at the last time point. ~~Concentrations of radioactivity~~Radioactivity levels in the spinal cord and brain in both male and female animals were low, indicating limited penetration through the blood-brain barrier (Figure 3 a, b and c).

BGA002 highly concentrates in tumors and correlates with efficacy in different xenograft mouse models

The pharmacokinetic (PK) profile of BGA002 in tumors was investigated in three tumor xenograft mouse models with MNA and MYCN-expression (NB, RMS and SCLC). ~~BGA002 was administered subcutaneously (SC) for 15 days (15 mg/kg/day for RMS and 10 mg/kg/day for NB) or 9 days (5 mg/kg/day and 15 mg/kg/day for SCLC), and its concentration in the tumor was compared with the liver (a healthy organ showing the highest BGA002 concentration in biodistribution studies).~~ ~~These~~Our results reveal that BGA002 can be found into the tumors in all the three mouse models (Figure 4 and table S6). In particular, in NB tumors BGA002 reached a concentration 3-fold higher

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compared to the liver (Figure 4 and table S7). The anti-tumor activity of BGA002 was evaluated *in vivo* in the same three xenograft murine models. In the RMS model, treatment with BGA002 (SC administration of 15 mg/kg/day for 15 days) resulted in tumor weight decrease ~~of~~by more than 70% (Figure 5 b). In addition, BGA002 (SC administration ~~for 9 days) at~~ of 5 and 15 mg/kg/day for 9 days) induced tumor weight decrease ~~of 15% and 59% (p<0.05), respectively~~ in the multidrug resistant SCLC mouse model of 15% and 59% (p<0.05), respectively (Figure 5 b~~)~~), highlighting ~~a trend of reduction as expected considering our~~agreement with previous data[13]. Similarly, reduction of the N-Myc protein was observed in the RMS model and multidrug resistant SCLC mouse (Figure 5 a and c). In this regarding, we intentionally decided to use immunohistochemistry (IHC) analysis for qualitative evaluation~~, only about~~ of the effect of BGA002 on protein ~~in SCLC mouse model (Figure 5 a).~~ Interestingly, this trend of reduction is ~~maintained~~observed in ~~western~~Western blot in both SCLC and RMS model (Figure 5 b). ~~Unfortunately,~~However, statistical significance appears only in RMS ($p < 0.05$). ~~While this observation are not fully supported by statistical analysis, we prefer to keep this information considering its value as qualitative result and considering that the focus of the paper lies in pharmacokinetics, rather than pharmacodynamics of BGA002.~~ Finally, a similar pattern of response was observed in a histological analysis of tumor vascularization, leading to tumor vessels elimination after treatment at 15 mg/kg/day (Figure S4).

DISCUSSION

~~It is well established that MYCN is a key oncogenic driver in many types of pediatric and adult cancers[1]. MYCN drives cancer cells towards a stem-like phenotype that affects many different processes, including induction of proliferation, cell growth, immune escape, invasion, metastases, alteration of metabolism, and inhibition of apoptosis[1]. In particular, MNA and/or overexpression is a key feature of highly aggressive and deadliest cancer subtypes, and the majority of these tumors is associated with a poor prognosis[1]. Considering the differences in the pattern of expression in normal cells and its role in MYCN positive tumors, MYCN can be considered tumor specific and a~~

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promising target for therapeutic interventions[8]. We have previously reported an alternative approach based on a selective MYCN gene expression inhibition at the level of chromosomal DNA, through a potent specific antigene PNA oligonucleotide named BGA002[2,4,11–14]. The BGA002 sequence is complementary to a sequence of MYCN gene in murine and in human genomes, showing uniqueness in the latter. In particular, compared to the first lead anti-MYCN antigene PNA BGA001[4,11,12], BGA002 demonstrated a more potent and specific inhibition of MYCN expression[2,13,14]. BGA002 caused the block of different MYCN-related tumorigenic elements that contribute to cell deregulations, a dose-dependent tumor growth inhibition and the induction of apoptosis in MYCN-positive NB and SCLC cells [2,14]; in vivo systemic treatment with BGA002 leads to potent anti-tumor activity in MNA-NB and MNA-SCLC[2,14].

In previous studies, the antigene PNA oligonucleotide approach (via persistent and specific block at the level of the target gene transcription) showed pharmacological advantages compared to the block of mRNA translation by antisense PNA oligonucleotide strategies[2,4,11,15]. PNAs have unique characteristics for antigene application, as their neutral backbone avoids the electrostatic repulsion normally encountered between the negatively charged double strand DNA. However, naked PNA oligonucleotides showed limited solubility, low cellular uptake, poor biodistribution, tend to exhibit low plasma protein binding and are rapidly excreted in the urine[26,41], that prevented their broad application as therapeutics. Because PNA oligonucleotides have a peptide backbone, the most straightforward approach to overcome these limitations and improve its physicochemical and biological properties is the conjugation of PNA to short synthetic carrier peptides. Different types of peptides have been evaluated, that conferred improved pharmacological properties, ranging from cell penetrating peptides focused to optimize the cellular delivery, to peptides that showed improvement to the PK and biodistribution[18,19].

Regarding the anti-MYCN BGA002, because of its pharmacological mechanism of action as an antigene PNA oligonucleotide, delivery in the cell nucleus is a key point for selection of an optimal peptide. Therefore, BGA002 contains a covalently linked NLS peptide that we previously selected

~~after experimental evaluation of different peptides. The NLS peptide sequence was previously reported by us for the anti-MYCN antigene PNA BGA001[4,11] and by others for an anti-MYC antigene PNA[20,21]. Unlike other reported NLS peptides linked to antigene PNAs[4,11,20,21], the NLS peptide in BGA002 is made by D-amino acids to improve its stability and limit degradation by proteases[2,13,14]. Our previous data showed that BGA002 has an efficient cellular and nuclear accessibility in cancer cells[2]. We previously showed that NLS peptide is important also to improve the pharmacodynamics properties for the target DNA sequence of the MYCN gene[42].~~

Here we report for the first time the development of a novel specific hybridization-based ELISA approach for the characterization of the ~~PK~~pharmacokinetic profile of the MYCN-inhibitor BGA002, and its quantification in ~~mouse and rabbit~~ biofluids, as well as in the organs of healthy mice and tumor xenografts.

~~PK~~

Pharmacokinetic analysis in plasma after single administration of BGA002 in mice showed dose independent, apparently linear pharmacokinetics across the dose range tested, with no evidence of sex differences between male and female animals. Results also show good bioavailability, as minor differences were observed between IV and SC route of administration. The similarity in the concentration range achieved after IV and SC ~~is relevant because it~~ provides evidence that SC administration does not contribute to interindividual variability in exposure. It also supports the use of SC route for further evaluation of efficacy, overcoming potential technical problems of daily IV administrations through tail vein in mice. Moreover, the relatively high bioavailability of BGA002 following SC administration also opens the possibility to use SC as a route of BGA002 administration in human clinical studies. This would ensure better adherence to treatment and lower the burden of treatment due to the requirements for IV administration.

The PK tissue biodistribution in mice after single subcutaneous administration of [^{14}C]-Ac-BGA002 ~~at several time-points from 1 h up to 7 days,~~ showed a broad biodistribution in several tissues and

organs, with ~~a principal exposure observed~~drug levels detected in the kidney, liver, spleen, bone marrow, lymph nodes and adrenals. This is in line with previous pharmacokinetic studies[19], which indicated that PNA conjugated to short synthetic peptide carriers were rapidly cleared from circulation and distributed to a variety of tissues, with highest concentration in liver, kidney, spleen, mesenteric lymph nodes and adipose tissue (a very similar set of organs to the one we found for BGA002, except to the adipose tissue which in our study is not one of the main organs where BGA002 accumulates). Overall, these data show that peptide-conjugated PNAs overcome the common issues associated with unmodified PNAs, which include poor biodistribution, and they represent a synthetically feasible approach to improve their physicochemical and biological properties.

The broad biodistribution of BGA002 in several organs and tissues could suggest its potential ~~capability~~ to reach primary tumor and metastasis in ~~several~~ different sites in the body. In this respect, it is ~~relevant~~worth mentioning that BGA002 ~~localized~~was also detected in the adrenal medulla (~~that is~~i.e., a preferential primary site of origin for NB), and in the bone marrow (~~that is~~i.e., a preferential site of metastasis for NB and SCLC). Moreover, the ~~broad~~observed biodistribution ~~of BGA002 to several organs and tissues, suggest~~profile suggests a wider ~~consideration that~~application of the class of ~~the~~ PNAs oligonucleotides covalently bond to the positively charged NLS peptide, which could ~~also~~be used to treat other tumors ~~in many different body sites~~, and even non-oncological diseases affecting these different body sites.

~~The~~ On the other hand, the low biodistribution of BGA002 in the spinal cord and brain~~,~~ indicate that the blood-brain barrier limits its access to the central nervous system (CNS), most probably because the NLS delivery peptide is positively charged (contain lysine and arginine amino acids). If required, for therapeutic indications requiring CNS biodistribution, naked PNA oligonucleotides for their neutral characteristics can cross the blood brain barrier and reach the CNS[43].

In the present work, we investigated the PK profiles of BGA002 in different MNA and MYCN-expressing tumors. We selected NB and RMS (as relevant examples for poor prognosis MYCN-

positive pediatric tumors), and multidrug resistant SCLC (among the poor prognosis MYCN-positive adult tumors). This specific choice was addressed by the model characteristics themselves, considering their aggressiveness and pathology affinities. In particular, event free survival (EFS) commonly shows an endpoint within 60 days[2,13]. While this value tends to be higher in SCLC model, where we also found evidence of high vascularization, NB model usually reaches endpoint value under 30 days, highlighting how ~~much~~relevant MNA ~~may be a strong element~~is for poor prognosis. Tumor growth rate behaves in a similar way, reaching early relevant volume (500 mm^3 within the first 2 week) with an exponential growth ~~following~~[2,13]. Positive staining to Ki67 and MYCN protein are also characteristics normally well retained in our models ~~transversally and allow to properly evaluating,~~ allowing proper evaluation of tumor evolution under treatment[2,4,13].

Variability ~~is~~was also high in our experimental protocols, but the pharmacological ~~response always showed~~effects were dose ~~dependency~~ and exposure-dependent [2,4,13], highlighting the feasibility of this model for ~~study involving both PD and PK~~further evaluation of pharmacokinetics and pharmacodynamics. In fact, we previously reported the *in vivo* efficacy of BGA002 in a MNA-NB murine model[2], where we found that SC administration for 15 days of 2.5, 5 or 10 mg/kg/day resulted in a potent dose ~~response~~-dependent tumor growth inhibition, that reached tumor elimination after treatment with 10 mg/kg/day. In this MNA-NB mouse model, treatment with BGA002 at 5 mg/kg/day yielded a consistent reduction of the N-Myc protein (evaluated by IHC) and of the tumor vessels[2].

Remarkably, data on the ~~tumor PK~~pharmacokinetic profile ~~showed that repeated systemic administration~~ of BGA002 ~~resulted~~ in ~~its localization in~~ tumor across all ~~the~~ three highly aggressive MNA tumor mouse models showed drug presence in significant concentrations , with a remarkable 3-fold higher concentration in NB tumor compared to liver. Moreover, the concentration of BGA002 in tumors was associated to an anti-tumor PD activity in all the three MNA tumors. ~~In particular,~~

~~among the three tumors~~In fact, the highest ~~PK~~ concentration found in NB was also associated to the highest anti-tumor activity[2]~~, indicating a correlation of BGA002 PK with efficacy in these tumors.~~.

In summary, MYCN plays a central role in driving tumor evolution, orchestrating several aggressive oncogenic features. Among them, ~~it is well defined~~ the role of MYCN in neovascularization[44,45]~~.~~ has been established. Therefore, evidence from our ~~findings about~~experimental protocols showing that BGA002 ~~concentration in~~distributes into MYCN-expressing tumor might be also linked ~~by~~to the MYCN role in promoting the neovascularization (and thus accessibility) of the tumors. In this respect, it is ~~relevant~~worth emphasizing that treatment ~~with BGA002 in the~~of multi-drug resistant SCLC ~~model~~tumors with BGA002 led to elimination of tumor vascularization, similarly to our previous findings in NB and SCLC[2,13].

~~As~~

From a clinical perspective, it should be highlighted that MNA tumors are characterized by a very high amount of N-Myc protein, and high-risk and metastatic conditions~~, we evaluated a daily~~. Therefore, the treatment schedule ~~in MNA mice model to face this dramatic scenario~~used here was based on daily administration of BGA002. For ~~the same reasons, in human clinical trials we~~ this reason, we would propose frequent administration~~, while considering the lower PK~~ also in human ~~compared to mice.~~

~~Preclinical toxicology studies of repeated administration of BGA002 (4 weeks daily) performed in healthy mice under GLP conditions by using concentrations higher than those reported in the present work, showed that it is well tolerated (data not shown).~~

clinical trials. BGA002 could be ~~proposed in clinical trials~~administered as a monotherapy or in combination with selected anti-cancer drugs. In ~~this context~~fact, BGA002 ~~already showed~~was shown to act in synergy with retinoic acid (RA) in MYCN expressing NB, by overcoming RA resistance and restoring RA efficacy[14]~~.~~

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~~There are only a limited number of preclinical works describing the PK behaviour of administered .~~ This may be feasible as evidence from preclinical GLP toxicology studies following repeated administration of BGA002 (4 weeks daily) at a higher exposure range than what was described in the current investigation showed that treatment is well tolerated (data not shown).

There is limited preclinical research on the pharmacokinetics of oligonucleotides in tumors in vivo, and none ~~evaluating~~ has evaluated the effect of systemic repeated ~~dose~~dosing treatment[46–48]. Oligonucleotide therapeutics are emerging as promising anti-cancer precision medicines, especially for the targeting of the large number of undruggable proteins (such as that codified by MYCN) and non-coding RNAs[49]. ~~Our new preclinical~~The current findings indicate that the MYCN-specific antigene PNA-peptide BGA002 highly concentrates in aggressive MYCN-related tumors ~~and generates~~, resulting in significant anti-tumor ~~efficacy~~activity. Our data reinforce the potential role of oligonucleotides in cancer therapy and pose the basis for the ~~first-in-human clinical trials of~~FTIH study with BGA002 in these highly aggressive MYCN-positive tumors. BGA002 already obtained orphan drug designation for NB by the Food and Drug Administration (FDA) (orphan register: DRU-2017-6085) and the European Medicines Agency (EMA) (orphan drug application: EMA/OD/020/12), for Soft Tissue Sarcomas (including RMS) by EMA (orphan drug application: EMA/OD/037/16) and for SCLC by FDA (orphan register: DRU-2018-6260).

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AUTHOR CONTRIBUTIONS

A.S. carried out data curation, project administration and participated in the writing, in study conceptualization and design. D.B. participated in study supervision, in the writing, validation, in formal analysis and in investigation. L.M. participated in developing the methodology, data acquisition, validation and reviewed the manuscript. S.B. participated in the investigation, in validation, in visualization and in the writing. S.A. and C.A. participated in administrative, technical and material support. G.N. and L.C. developed the chemical synthesis of the compound. S.O. and O.DP. revised the manuscript and participated in administrative, technical, and material support. P.H. participated in funding acquisition, manuscript revision and study supervision. R.T. participated in study design and conceptualization, funding acquisition, manuscript revision and study supervision.

AUTHORS' DISCLOSURE

R. Tonelli is BIOGENERA shareholder. A.L. Scardovi, worked for BIOGENERA at the time of the studies, she is currently working for Ritrova Therapeutics, Inc. D. Bartolucci, S. Bortolotti, S. Angelucci, C. Amadesi, G. Nieddu, and L. Cerisoli are working at BIOGENERA. No potential conflicts of interest were disclosed by the other authors.

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TABLES

Table 1 Hybridization-based ELISA assay parameters in mouse and rabbit tissue omogenate.

Table reporting complete hybridization-based ELISA assay parameters calculated in mice and rabbits tissue omogenate. In order are reported data for the four different matrix used in the validation. The table cover both GLP-Like and GLP data. Abbreviation: lower limit of quantification (LLOQ), upper limit of quantification (ULOQ).

		GLP-Like study		GLP study	
Parameter	Unit	Mouse Tumor	Mouse Liver	Mouse Plasma	Rabbit Plasma
LLOQ	ng/mL	200	100	20	20
ULOQ	ng/mL	3200	3200	200	200
Intra-assay precision	%CV	≤7.7	≤5.0	≤8.9	≤17.0
Inter-assay precision	%CV	≤8.7	≤11.6	≤6.2	≤17.1
Accuracy	%bias	-15.7 to 2.4	-29.0 to 19.6	-8.7 to 26.1	-18.3 to 15.0

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Table 2 Pharmacokinetics parameters in mouse ~~and rabbit~~.

Table reporting complete PK parameters calculated in mice ~~and rabbits.~~ In order are reported data after subcutaneous (SC) or intravenous (IV) single dose administration in mouse and single versus repeated subcutaneous (SC) administration in mouse. Each ~~value~~esimate is reported as mean of multiple single values. Abbreviation: time to reach maximum concentration (T_{max}), ~~highest~~peak concentration (C_{max}), area under the concentration vs time curve (AUC_{0-t}), AUC from the time of dosing extrapolated to infinity (AUC_{inf}), elimination half-time ($t_{1/2\ elim}$), volume of distribution (V_d), total body clearance (Cl_b), bioavailability (F).

			Mouse single administration					Mouse repeated administration (SC)		
BGA002 Dose (mg/kg):			5.0		15.0			2.5	7.5	25
Parameter	Unit	Administration route	M	F	M	F	Day(s)	F	F	F
T_{max}	h	SC	0.16	0.50	0.50	0.16	1 Day	0.50	0.50	0.50
C_{max}	ng/mL		259	296	487	558		198	410	1031
$AUC_{(0-t)}$	h*ng/mL		566.07	408.80	1527.29	1545.21		478.17	1331.15	3433.62
AUC_{inf}	h*ng/mL		711.09	586.01	1775.22	1848.95		766.46	1601.45	3951.81
$t_{1/2\ elim}$	h		4.10	1.88	3.06	3.33		7.48	3.28	2.76
V_d	mL/kg		41621.48	23155.77	37342.78	38928.76		35218.41	22169.39	25217.22
Cl_b	mg/h/kg		7031.42	8532.22	8449.65	8112.69		3261.74	4683.26	6326.21
F	%		85.81	59.29	111.01	108.05				
T_{max}	h	IV	0.25	0.08	0.50	0.08	28 Days	0.50	0.17	0.17
C_{max}	ng/mL		520	571	492	557		182	356	1284
$AUC_{(0-t)}$	h*ng/mL		659.69	689.55	1375.77	1430.13		428.83	1208.7	2801.54
AUC_{inf}	h*ng/mL		742.48	761.47	1643.21	1601.74		652.03	1512.09	3378.25
$t_{1/2\ elim}$	h		1.43	1.31	3.75	2.80		6.19	3.42	3.20
V_d	mL/kg		13939.52	12363.09	49321.23	37815.86		34231.43	24468.71	34142.64
Cl_b	mg/h/kg		6734.15	6566.21	9128.45	9364.78		3834.19	4960.02	7400.28

FIGURE LEGENDS

Figure 1. Hybridization-based ELISA approach for the quantification of BGA002. (A) Schematic representation of the single probes and elements and assembled version of ELISA assay. (B) Calibration standard curves selected from research and/or GLP study. The R^2 for each curve follows: Mouse tumor R^2 = 0.9959; Mouse Liver R^2 = 0.9983; Mouse Plasma R^2 = 0.9986; Rabbit Plasma R^2 = 0.9985. Standard deviation are too low for graphical representation.

Figure 2. BGA002 pharmacokinetics in ~~murine~~mice. (A) Plasma concentration–time profiles of BGA002 over 8 hours in male and female murine blood, after a single intravenous injection with BGA002 at 15 mg/kg. (B) Plasma concentration–time profiles of BGA002 over 8 hours in male and female murine blood, after a single subcutaneous injection with BGA002 at 15 mg/kg. (C) Comparison of plasma concentration–time profiles between IV and SC administration routes in murine blood, after single treatment with BGA002 at 15 mg/kg. (D) Comparison of plasma concentration–time profiles in murine blood obtained after one day and after 28 days of once-daily repeated SC administration of BGA002 at 2.5 mg/kg. (E) Plasma concentration–time profiles in murine blood over 8 hours after single SC administration with BGA002 at 2.5 mg/kg, 7.5 mg/kg and 25 mg/kg. In all the studies, ~~due to technical sample limitation in juvenile animals,~~ for each timepoint the samples from three different animals were pooled in a single tube and analyzed through hybridization-based ELISA.

Figure 3. BGA002 biodistribution in male and female mice after single SC administration. (A) Biodistribution in wild type mice up to seven days after SC administration of [^{14}C]-Ac-BGA002 (15 mg/kg). Organs and tissues are divided in three groups. BGA002 concentration is reported as percentage of ug equiv/g normalized within each group. Male mice at 1 h and 48 h are out of analysis due to procedure issues (animals managed to reach the injection site and ingested some of the dose or the dose was partially administered intradermally, in both cases resulting in an anomalous radioactivity distribution profile). (B) Biodistribution of [^{14}C]-Ac-BGA002 is reported as mean from

male and female values for specific organ of interest. As expected, higher values can be found in kidney and liver, where oligonucleotides are commonly accumulated. Time points at 1h and 48h have one single measure due to procedure issues (for these time points only the female result is reported, thus standard deviations are missing). Red dots represent single values of female mice, blue dots represent single values of male mice. (C) Example of radiographic section image of biodistribution in mouse (female) after eight hours after single SC administration of [^{14}C]-Ac-BGA002 (15 mg/kg).

Figure 4. BGA002 ~~localizes~~is localized in different MNA-positive tumors. ~~PK concentration~~ BGA concentrations in tumor and liver in MNA-positive SCLC, NB and RMS mouse models~~,~~ after systemic SC administration ~~with BGA002 at~~of 15 mg/kg BGA002 for 9 days (~~in~~ SCLC), 10 mg/kg for 15 days (~~in~~ NB) and 15 mg/kg for 15 days (~~in~~ RMS). Mean values for SCLC are respectively 120 µg/g and 273 µg/g (*p*-value = 0.0072); Mean values for NB are respectively 360.6 µg/g and 111.9 µg/g (*p*-value = 0.0424); Mean values for RMS are respectively 121.4 µg/g and 754.6 µg/g (*p*-value = 0.1). Mann-Whitney test was performed for statistical analysis (ns = *p*-value > 0.05; * = *p*-value ≤ 0.05; ** = *p*-value ≤ 0.01).

Figure 5. BGA002 induces reduction in tumor weight and N-Myc protein in MNA-positive tumor mouse models. (A) ~~Evaluation by bioluminescence~~Bioluminescence and immunohistochemistry (reconstruction and magnification) evaluation of tumor growth after ~~15 days of once daily~~ systemic (IV) administration of BGA002 at 5 or 15 mg/kg/days for 15 days in MNA-positive SCLC mouse model. ~~Data are reported at~~ Reported data refers to the end of the experiment ~~end.~~ (B). ~~Tumor~~Similarly, tumor weight is reported at the end of experiment in SCLC and RMS, after ~~15 days of once daily~~ SC administration of BGA002 for 15 days. Mean values for SCLC are respectively 313.1 mg, 267.0 mg (*p*-value = 0.89) and 128.7 (*p*-value = 0.032); Mean values for RMS are respectively 303.6 mg and 90.8 mg (*p*-value = 0.06). (C) N-Myc protein levels are reported at the end of experiment in SCLC (*p*-value = 0.19 and 0.14) and RMS (*p*-value = 0.017), after treatment with

BGA002. Mann-Whitney test was performed for statistical analysis (ns = *p*-value > 0.05; * = *p*-value ≤ 0.05).