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1 **Novel Cationic Bis(acylhydrazones) as Modulators of Epstein–Barr**
2 **Virus Immune Evasion Acting through Disruption of Interaction**
3 **between Nucleolin and G-quadruplexes of EBNA1 mRNA**

4
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21
22 **HIGHLIGHTS**

- 23 • Interaction of NCL with G4 of EBNA1 mRNA is involved into immune evasion of EBNA1 and EBV
24 • Cationic bis(acylhydrazones) were developed as novel ligands binding to G4 of EBNA1 mRNA
25 • SAR of novel ligands with respect to *in vitro* binding to G4 of EBNA1 mRNA is discussed
26 • Two novel ligands enhance the expression of EBNA1 and antigen presentation
27 • Novel ligands disrupt the NCL–EBNA1 mRNA interaction *in cellulo* as shown by PLA

1 **ABSTRACT**

2 The oncogenic Epstein–Barr virus (EBV) evades the immune system through limiting the expression of
3 its highly antigenic and essential genome maintenance protein, EBNA1, to the minimal level to ensure
4 viral genome replication, thereby also minimizing the production of EBNA1-derived antigenic peptides.
5 This regulation is based on inhibition of translation of the virally-encoded EBNA1 mRNA, and involves
6 the interaction of host protein nucleolin (NCL) with G-quadruplex (G4) structures that form in the
7 glycine–alanine repeat (GAR)-encoding sequence of the EBNA1 mRNA. Ligands that bind to these G4-
8 RNA can prevent their interaction with NCL, leading to disinhibition of EBNA1 expression and antigen
9 presentation, thereby interfering with the immune evasion of EBNA1 and therefore of EBV (M. Lista et
10 al., *Nature Commun.*, **2017**, *8*, 16043). In this work, we synthesized and studied a series of 20 cationic
11 bis(acylhydrazone) derivatives designed as G4 ligands. The *in vitro* evaluation showed that most
12 derivatives based on central pyridine (Py), naphthyridine (Naph) or phenanthroline (Phen) units were
13 efficient G4 binders, in contrast to their pyrimidine (Pym) counterparts, which were poor G4 binders
14 due a significantly different molecular geometry. The influence of lateral heterocyclic units (*N*-
15 substituted pyridinium or quinolinium residues) on G4-binding properties was also investigated. Two
16 novel compounds, namely **PyDH2** and **PhenDH2**, when used at a 5 μM concentration, were able to
17 significantly enhance EBNA1 expression in H1299 cells in a GAR-dependent manner, while being
18 significantly less toxic than the prototype drug **PhenDC3** (GI₅₀ > 50 μM). Antigen presentation, RNA
19 pull-down and proximity ligation assays confirmed that the effect of both drugs was related to the
20 disruption of NCL–EBNA1 mRNA interaction and the subsequent promotion of GAR-restricted antigen
21 presentation. Our work provides a novel modular scaffold for the development of G-quadruplex-
22 targeting drugs acting through interference with G4-protein interaction.

23

24 **KEYWORDS**

25 Epstein–Barr virus • G-quadruplex RNA • G-quadruplex ligands • Nucleolin • Immune evasion

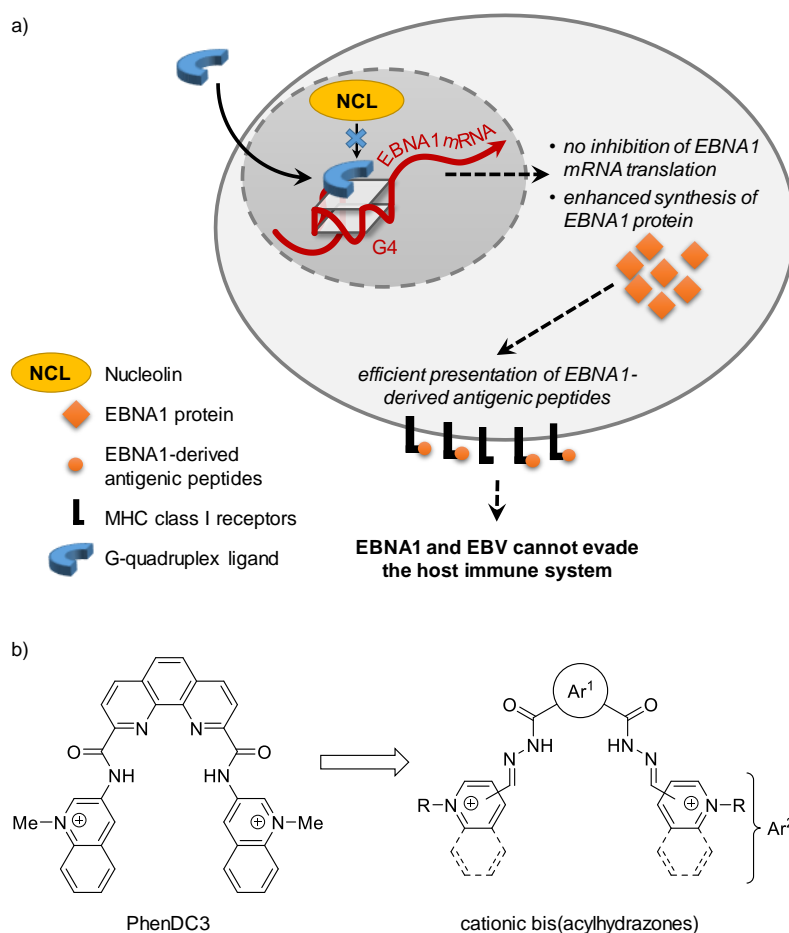
1 1. INTRODUCTION

2 The Epstein–Barr virus (EBV) is the first oncogenic virus discovered in human. Most of world’s adult
3 population (>90%) is infected with the virus but do not develop any pathology [1]. However, a small
4 proportion of infected individuals develops EBV-linked cancers, which include Burkitt and Hodgkin
5 lymphomas, and up to 10% gastric carcinomas, as well as nasopharyngeal carcinoma, particularly
6 frequent among men in China and Tunisia [1,2]. Like all gamma-herpesviruses, EBV effectively evades
7 the host immune system but has an Achilles heel, namely its genome maintenance protein EBNA1.
8 EBNA1 is essential for EBV genome replication and maintenance and, as such, is expressed in all
9 dividing EBV-infected cells. On the other hand, EBNA1 is highly antigenic, and CD8⁺ T cells directed
10 towards EBNA1 epitopes are present in all infected individuals. Hence, EBV has seemingly evolved a
11 mechanism to limit EBNA1 production to the minimal level required for the viral genome replication
12 thereby, at the same time, minimizing the production of EBNA1-derived antigenic peptides presented
13 to the cytotoxic T cells through the major histocompatibility complex (MHC) class I pathway. The
14 central glycine–alanine repeat (GAR) of EBNA1 plays a critical role in this mechanism of immune
15 evasion, as it is able to self-inhibit the translation of its own mRNA *in cis*. Remarkably, infection by an
16 EBV strain encoding a truncated version of EBNA1 in which GAR has been deleted (EBNA1ΔGAR) leads
17 to high level of EBNA1 protein and to an efficient T cell response, which demonstrates the critical role
18 of GAR in EBNA1 immune evasion [3]. GAR-based EBNA1 immune evasion is considered a relevant
19 therapeutic target in the treatment of EBV-related cancers, since most tumor cells from these cancers
20 are infected by EBV whereas, in healthy individuals, the latent infection by EBV is primarily restricted
21 to a specific small pool of memory B cells. Hence, overcoming the GAR-based self-inhibition of EBNA1
22 translation should unveil EBV-carrying tumor cells to cytotoxic T cells without having significant effect
23 on the vast majority of healthy host cells. Of note, GAR is polymorphic with respect to its length and,
24 importantly, the effect of GAR is length-dependent: longer domains display a stronger inhibitory effect
25 on both mRNA translation and antigen presentation [4–6].

26 The GAR-encoding mRNA sequence is GC-rich and is capable of forming multiple G-quadruplex (G4)
27 structures, which were shown to be implicated in the regulation of EBNA1 synthesis *in vitro* [7]. G4s
28 are particular secondary structures of nucleic acids formed through the stacking of G-quartets, that is,
29 planar arrangements of four guanines connected by Hoogsteen hydrogen bonds. G4 structures within
30 G-rich DNA or RNA sequences have been implicated in gene regulation where they can affect
31 transcription, splicing and translation [8,9]. The mechanisms of G4-based regulation are still poorly
32 understood, but cellular factors that interact with these structures are rapidly emerging [10,11].
33 Moreover, an increasing number of pathologies, that include cancer, neurodegenerative disorders,

1 bacterial and viral diseases, have been associated with G4-based regulation, illustrating the potential
2 importance of these structures as therapeutic targets [12–17].

3 Along these lines, we have previously developed a yeast (*Saccharomyces cerevisiae*)-based assay
4 reproducing all the aspects of the GAR-based inhibition of translation, including the GAR-length
5 dependency [18]. This allowed us to decipher the mechanisms of GAR-mediated mRNA translation
6 suppression *in cis* and, in particular, to identify the cellular factors involved [19,20]. The yeast assay
7 was successfully used first to identify small molecular-weight compounds that can stimulate EBNA1
8 expression both in yeast and in mammalian cells and relieve the GAR-based limitation of antigen
9 presentation [18,20]. More recently, this model was employed for a genetic screen that aimed at
10 identifying host cell genes involved in the GAR-mediated inhibition of translation. This enabled us to
11 identify the yeast *NSR1* gene encoding the ortholog of human nucleolin (NCL) [21], and to demonstrate
12 that NCL is critically involved in the GAR-based limitation of EBNA1 translation and antigen
13 presentation, and thus in the immune evasion of EBV. Specifically, we showed that NCL directly
14 interacts in the nucleus (or in close vicinity of the nucleus) with G4s that form in the GAR-encoding
15 sequence of EBNA1 mRNA and inhibits its translation, thereby limiting the production of EBNA1-
16 derived antigenic peptides which, in turn, favors immune evasion of EBV-infected cells [22].
17 Consequently, the interaction of NCL with G4 of EBNA1 mRNA appears a relevant therapeutic target
18 for the treatment and/or prevention of EBV-related cancers, as drugs that disrupt this interaction
19 could, in principle, overcome the GAR-based EBNA1 immune evasion of EBV. In line, we demonstrated
20 that the benchmark G4 ligand **PhenDC3** is able to prevent NCL from binding to G4s formed in the GAR
21 mRNA sequence, and to stimulate GAR-limited translation and antigen presentation (Fig. 1a) [22].
22 Importantly, this ability of **PhenDC3** is not a general property of all the G4-ligands since the other
23 benchmark G4 ligand, pyridostatine (**PDS**), has no effect on NCL binding nor on GAR-based inhibition
24 of translation. Hence, it appears that active G4 ligands not only need to bind efficiently to G4 that form
25 in the GAR-encoding sequence of EBNA1 mRNA, but they should also be able to interfere with NCL
26 binding, which does not appear as a general property of all G4 ligands.



1
 2 **Fig. 1.** a) Effect of G-quadruplex ligands on the nucleolin (NCL)- and GAr-dependent limitation of EBNA1 synthesis.
 3 NCL binds directly to G-quadruplexes (G4s) formed in the GAr-encoding sequence of EBNA1 mRNA, thus
 4 inhibiting its translation and thereby leading to a weak production of EBNA1 protein and EBNA1-derived
 5 antigenic peptides. This allows EBNA1 and EBV to evade the immune system. Some G4 ligands like **PhenDC3**
 6 compete with NCL for binding to G4s of EBNA1 mRNA, thereby relieving the inhibitory effect of GAr and NCL on
 7 both translation and antigen presentation. b) Design of cationic bis(acylhydrazone) ligands featuring shape
 8 similarity to **PhenDC3**.

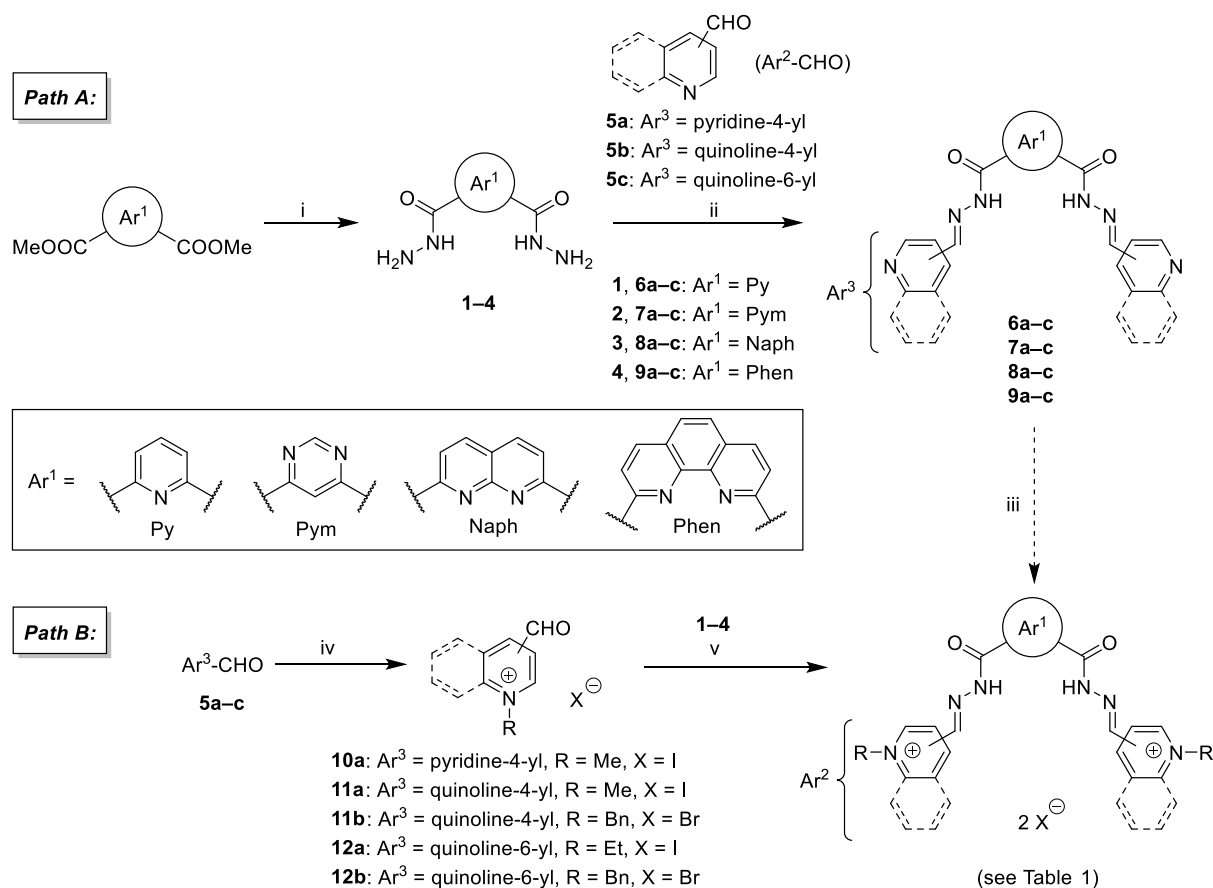
9 With the aim to explore new pharmaceutical scaffolds as putative G4 ligands and disruptors of the
 10 NCL–EBNA1 G4-mRNA interactions, we designed a series of cationic bis(acylhydrazone) derivatives
 11 representing shape analogues of **PhenDC3** (Fig. 1b). These U-shaped scaffolds are expected to provide
 12 an optimal overlap with G-tetrads, as demonstrated by the structural model of **PhenDC3** bound to a
 13 parallel-stranded G4-DNA substrate [23]. In addition, the *N*-acylhydrazone group has been identified
 14 as a privileged scaffold in drug design, due to a combination of hydrogen-bond acceptor and donor
 15 sites capable of interactions with a wide range of biomolecules. Despite the fact that acylhydrazone
 16 derivatives are rapidly metabolized, this motif is encountered in several approved drugs (e.g.,
 17 nifuroxazide and dantrolene) [24] and drug candidates [25–27]. The facile synthetic availability of
 18 acylhydrazone derivatives, which allows furnishing congeneric series of compounds with distinct

1 physico-chemical properties and bioactivities, makes it a promising scaffold in the drug discovery field
2 [28–30]. Herein, we describe the synthesis of novel derivatives and the determination of their *in vitro*
3 and *in cellulo* ability to bind to G4 of EBNA1 mRNA, as well as their effect on NCL-mediated GAR-
4 dependent limitation of EBNA1 expression and antigen presentation.

5 **2. RESULTS**

6 **2.1. Chemistry**

7 The design of cationic bis(acylhydrazones) features a central heterocyclic core Ar¹, i.e., a derivative of
8 pyridine (Py), pyrimidine (Pym), 1,8-naphthyridine (Naph), or 1,10-phenanthroline (Phen), connected
9 via two acylhydrazone linkages to lateral, *N*-substituted cationic heterocycles (Ar² = pyridinium or
10 quinolinium, R = Me, Et, or Bn). Our initial approach to the synthesis of these derivatives relied on the
11 formation of neutral bis(acylhydrazone) precursors, followed by quaternization of lateral heterocyclic
12 residues (Scheme 1, path A). Thus, four heterocyclic bis(acylhydrazides) **1–4**, obtained by a
13 hydrazinolysis of the corresponding dimethyl esters, were made to react with three heteroaromatic
14 aldehydes **5a–c**, to give the 11 corresponding bis(acylhydrazones) **6a–c**, **7a–c**, **8a–c** and **9a–c**, with
15 yields ranging from 55 to 99% (Experimental Part). Subsequent quaternization using excess alkyl or
16 benzyl halide gave the corresponding cationic bis(acylhydrazones). However, while some products
17 (e.g., **PyDH1**, **PyDH2**) could be obtained in a pure form after a single recrystallization, several other
18 ones (e.g., **PymDH1**, **PymDH2**, **NaphDH2**) contained up to 20% (as per ¹H NMR) of mono-alkylated
19 product as an impurity, which could not be removed even after repeated recrystallizations. Therefore,
20 we elaborated an alternative synthesis of these derivatives (Scheme 1, path B), relying on *N*-alkylation
21 of heteroaromatic aldehydes in a first step, and obtained five quaternized aldehydes (**10a**, **11a–b**, **12a–**
22 **b**) isolated as stable, but hygroscopic salts in good yields (73–94%). The subsequent condensation of
23 the latter with bis(acylhydrazides) **1–4** gave a series of 20 cationic bis(acylhydrazones) (**PyDH1–5**,
24 **PymDH1–5**, **NaphDH1–5**, and **PhenDH1–5**, Table 1), all obtained as iodide or bromide salts in good
25 yields (50–97%) and excellent purity after a single recrystallization. The identity and purity of all final
26 compounds were confirmed using spectroscopic and chromatographic methods and combustion
27 analysis.



- 1
- 2
- 3 **Scheme 1.** Synthesis of cationic bis(acylhydrazones). Reagents and conditions: (i) N₂H₄·H₂O, EtOH, reflux, 18 h;
- 4 (ii) EtOH, reflux, 18 h; (iii) RX, DMF, 40 °C or 60 °C, 18 h; (iv) RX, DCM, r.t., 72 h or acetone, reflux, 18 h; (v) DMF,
- 5 80 °C or 100 °C, 2 h.

6 **Table 1.** Structures of novel cationic bis(acylhydrazones)

Ar ¹ \ Ar ²					
	PyDH1	PyDH2	PyDH3	PyDH4	PyDH5
	PymDH1	PymDH2	PymDH3	PymDH4	PymDH5
	NaphDH1	NaphDH2	NaphDH3	NaphDH4	NaphDH5
	PhenDH1	PhenDH2	PhenDH3	PhenDH4	PhenDH5

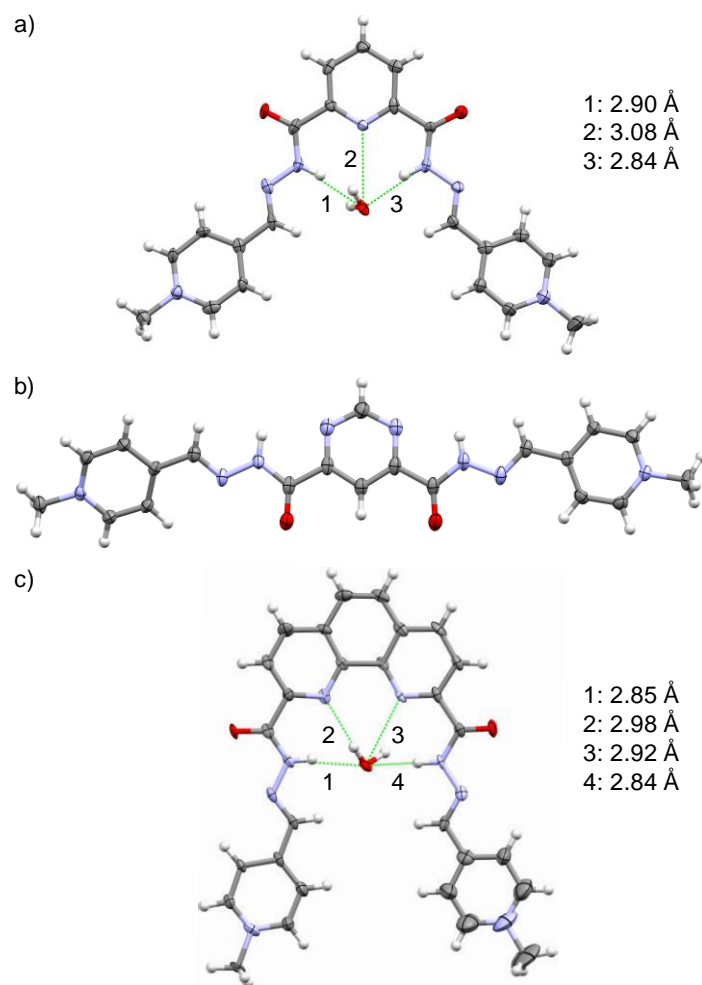
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2 2.2. Structural characterization

3 X-ray quality crystals of **PyDH1** ($2I^-$), **PymDH1** ($2I^-$), and **PhenDH1** ($2I^-$) could be obtained from
4 acetonitrile–water (in the case of **PymDH1** ($2I^-$), a simultaneous formation of yellow and orange
5 polymorphs was observed, both belonging to the $\bar{P}1$ space group but differing by the packing of
6 cations). Single-crystal diffraction analysis revealed that in all three compounds the organic cations
7 were essentially planar, with minimal twisting at the level of terminal heterocyclic rings breaking the
8 two-fold molecular symmetry (Fig. 2). In all cases, both *N*-acylhydrazone groups adopted *E*-
9 *antiperiplanar* conformation. Remarkably, **PyDH1** and **PhenDH1** revealed co-crystallized water
10 molecules located in between the two acylhydrazone groups and forming hydrogen bonds with both
11 amide NH groups (**PyDH1**: $d(\text{NH}\cdots\text{O}) = 2.84$ and 2.90 Å, $\alpha(\text{N-H}\cdots\text{O}) = 150^\circ$; **PhenDH1**: $d(\text{NH}\cdots\text{O}) = 2.85$
12 and 2.84 Å, $\alpha(\text{N-H}\cdots\text{O}) \approx 170^\circ$). In addition, in **PyDH1** the water molecule formed a weak hydrogen
13 bond with the pyridine nitrogen ($d(\text{N}\cdots\text{O}) = 3.08$ Å, Fig. 2, a), whereas in **PhenDH1** it formed a stronger,
14 bifurcated hydrogen bonds with both phenanthroline nitrogens (mean $d(\text{N}\cdots\text{O}) = 2.95$ Å, Fig. 2c). The
15 coordination of water molecules thus determines the inward-folded, V-shaped conformation of
16 **PyDH1**, similar to what was observed with related, charge-neutral pyridine-2,6-bis(acylhydrazones)
17 [31], as well as the U-shaped conformation of **PhenDH1** which is further stabilized due to the number
18 and favorable arrangement of hydrogen bonds. In contrast, **PymDH1** (Fig. 2b) was found to adopt a
19 linear conformation, partially stabilized by intramolecular bonds between the amide NH groups and
20 nitrogen atoms of the pyrimidine core ($d(\text{N}\cdots\text{N}) = 2.66$ Å). Although this compound also crystallized
21 along with water molecules, the latter were found to coordinate to amide CO groups, on one hand,
22 and iodide anions, on the other hand (not shown).

23 It may be suggested that the described interactions with water molecules persist in aqueous solutions,
24 governing the molecular shape of bis(acylhydrazone) ligands. In this context, V- or U-shaped
25 derivatives **PyDHn** and **PhenDHn**, presumably, have a more favorable structure for binding to G4-
26 quadruplex structures due to a more complete overlap with G-tetrads, as compared to linear
27 analogues **PymDHn**.

28

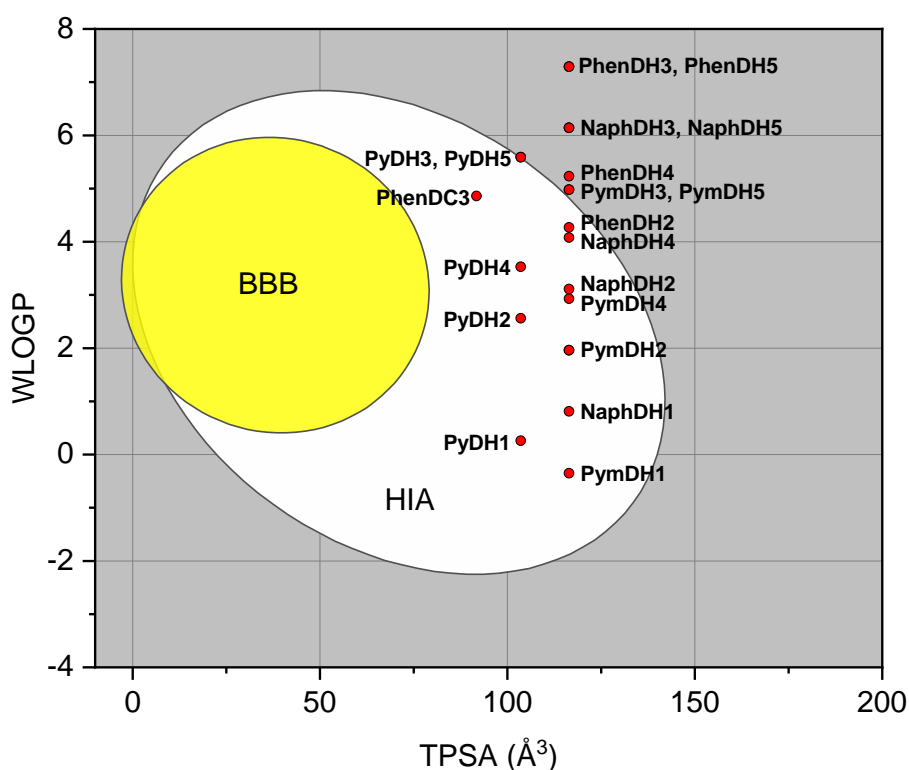


1
2 **Fig. 2.** Solid-state structures of a) **PyDH1** ($2I^-$) \times 2 H_2O , b) **PymDH1** ($2I^-$) \times 2 H_2O \times MeCN (yellow form), and c)
3 **PhenDH1** ($2I^-$) \times 2 H_2O \times MeCN, from single-crystal X-ray diffraction analysis. Non-bound water, acetonitrile
4 molecules and counter-ions were omitted for clarity. CPK atom colors; green lines and labels indicate
5 intermolecular hydrogen bonds with crystallized water molecules and the corresponding N...O distances.

6 **2.3. *In silico* evaluation**

7 The drug-like properties of cationic bis(acylhydrazones) were evaluated using SwissADME, a free tool
8 allowing to assess the physicochemical descriptors, pharmacokinetics (ADME), and drug-likeness of
9 small molecules [32]. It should be noted that certain methods of *in silico* evaluation are poorly suitable
10 for cationic compounds as the ones described in this work, as illustrated by significant variance of
11 physico-chemical properties predicted by different algorithms for the same compound (e.g. for **PyDH1**,
12 cLogP from -7.75 to 1.83 , Table S1). According to the established practices, consensus estimation (i.e.,
13 the average of five models) was used in evaluation of drug-likeness of compounds [32]. The results of
14 *in silico* assessment (Fig. S1 and Table S1) indicated satisfactory bioavailability for most
15 bis(acylhydrazones), as given by the combination of six physicochemical descriptors (lipophilicity,
16 molecular weight, polarity, solubility, saturation and number of rotatable bonds). Among those,
17 insufficient saturation (i.e., low fraction of sp^3 carbons) could be identified as the limiting factor for

1 bioavailability of most derivatives, as well as high molecular weight for the derivatives **NaphDH3**,
2 **NaphDH5**, **PhenDH3** and **PhenDH5** ($M > 700$ Da). With the exception of the latter four derivatives, all
3 bis(acylhydrazones) satisfied the Lipinski's rule, and most derivatives also satisfied the Muegge's rule
4 [33], indicating a high potential to serve as drugs (Table S1). Finally, we employed a gastrointestinal
5 absorption predictor calculated by the BOILED-egg model (Fig. 3) [34]. According to this model, most
6 compounds, again with the exception of **NaphDH3**, **NaphDH5**, and **PhenDH3–5**, had a high rate of
7 passive gastrointestinal absorption; however, none of compounds was expected to cross the blood-
8 brain barrier (Fig. 3). In conclusion, the results of *in silico* evaluation indicate that the cationic
9 bis(acylhydrazone) scaffolds are compatible with their use as RNA-targeting drugs, while heavy and
10 aromatic substitutes (e.g., R = Bn) should be avoided for the sake of bioavailability, unless
11 indispensable for RNA binding.



12
13 **Fig. 3.** BOILED-egg evaluation [34] of passive gastrointestinal absorption (HIA) and brain (BBB) penetration of
14 bis(acylhydrazones). The white region indicates high probability of passive gastrointestinal absorption, and the
15 yellow region indicates high probability of BBB penetration.

16 The compounds were also assessed for the presence of fragments associated with pan-assay
17 interfering compounds (PAINS). The presence of quinolinium fragments was identified as potentially
18 troublesome in some (but not all) compounds (Table S1); however, an inspection of the original screen
19 revealed that this fragment was mainly associated with cationic dyes, potentially interfering with
20 colorimetric or fluorimetric assays [35]. Since bis(acyhydrazones) are colorless or faintly colored

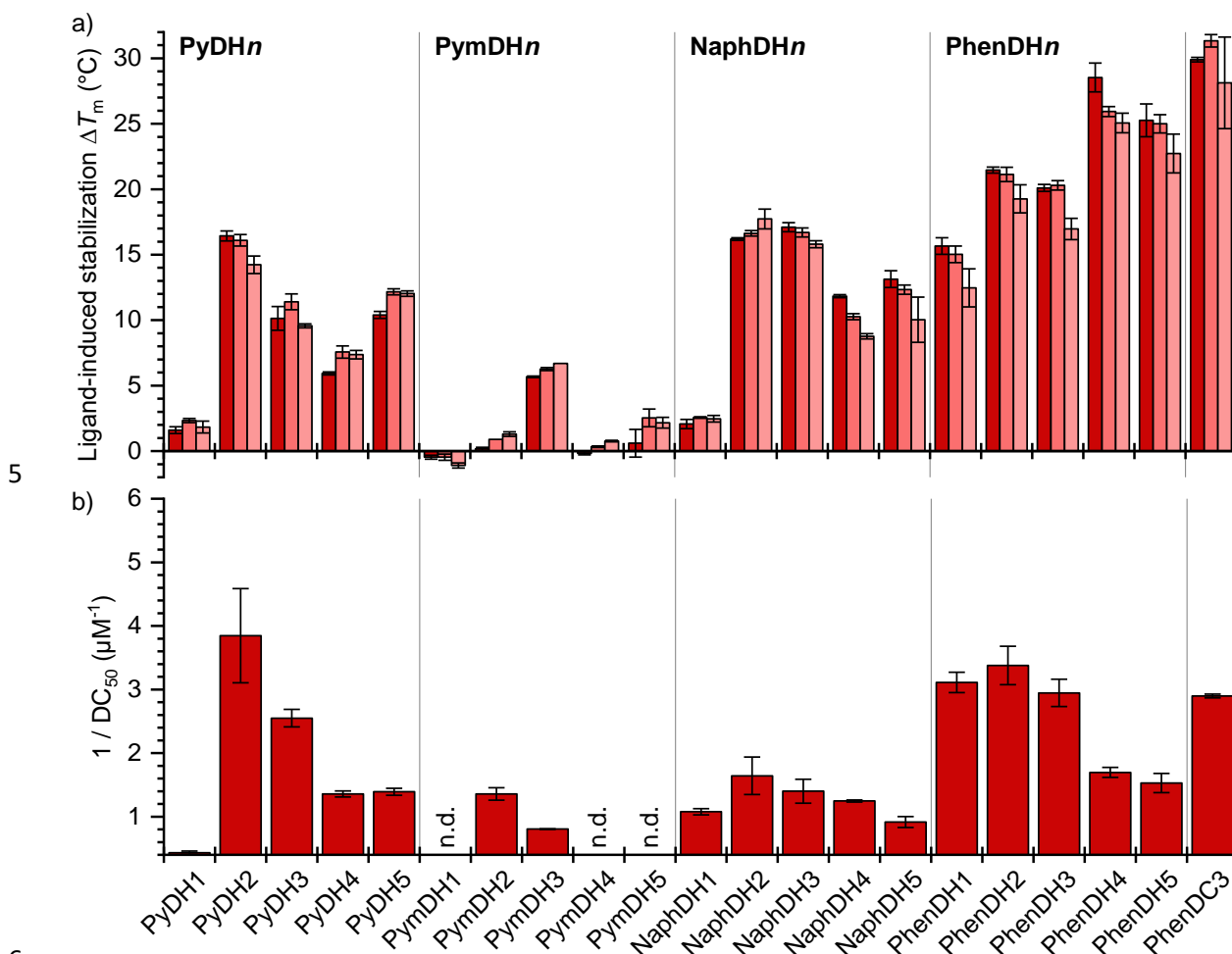
1 compounds, this filter was not applied and all compounds were systematically assessed by biophysical
2 and biological tests.

3 **2.4. Biophysical studies of interaction of novel ligands with G4 of EBNA1 mRNA and G4-DNA**

4 The interaction of novel bis(acylhydrazone) derivatives with the most frequent G4-forming motif
5 encountered in GAr-encoding sequence of the EBNA1 mRNA (g4-EBNA1, 5'-r(GGGGCAGGA-
6 GCAGGAGGA)-3') was studied *in vitro* using two widely established methods, namely fluorescence-
7 monitored thermal denaturation (fluorescence melting) and fluorescent indicator displacement (FID)
8 assay. In the first method, the binding of ligands is manifested by an increase of denaturation
9 temperature (ΔT_m) of oligoribonucleotide F-g4-EBNA1-T, bearing a fluorophore (5', 6-FAM) and a
10 quencher (3', TAMRA). In our conditions (10 mM KAsO₂Me₂, 10 mM KCl, 90 mM LiCl buffer, pH 7.3)
11 and in the absence of ligands, F-g4-EBNA1-T denatured at $T_m^0 = 58.6$ °C, which is slightly higher than
12 the reported value (54 °C) [7]. In addition to the ranking of ligands with respect to their ability to
13 stabilize the G4 substrate, fluorescence melting experiments provide information on G4-RNA vs. ds-
14 DNA selectivity of ligands, namely through analysis of the drop of ΔT_m values observed in the presence
15 of unlabeled, double-stranded DNA competitor (ds26, 15 or 50 molar equivalents) [36]. The results
16 (Fig. 4, a) demonstrate that bis(acylhydrazone) derivatives display great variability with respect to their
17 capacity to bind to and stabilize g4-EBNA1, revealing interesting structure–activity relationships
18 discussed in details below. Specifically, most derivatives of the PyDH and NaphDH families, as well as
19 PhenDH1, demonstrated significant stabilization of g4-EBNA1 ($\Delta T_m = 10$ to 20 °C), and further
20 phenanthroline derivatives (**PhenDH2–5**) demonstrated even higher stabilization of the substrate (ΔT_m
21 = 20 to 30 °C), comparable to the result obtained with **PhenDC3** ($\Delta T_m = 30.0$ °C). In contrast, all
22 derivatives of the PymDH family, as well as compounds **PyDH1** and **NaphDH1**, demonstrated low or
23 very low stabilization of G4-RNA ($\Delta T_m < 10$ °C), illustrating the importance of the nature of heterocyclic
24 residues on the G4-RNA binding properties of ligands. Finally, most derivatives that stabilized g4-
25 EBNA1 also displayed significant level of selectivity with respect to ds-DNA, as their stabilizing effect
26 was almost unaffected by the presence of ds-DNA competitor.

27 To assess the selectivity of novel ligands for G4 of EBNA1 mRNA, additional fluorescence melting
28 experiments were performed with two G4-DNA substrates, namely F-24TTG-T (predominantly a
29 hybrid-1 G4 structure, adopted by a variant of human telomeric DNA sequence) [37] and F-myc22-T
30 (parallel-stranded G4-DNA, adopted by variant of the G4-forming sequence from the promoter region
31 of *c-Myc* oncogene) [38]. The results (Fig. S2) demonstrate no preferential stabilization of one or
32 another substrate, since all compounds that stabilized g4-EBNA1 (**PyDH2–5**, **NaphDH2–5**, and all
33 PhenDH derivatives) also strongly stabilized both G4-DNA substrates (with $\Delta T_m = 15$ to 30 °C). A similar
34 behavior was observed with PhenDC3, which stabilized both G4-DNA with ΔT_m of over 35 °C. The lack

1 of selectivity is not surprising given the structural similarity of novel ligands to **PhenDC3** (a polyvalent
 2 G4 binder) and indicates a similar binding mode, relying on compound stacking with terminal G-tetrads
 3 of G4 structures. Obviously, the substituents explored in this work (ethyl or phenyl groups) are not
 4 sufficient for achieving a significant level of selectivity between different G4 structures.



6
 7 **Fig. 4.** *In vitro* binding of tested compounds to g4-EBNA1. a) Thermal stabilization F-g4-EBNA1-T (0.2 μM) by
 8 tested compounds (1.0 μM), assessed by fluorescence melting experiments in the absence (dark red bars) or in
 9 the presence of duplex DNA competitor ds26 (red bars: 3 μM, pale red bars: 10 μM). The experiments were
 10 performed in K10 buffer (10 mM LiAsO₂Me₂, 10 mM KCl, 90 mM LiCl, pH 7.3); data are means ± s.d. from three
 11 technical replicates. b) Ligand-induced displacement of TO (0.5 μM) from g4-EBNA1 (0.25 μM). Data are means
 12 ± s.d. from three technical replicates; n.d. = no displacement (DC₅₀ > 2.5 μM). The experiments were performed
 13 in K100 buffer (10 mM LiAsO₂Me₂, 100 mM KCl, 1 mM EDTA, 1% v/v DMSO).

14 A complementary information about ligand affinity for g4-EBNA1 was obtained from the FID assay,
 15 which assesses the binding of ligands through displacement of a fluorescent probe (Thiazole Orange,
 16 TO). In this assay, the apparent ligand affinity is given by the concentration required to displace 50%
 17 of the bound probe (DC₅₀) [39]. The results of the FID assay (Fig. 4b and Table S2) indicated high g4-
 18 EBNA1 affinity (DC₅₀ < 0.5 μM) for compounds **PyDH2**, **PyDH3**, as well as **PhenDH1–3**, which was

1 comparable to the result obtained for **PhenDC3** ($DC_{50} = 0.31 \mu\text{M}$). In contrast, the derivatives **PyDH1**,
 2 **PymDH1**, **PymDH4** and **PyDH5** were not able to displace TO from g4-EBNA1 ($DC_{50} > 2 \mu\text{M}$), giving
 3 evidence of low affinity, whereas other derivatives demonstrated moderate affinity ($DC_{50} = 0.5$ to
 4 $2 \mu\text{M}$). Of note, none of tested ligands was able to induce displacement of TO from the double-
 5 stranded DNA substrate ds26 ($DC_{50} > 2.5 \mu\text{M}$ in all cases). Globally, the results obtained with both
 6 biophysical methods were in a good agreement, except for a few discrepancies (compounds **PyDH5**,
 7 **NaphDH2–5** and **PhenDH4–5** which demonstrated high thermal stabilization of g4-EBNA but only
 8 moderate capacity to displace TO, $DC_{50} = 0.5$ to $1 \mu\text{M}$). Altogether, the results of biophysical studies
 9 point to pyridine derivatives **PyDH2** and **PyDH3**, as well as all derivatives of the phenanthroline series,
 10 as most promising ligands for g4-EBNA.

11 2.5. Biological tests

12 2.5.1. Effect of compounds on GAR-dependent protein expression

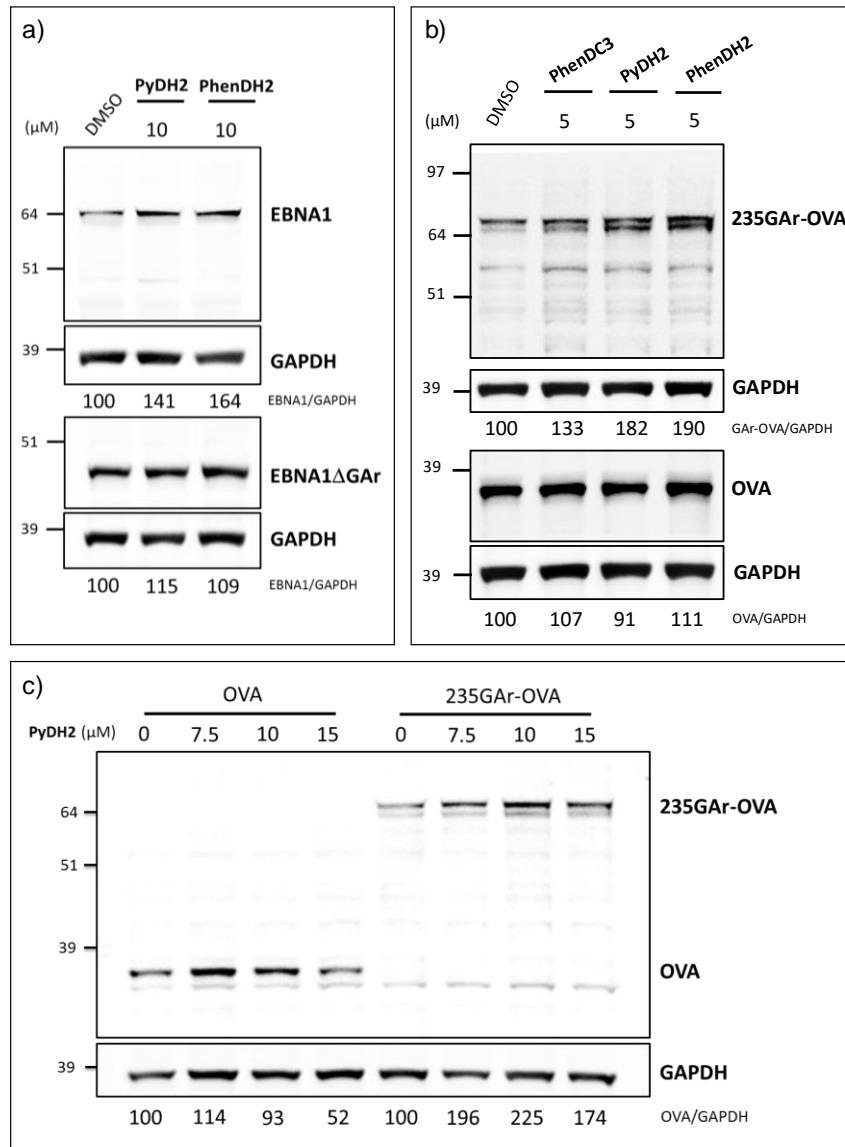
13 To assay the biological activity of the various compounds bis(acylhydrazones), we first determined
 14 their effect on the GAR-dependent limitation of EBNA1 expression, which represents a mechanism at
 15 the basis of EBNA1/EBV immune evasion. For this purpose, human lung carcinoma H1299 cells were
 16 transfected with EBNA1 or EBNA1 Δ GAr constructs and treated with $10 \mu\text{M}$ of the indicated compounds
 17 or, as a control, with DMSO (the vehicle). Then, the levels of EBNA1 or EBNA1 Δ GAr was assessed by
 18 western blot analysis using an antibody raised against EBNA1 and, as a loading control, an antibody
 19 raised against GAPDH. **PhenDC3** was used as a positive control and DMSO as a negative control. In this
 20 assay, three types of results were observed (Table 2): (i) most of the compounds had no effect
 21 (indicated by a “0”); (ii) two compounds, namely **PyDH2** and **PhenDH2**, similarly to **PhenDC3**, led to a
 22 GAR-dependent increase in EBNA1 expression, indicated by a “+” (i.e., they increased EBNA1 level while
 23 having no effect on EBNA1 Δ GAr); and finally (iii) three compounds (**PyDH3**, **PymDH5**, and **PhenDH1**)
 24 increased EBNA1 level in a GAR-independent manner indicated by a “x” (i.e., they increased both
 25 EBNA1 and EBNA1 Δ GAr levels). The western blot results obtained with compounds **PyDH2** and
 26 **PhenDH2** are presented on Fig. 5a. As we were interested in compounds able to interfere with the
 27 GAR-dependent limitation of EBNA1 expression and antigen presentation, a mechanism at the basis of
 28 EBNA1 immune evasion, we focused only on the compounds that increase EBNA1 level while having
 29 no effect on EBNA1 Δ GAr, namely **PhenDH2** and **PyDH2**.

30 **Table 2.** Effect of compounds (all tested at $5 \mu\text{M}$) on EBNA1 expression in H1299 cells.

Compound	Effect ^a	Compound	Effect ^a	Compound	Effect ^a	Compound	Effect ^a
PyDH1	0 (106/93) ^b	PymDH1	0 (80/92) ^b	NaphDH1	0 (112/99) ^b	PhenDH1	x (78/76) ^{b,c}
PyDH2	+ (162/101) ^b	PymDH2	0 (89/103) ^b	NaphDH2	0 (95/100) ^b	PhenDH2	+ (189/96) ^b

PyDH3	× (134/149) ^b	PymDH3	0 (108/106) ^b	NaphDH3	0 (106/93) ^b	PhenDH3	0 (96/114) ^b
PyDH4	0 (86/98) ^b	PymDH4	× (48/47) ^{b,c}	NaphDH4	0 (82/111) ^b	PhenDH4	0 (80/96) ^b
PyDH5	× (58/70) ^{b,c}	PymDH5	×(139/137) ^b	NaphDH5	0 (104/108) ^b	PhenDH5	0 (111/94) ^b

1 ^a 0: No effect ; +: GAR-dependent increase in EBNA1 expression; ×: GAR-independent effect. ^b Number between
2 brackets indicate the quantification of respectively GAR-OVA (numerator) and OVA (denominator) levels, as
3 compared to their respective levels in DMSO-treated cells. ^c Values in italics, which are significantly smaller than
4 100, point to compounds that are toxic to both GAR-OVA- and OVA-expressing cells.



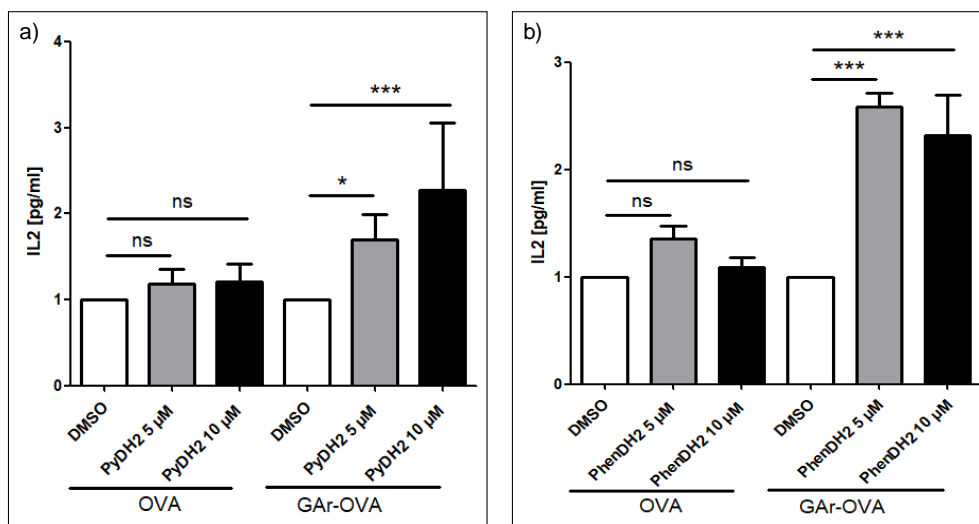
5
6 **Fig. 5.** a) Expression of EBNA1 (top panel) or EBNA1ΔGAR (bottom panel) in transfected H1299 cells treated with
7 DMSO (control), **PyDH2** (10 μM), or **PhenDH2** (10 μM) 40 h post-transfection. b) Expression of 235GAR-OVA (top
8 panel) or OVA (bottom panel) in transfected H1299 cells treated with DMSO (control), **PhenDC3**, **PyDH2**, or
9 **PhenDH2** (all at 10 μM) 40 h post-transfection. c) Expression of 235GAR-OVA or OVA in transfected H1299 cells
10 treated with **PyDH2** used at 0 (control), 7.5, 10, or 15 μM concentration. Protein (EBNA1, EBNA1ΔGAR, 235GAR-

1 OVA or OVA) levels were normalized with respect to GAPDH (loading control) and the resulting values indicated
2 below the gels.

3 On the basis of the compounds effect on the expression of EBNA1 and *in silico* evaluation of their drug-
4 likeness, **PyDH2** and **PhenDH2** were selected for further biological studies that include antigen
5 presentation assay and proximity ligation assay (PLA). For this purpose, we first evaluated the effect
6 of various concentrations of both compounds on expression of ovalbumin (OVA) and 235GAR-OVA in
7 H1299 cells using the same procedure as described above for EBNA1. The OVA/235GAR-OVA system
8 allows to assess the ability of GAR to limit both protein expression and antigen presentation since
9 235GAR (a full-length, 235 amino-acid GAR domain), when fused to OVA, strongly limits both its
10 expression and its antigen presentation by the MHC class I pathway. In this way, this model
11 recapitulates the effect of GAR on EBNA1 expression and antigen presentation [18]. Similar to what has
12 been observed with **PhenDC3** [22], compounds **PyDH2** and **PhenDH2** increased 235GAR-OVA level in a
13 dose-dependent manner, whereas having no significant effect on OVA expression (Fig. 5b,c). Together
14 with the data on the effect of compounds on EBNA1 expression, these results confirm the suggested
15 mechanism of biological activity of both compounds, namely, relieving of the GAR-dependent inhibition
16 of protein expression.

17 **2.5.2 Antigen presentation assay**

18 We subsequently studied the ability of compounds **PyDH2** and **PhenDH2** to interfere with immune
19 evasion of EBV, via the assessment of the GAR-dependent suppression of antigen presentation. For this
20 purpose, H1299 cells were transfected with murine MHC class I Kb and 235GAR-OVA or OVA plasmid
21 constructs and co-cultured with naive OVA₂₅₇₋₂₆₄ (SL8 peptide)-specific CD8⁺ T-cells from OT-1 mice.
22 Since IL-2 is a potent growth factor stimulating T-cell proliferation, the levels of antigen presentation
23 were determined by measuring the release of IL-2 in the supernatant by ELISA. IL-2 release following
24 the treatment of cells with compounds **PyDH2** or **PhenDH2**, as compared to DMSO-treated cells (Fig.
25 6). These data indicate that **PyDH2** used at a concentration of 10 μ M, as well as **PhenDH2** used at a
26 concentration of 5 or 10 μ M significantly increased the presentation of antigenic peptides from GAR-
27 OVA (more than a two-fold increase), while having no effect on cells expressing OVA. Thus, both
28 compounds increased antigen presentation levels in a GAR-dependent manner, demonstrating their
29 ability to significantly interfere with the immune evasion of EBNA1.



1
 2 **Fig. 6.** IL2 concentration (pg mL⁻¹) determined following the treatment of H1299 cells expressing OVA (left) or
 3 GAR-OVA (right) with a) DMSO (control), 5 μM and 10 μM of **PyDH2**; b) DMSO (control), 5 μM and 10 μM of
 4 **PhendH2**. *, $p < 0.05$; ***, $p < 0.001$; ns, not significant.

5 2.5.3. Cell viability

6 The toxicity of compounds **PyDH2** and **PhendH2** for Mutu-1 (Epstein-Barr virus-related Burkitt
 7 lymphoma) cells in a concentration range of 0.5 to 100 μM was assessed using the classical MTT assay
 8 and compared to the result obtained with cells treated by DMSO (compound vehicle). Upon 24 h
 9 treatment, both compounds displayed a relatively low toxicity for Mutu-1 cells (**PyDH2**: $GI_{50} > 100$ μM,
 10 **PhendH2**: $50 < GI_{50} < 100$ μM, Fig. 7). Moreover, the toxicity of both compounds was only slightly
 11 higher upon 48 h treatment (**PyDH2**: $GI_{50} \geq 100$ μM, **PhendH2**: $GI_{50} \approx 20$ μM, cf. Supporting
 12 Information, Fig. S3). Thus, both compounds are not significantly toxic when used at a concentration
 13 range in which they increase the expression of EBNA1 and the production of EBNA1-derived antigenic
 14 peptides (i.e., 5–10 μM), in contrast to the prototype drug **PhenDC3**, which induced significant toxicity
 15 when used at concentrations > 2.5 μM in identical conditions [22]. Thus, as compared to the prototype
 16 G4 ligand **PhenDC3**, these novel acylhydrazone ligands present the advantage of being significantly
 17 less toxic.

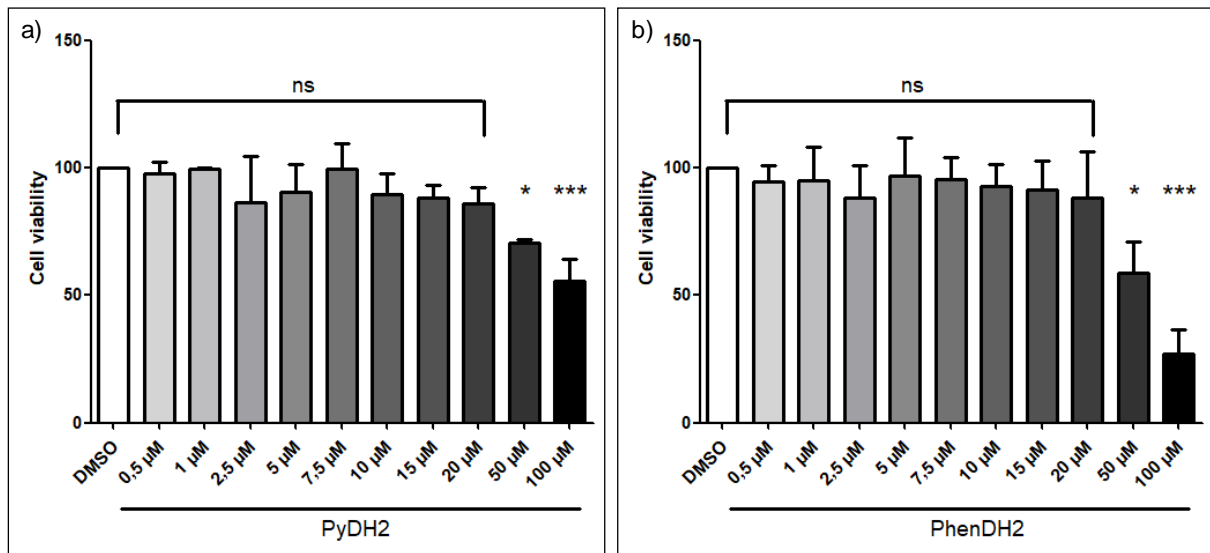


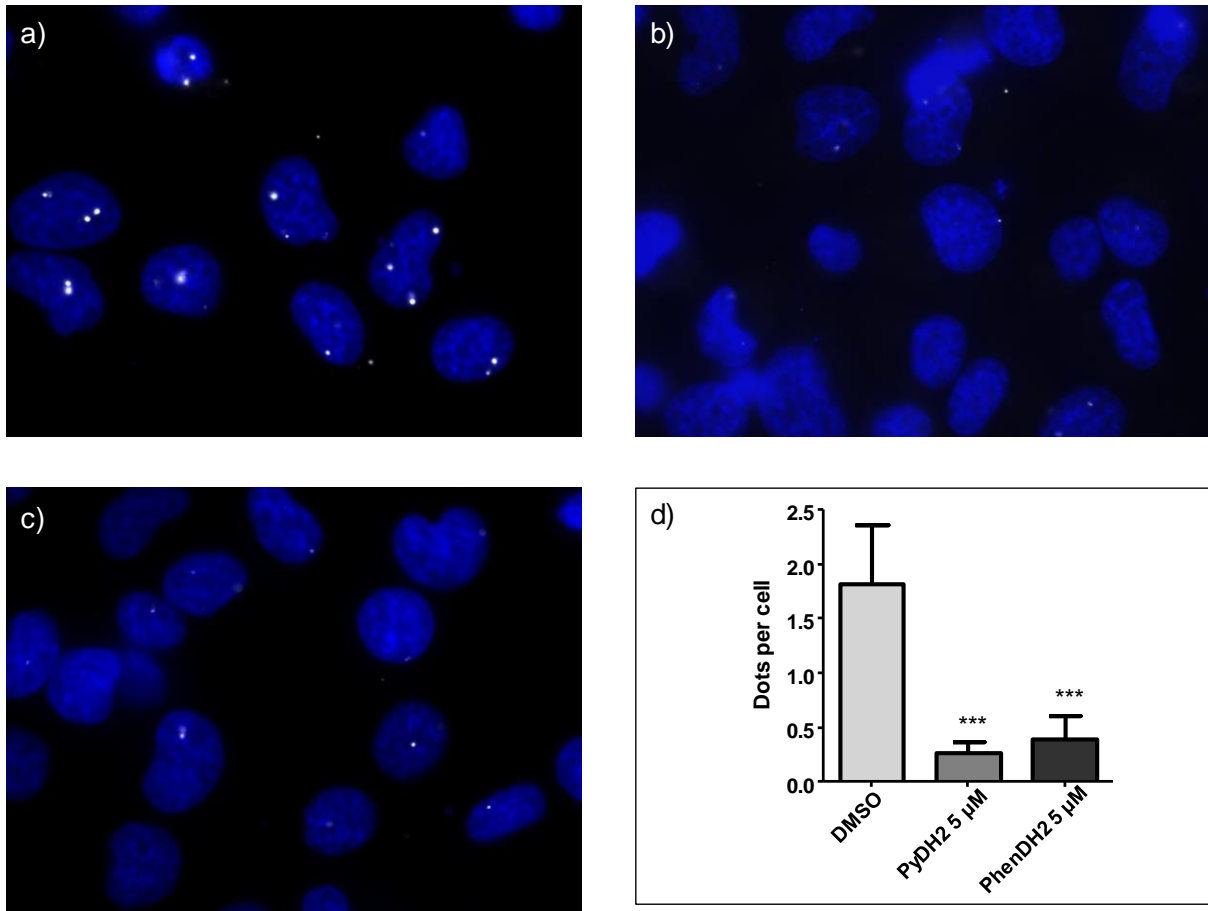
Fig. 7. Viability (%) of Mutu-1 cells assessed after a 24 h treatment with DMSO (control) or various concentrations of a) **PyDH2** or b) **PhenDH2**. *, $p < 0.05$; ***, $p < 0.001$; ns, not significant.

2.5.4. Inhibition of NCL binding to G4 in EBNA1 mRNA: proximity ligation assay (PLA) and RNA pull-down assay

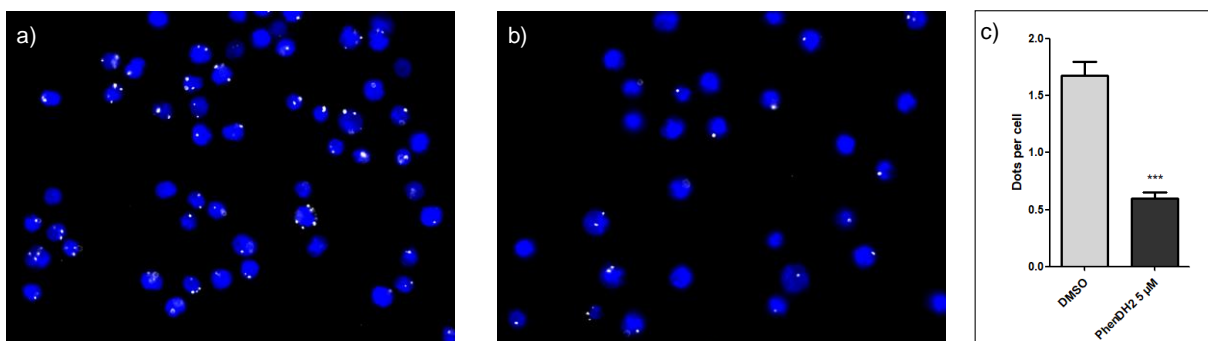
We employed the proximity ligation assay (PLA) [40] to verify that compounds **PyDH2** and **PhenDH2** prevent nucleolin (NCL) to interact with the G4s of the GAR-encoding sequence of EBNA1 mRNA, the mechanism at the basis of the GAR-dependent limitation of EBNA1 expression and antigen presentation. Briefly, PLA is a technique originally developed to detect proteins in close proximity (theoretically, at a maximum distance of 40 nm) and which is based on the use of a pair of antibodies raised in two different species, each targeting one of the two protein of interest. By using labelled oligomers, PLA has been adapted to the study of protein–DNA [41] and protein–RNA interactions. In the latter case, mRNA of interest (i.e., EBNA1) is tagged through *in situ* hybridization with a digoxigenin-labelled DNA probe, followed by incubation with a mouse anti-digoxigenin, whereas NCL is tagged with rabbit anti-nucleolin. If both are located in a close proximity, the subsequent incubation with DNA probe-conjugated anti-rabbit (*plus* probe) and anti-mouse (*minus* probe) antibodies and two connector oligonucleotide probes induces rolling circle amplification, as schematically shown on Fig. S4 (Supporting Information). The latter generates a concatemeric DNA product, which is finally detected through hybridization with a fluorescently labelled oligonucleotide probe as a distinct bright spot on a micrograph [42]. This way, we previously demonstrated that NCL interacts with G-quadruplexes formed in the GAR-encoding sequence of EBNA1 mRNA, and that **PhenDC3** inhibits this interaction in Mutu-1 cells as well as in H1299 cells transiently expressing EBNA1 [22,42]. Herein, we first exploited H1299 cells transiently expressing EBNA1 following transfection with EBNA1 plasmid. Cells treated with DMSO control demonstrated high level of PLA signals (1.83 ± 0.54 per cell) further confirming that the EBNA1 mRNA–NCL interaction takes place in, or at the close vicinity of the nucleus

1 (Fig. 8a), whereas non-transfected cells did not display PLA signals (Supplementary Fig. S5). Treatment
2 with compounds **PyDH2** or **PhenDH2** both at a concentration of 5 μ M significantly reduced both the
3 number (0.27 ± 0.11 and 0.40 ± 0.21 per cell, in cells treated with **PyDH2** and **PhenDH2**, respectively)
4 and the intensity of PLA signals (Fig. 8b–d), hence confirming the ability of these new derivatives to
5 disrupt the EBNA1 mRNA–NCL interaction, *in fine* leading to the GAR-dependent inhibition of EBNA1
6 expression and presentation of EBNA1-derived antigenic peptides. A similar effect was observed in
7 Mutu-1 cells (EBV-infected Burkitt lymphoma B cells expressing endogenous EBNA1): treatment with
8 5 μ M **PhenDH2** decreased the average number of PLA signals per cell from 1.68 ± 0.44 to 0.59 ± 0.20
9 (Fig. 9 and Supplementary Fig. S6).

10 Finally, to confirm that both hit compounds interfere with NCL binding to G4 structures in EBNA1
11 mRNA, we performed *in vitro* RNA pull-down assay using biotin-tagged RNA oligonucleotide g4-EBNA1
12 and recombinant NCL. Experiments performed in the presence of **PyDH2** or **PhenDH2** (10 μ M)
13 demonstrated that both compounds reduced the amount of RNA-bound NCL almost 10-fold with
14 respect to DMSO control (Fig. 10a); in identical conditions, **PhenDC3** employed at the same
15 concentration reduced the amount of bound NCL only two-fold [22]. Of note, negligible binding of NCL
16 was detected with a non-G4-forming RNA oligonucleotide GM (obtained from g4-EBNA1 by
17 replacement of guanines critical for G4 formation with A or U residues), demonstrating that RNA
18 binding of NCL is G4-specific (Fig. 10b). Collectively, these results strongly suggest that **PyDH2** and
19 **PhenDH2**, similarly but more efficiently than **PhenDC3**, act by preventing NCL to interact with G4s that
20 form in the GAR-encoding sequence of EBNA1 mRNA.

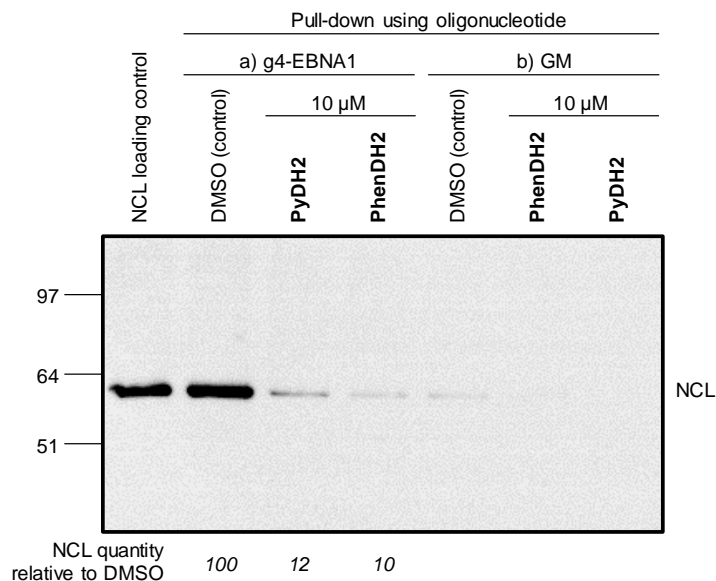


1
 2 **Fig. 8.** Proximity ligation assay (PLA) performed in H1299 cells transiently expressing EBNA1. a–c) Microscopy
 3 images of cells treated with a) DMSO (compound vehicle, negative control), b) **PyDH2** (5 μM), and c) **PhenDH2**
 4 (5 μM). Nuclei were revealed by DAPI staining and appear in blue; white dots (PLA signals) indicate interaction
 5 between NCL and G4 of EBNA1 mRNA. As previously observed, this interaction mostly takes place in, or at the
 6 close vicinity of the nucleus [22,42]. d) Number of nuclear PLA signals (dots) per cell in H1299 cells expressing
 7 EBNA1 and treated with DMSO (control), **PyDH2** (5 μM) or with **PhenDH2** (5 μM). Data from two biological
 8 replicates, 100 cells per sample were analyzed. ***, $p < 0.001$.



9
 10 **Fig. 9.** Proximity ligation assay (PLA) performed in Mutu-1 cells natively expressing EBNA1. a–b) Microscopy
 11 images of cells treated with a) DMSO (compound vehicle, negative control) and b) **PhenDH2** (5 μM). Nuclei were
 12 revealed by DAPI staining and appear in blue; white dots (PLA signals) indicate interaction between NCL and G4

1 of EBNA1 mRNA. c) Number of nuclear PLA signals (dots) per cell in Mutu-1 cells treated with DMSO (control) or
 2 with **PhenDH2** (5 μ M). Data from two biological replicates, ≥ 200 cells per sample were analyzed. ***, $p < 0.001$.



3
 4 **Fig. 10.** RNA pull-down assay to identify the capacity of ligands to prevent NCL binding to G4-RNA. Recombinant
 5 NCL was applied to streptavidin-coupled agarose beads and biotin-tagged RNA oligonucleotides: a) g4-EBNA1; b)
 6 GM (non-G4-forming 18-mer sequence) in the presence of **PyDH2** or **PhenDH2** (at a concentration of 10 μ M) or
 7 DMSO (vehicle) as indicated. The NCL protein still bound to the beads after washing with 800 mM KCl was eluted
 8 (SDS buffer) and analyzed by SDS-PAGE and western blot.

9 3. DISCUSSION

10 3.1. *In vitro* binding to g4-EBNA1: structure–properties relationships

11 A systematic comparison of 20 derivatives belonging to the same bis(acylhydrazone) family allowed us
 12 to reveal several interesting relationships between the structure of ligands and their affinity to g4-
 13 EBNA1. Firstly, the core heterocycle (Ar^1) proved to play a crucial role, as all PymDH derivatives
 14 exhibited poor ability to bind and stabilize g4-EBNA1, as demonstrated by the results of both
 15 biophysical techniques (FID assay and fluorescence melting). As evidenced by X-ray structural analysis
 16 (Fig. 2), this is due to the extended linear shape of PymDH derivatives, which ostensibly hampers their
 17 efficient stacking with G-quartets. Considering the whole set of ligands, the following trend can be
 18 deduced with respect to the impact of Ar^1 on G4-binding properties: PhenDH > NaphDH \approx PyDH \gg
 19 PymDH. A similar trend, albeit not comprising pyrimidine derivatives, was also observed in other series
 20 of related cationic dicarboxamide derivatives [43,44]. Secondly, within each sub-family of ligands,
 21 derivatives with lateral pyridinium residues (i.e., **PyDH1**, **PymDH1**, **NaphDH1**, and **PhenDH1**)
 22 systematically demonstrated less efficient binding to G4 structures, in contrast to the derivatives
 23 bearing lateral quinolinium heterocycles. Consistently with literature data on related derivatives [43],

1 this fact can be attributed to the limited π -stacking surface of pyridinium derivatives. With respect to
2 the substitution pattern of lateral quinolinium rings, 4-substituted derivatives (**PyDH3** etc.) appeared
3 more efficient g4-EBNA1 binders than analogous, 6-substituted derivatives (**PyDH5** etc.) according to
4 the FID assay, but this trend was not supported by the results of the fluorescence-melting assay.
5 Finally, side-chain substituents (R) seem to have little influence on G4-binding properties, even though
6 their impact could not be comprehensively evaluated due to the limited size of our combinatorial
7 matrix. On the other hand, the nature of these substituents plays a crucial role for bioavailability of
8 bis(acylhydrazones), with benzyl substituents are detrimental to drug-like properties due to imparted
9 high molecular weight and insaturation. Of note, the same structure-properties relationships were
10 observed with respect to ligand-induced stabilization of two G4-DNA substrates, myc22 (parallel G4-
11 DNA) and 25TAG (hybrid G4-DNA), demonstrating the polyvalence of G4-binding properties of the
12 novel ligands. Thus, the *N*-acylhydrazone group appears to be essentially as efficient as the
13 carboxamide group in the design of cationic poly-heteroaromatic G4 ligands, as can be evidenced from
14 comparison of the results obtained with **PhenDC3** and its acylhydrazone analogue **PhenDH2** (the
15 former appears as a slightly more efficient binder in fluorescence melting assay, but not in the FID
16 assay). Taken together, these data demonstrate that cationic bis(acylhydrazones) represent a
17 promising scaffold for G4-DNA and G4-RNA binders, providing wide possibilities for further
18 functionalization and modulation of physico-chemical properties.

19 **3.2. Effect of compounds on the GAR-dependent synthesis of EBNA1 and immune evasion of EBV**

20 Among the 20 tested bis(acylhydrazones, **PyDH2** and **PhenDH2** were found to increase EBNA1
21 expression in a GAR-dependent manner in H1299 cells (Table 2). Remarkably, both compounds
22 demonstrated high affinity to g4-EBNA1 according to two biophysical methods (fluorescence melting
23 and FID assay, Fig. 4). These results speak in favor of the expected mechanism for their biological
24 activity, namely, interference with the NCL-based inhibition of mRNA translation by preventing the
25 interaction between NCL and G4 structures in EBNA1 mRNA, as further confirmed by PLA and RNA pull-
26 down assays. Considering the absence of selective targeting of G4-RNA with respect to G4-DNA *in vitro*,
27 the cellular activity of the two compounds can be attributed to the fact that GAR repeat of EBNA1
28 mRNA contains a cluster of multiple (≈ 13) G4-forming sequences, which may be particularly
29 susceptible to ligand-induced effects such as NCL displacement. Moreover, G4-DNA structures form
30 only transiently during DNA transactions such as replication, transcription and recombination, and are
31 otherwise disfavored in the double-stranded DNA context; this is not the case with RNA whose single-
32 stranded nature favors the formation of long-lived secondary motifs such as G4. PLA experiments
33 provided further support this mechanism, demonstrating the ability of compounds to disrupt the
34 interaction between NCL and the GAR-encoding sequence of EBNA1 mRNA in cells. Consistently, both

1 compounds were found to increase expression of 235GAR-OVA and the level of antigen presentation
2 in the GAR-OVA model, while having no effect on cells expressing OVA. Considering their low intrinsic
3 toxicity with respect to Mutu-1 cells (**PyDH2**: $GI_{50} > 100 \mu\text{M}$, **PhenDH2**: $50 > GI_{50} > 100 \mu\text{M}$), both
4 compounds may be employed in order to overcome the immune evasion of EBV through reversion of
5 NCL and GAR-based repression of EBNA1 expression.

6 Interestingly, four naphthyridine derivatives (**NaphDH2–5**) demonstrated significant stabilization of
7 g4-EBNA1, according to fluorescence melting experiments (Fig. 4a), but had not influence on EBNA1
8 expression (Table 2). This behavior is not without precedent, as it was also observed with pyridostatin
9 (PDS), another well-studied G4 binder [22], and indicates that ligand-induced stabilization of a G4
10 structure is not a prerequisite to prevent its interaction with NCL by a competitive mechanism. In fact,
11 one can imagine that stabilization of G4 structure could even favor their interaction with NCL (cf. Fig.
12 S3, Supporting Information). Hence, G4 stabilization and ability to interfere with NCL–G4 interaction
13 may be two independent events. In line, all NaphDH derivatives were significantly less active in
14 displacing the fluorescence probe TO from g4-EBNA1 ($DC_{50} > 0.5 \mu\text{M}$), in contrast to the two biologically
15 active derivatives, **PyDH2** and **PhenDH2** (Fig. 4b). This also indicates that FID assay is potentially better
16 suited for identification of G4-targeting drugs acting through interference with protein binding to G4
17 structures. Therefore, the different compounds described here, in addition to constitute promising
18 scaffolds for drugs able to unveil EBV-related tumors to the immune system and more generally to
19 interfere with G4-RNA binders, may also represent useful tools to decipher the mode of interaction
20 between cellular factors and G4.

21 The final aspect of his works is related to the potential applications of G4 ligands in the context of EBV-
22 related cancers. Indeed, since most of EBV-infected individuals does not develop EBV-linked cancers,
23 it is important to consider the putative effect of drugs on the virus latency, irrespectively of the obvious
24 application for treating cancers associated to EBV infection. However, in healthy individuals, the latent
25 infection by EBV is primarily restricted to a specific small pool of memory B cells. Hence, overcoming
26 the GAR-based inhibition of EBNA1 translation, in addition to unveil EBV-carrying tumor cells to
27 cytotoxic T cells, should also unveil just a few non-tumoral cells, therefore having no effect on the vast
28 majority of healthy host cells. The second point is that, because our compounds should unveil all EBV-
29 infected cells to the immune system (given that EBNA1 is expressed in all EBV-infected cells as it is
30 essential for viral genome replication and maintenance), they would most certainly have applications
31 as anti-viral agents, thereby probably presenting interesting medical application in fields other than
32 EBV-related cancers. For example, they may find applications in grafted patients that all receive
33 immunosuppressive treatments after the graft, which may lead to reactivation of EBV thereby leading

1 to lympho-proliferation. In these patients, elimination of EBV-infected cells prior to the graft may thus
2 present a clear therapeutic interest.

3 **4. CONCLUSION**

4 In this work, we designed and synthesized a novel series of cationic derivatives representing
5 bis(acylhydrazone) analogues of the well-studied G-quadruplex ligands PDC (360A) and **PhenDC3**. We
6 demonstrated that harnessing the bis(acylhydrazone) motif allows facile generation of series of
7 derivatives differing in terms of their physico-chemical properties, drug-like character, G4-binding
8 properties, and biological activity. Specifically, our results demonstrate that the acylhydrazone group
9 does not significantly impart the G4-binding properties of compounds, as compared with carboxamide
10 analogues: instead, the binding to G4 structures seems mostly governed by the nature of central and
11 lateral heterocyclic residues, revealing interesting SARs that can be interpreted in terms of molecular
12 structure and preorganization of ligands. Even though the modifications of the scaffold explored in this
13 work were not sufficient to achieve preferential targeting of viral G4-RNA with respect to G4-DNA *in*
14 *vitro*, the results of biological assays demonstrate that two compounds, namely **PyDH2** and **PhenDH2**,
15 displayed promising biological activity in EBV-related cellular models, being able to interfere with the
16 GAR-dependent limitation of protein expression and antigen presentation. Moreover, both compounds
17 were significantly less toxic than the prototype drug **PhenDC3** when used at concentrations required
18 for boosting the production of EBNA1-derived antigenic peptides (i.e., 5–10 μ M). Therefore, these
19 compounds represent promising drug candidates for interfering with the immune evasion of EBV. Last
20 but not least, the modular bis(acylhydrazone) scaffold presented here represents a promising platform
21 for the development of novel ligands targeting other therapeutically important G4-RNA and/or G4-
22 DNA structures.

23 **5. EXPERIMENTAL SECTION**

24 **5.1. Chemistry**

25 **Synthesis and characterization:** All commercially available chemicals were reagent grade and used
26 without further purification. NMR spectra were measured with a Bruker Avance 300 spectrometer (1 H:
27 300 MHz, 13 C: 75 MHz) at 25 $^{\circ}$ C; chemical shifts are given in ppm (δ) values. Multiplicities of 13 C NMR
28 signals were determined from DEPT-135 experiments. The melting points were determined in open-
29 end capillaries with a digital melting point instrument (SMP30, Stuart). Elemental microanalysis of all
30 novel compounds was performed by the *Service de Microanalyse*, CNRS–ICSN, Gif-sur-Yvette, France.
31 The purity of final compounds was assessed by LC/MS analysis (Waters Alliance 2695 equipped with a
32 Waters XBridge C_{18} -3.5 μ m column and a photodiode array detector; eluent A: water with 0.05% TFA,
33 eluent B: MeCN with 0.05% TFA, gradient elution with 2 to 100% of eluent B). Mass spectra (MS, ESI in

1 the positive-ion mode) were recorded with a Waters ZQ instrument (cone voltage: 30 V). In the
2 assignment of MS of salts, *M* always refers to the organic dication.

3

4 **General procedure for the synthesis of bis(acylhydrazides) (1–4):** A solution of the corresponding
5 dimethyl ester (1.95 g, 10 mmol) and hydrazine hydrate (10.9 mL, 11.2 g, 220 mmol) in ethanol (150
6 mL) was heated under reflux for 18 h and then cooled to room temperature. The precipitate was
7 filtered, washed twice with ethanol, once with ether, and dried, to give the bis(acylhydrazide) which
8 was sufficiently pure and employed without further purification.

9 *Pyridine-2,6-dicarbohydrazide (1)* [45]: Yield 1.73 g (89%). White solid, m.p. 285–286 °C (lit. 285 °C); ¹H
10 NMR (300 MHz, DMSO-*d*₆): δ 10.63 (s, 2H), 8.13 (s, 3H), 4.63 (br s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ
11 161.9 (C_q), 148.4 (C_q), 139.3 (CH), 123.7 (CH); MS (ESI⁺): *m/z* = 196.2 [*M* + H]⁺.

12 *Pyrimidine-4,6-dicarbohydrazide (2)*: Yield 1.81 g (92%). Pale-yellow solid, m.p. (decomp.) 280 °C; ¹H
13 NMR (300 MHz, DMSO-*d*₆): δ 10.41 (s, 2H), 9.35 (d, *J* = 1.1 Hz, 1H), 8.35 (d, *J* = 1.1 Hz, 1H), 4.77 (br s,
14 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 160.1 (C_q), 158.4 (C_q), 157.2 (CH), 114.7 (CH); MS (ESI⁺): *m/z* =
15 197.1 [*M* + H]⁺.

16 *1,8-Naphthyridine-2,7-dicarbohydrazide (3)*: Yield 2.45 g (97%). Pale-yellow solid, m.p. > 290 °C; ¹H
17 NMR (300 MHz, DMSO-*d*₆): δ 10.00 (s, 2H), 8.74 (d, *J* = 8.4 Hz, 2H), 8.25 (d, *J* = 8.4 Hz, 2H), 4.74 (br s,
18 4H); ¹³C NMR (75 MHz, CD₃CO₂D): δ 164.7 (C_q), 153.1 (C_q), 141.1 (CH), 126.7 (C_q), 122.0 (CH); MS (ESI⁺):
19 *m/z* = 247.1 [*M* + H]⁺.

20 *1,10-Phenanthroline-2,9-dicarbohydrazide (4)* [46]: Yield 2.38 g (80%). Pale yellow solid, m.p. > 290 °C
21 (lit. 318–325 °C); ¹H NMR (300 MHz, DMSO-*d*₆): δ 10.77 (s, 2H), 8.69 (d, *J* = 8.3 Hz, 2H), 8.40 (d, *J* = 8.3
22 Hz, 2H), 8.14 (s, 2H), 4.78 (br s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 162.9 (C_q), 149.5 (C_q), 144.0 (C_q),
23 137.9 (CH), 130.1 (C_q), 127.8 (CH), 121.0 (CH); MS (ESI⁺): *m/z* = 297.2 [*M* + H]⁺.

24

25 **Typical procedure for the synthesis of bis(acylhydrazone) precursors (6a–c, 7a–c, 8a–c, and 9a–c):** A
26 solution of a bis(acylhydrazide) (1–4, 2.0 mmol) and a heteroaromatic aldehyde (5a–5c, 4.4 mmol) in
27 ethanol (10 mL) was heated under reflux for 18 h. After cooling, the precipitate was collected by
28 filtration, thoroughly washed with ethanol, and dried, to give the corresponding bis(acylhydrazone)
29 which was sufficiently pure and employed without further purification.

30 *N*²,*N*⁶-Bis(pyridin-4-ylmethylene)pyridine-2,6-dicarbohydrazide (6a): Yield 90%; white powder, m.p. >
31 290 °C; ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.54 (s, 2H), 8.79 (s, 2H), 8.72 (d, *J* = 5.9 Hz, 4H), 8.44–8.25

1 (m, 3H), 7.77 (d, $J = 6.0$ Hz, 4H); ^{13}C NMR (75 MHz, DMSO- d_6): δ 159.8 (C_q), 150.4 (CH), 148.0 (C_q), 147.6
2 (CH), 141.3 (C_q), 140.2 (CH), 125.9 (CH), 121.1 (CH); MS (ESI⁺): $m/z = 374.2$ [$M + \text{H}$]⁺, 187.7 [$M + 2\text{H}$]²⁺.

3 *N*²,*N*⁶-Bis(quinolin-4-ylmethylene)pyridine-2,6-dicarbohydrazide (**6b**): Yield 93%; pale-yellow solid,
4 m.p. > 290 °C; ^1H NMR (300 MHz, DMSO- d_6): δ 12.65 (s, 2H), 9.48 (s, 2H), 9.07 (d, $J = 4.5$ Hz, 2H), 8.91
5 (dd, $J = 8.4, 0.7$ Hz, 2H), 8.53–8.33 (m, 3H), 8.16 (dd, $J = 8.3, 0.7$ Hz, 2H), 7.99 (d, $J = 4.5$ Hz, 2H), 7.92–
6 7.79 (m, 4H); ^{13}C NMR (75 MHz, DMSO- d_6): δ 159.7 (C_q), 150.5 (CH), 148.5 (C_q), 148.0 (C_q), 147.3 (CH),
7 140.3 (CH), 137.1 (C_q), 129.9 (CH), 129.8 (CH), 127.7 (CH), 125.9 (CH), 124.8 (C_q), 124.4 (CH), 120.0 (CH);
8 MS (ESI⁺): $m/z = 474.3$ [$M + \text{H}$]⁺.

9 *N*²,*N*⁶-Bis(quinolin-6-ylmethylene)pyridine-2,6-dicarbohydrazide (**6c**): Yield 80%; pale-yellow solid,
10 m.p. > 290 °C; ^1H NMR (300 MHz, DMSO- d_6): δ 12.50 (s, 2H), 9.01–8.94 (m, 4H), 8.52 (d, $J = 7.8$ Hz, 2H),
11 8.46–8.39 (m, 2H), 8.39–8.28 (m, 5H), 8.13 (d, $J = 8.8$ Hz, 2H), 7.63 (dd, $J = 8.3, 4.3$ Hz, 2H); ^{13}C NMR (75
12 MHz, DMSO- d_6): δ 159.6 (C_q), 151.5 (CH), 149.2 (CH), 148.6 (C_q), 148.2 (C_q), 140.1 (CH), 136.5 (CH),
13 132.3 (C_q), 129.8 (CH), 129.0 (CH), 128.0 (C_q), 126.4 (CH), 125.6 (CH), 122.2 (CH); MS (ESI⁺): $m/z = 474.3$
14 [$M + \text{H}$]⁺, 237.7 [$M + 2\text{H}$]²⁺.

15 *N*⁴,*N*⁶-Bis(pyridin-4-ylmethylene)pyrimidine-4,6-dicarbohydrazide (**7a**): Yield 99%; white solid, m.p. >
16 290 °C; ^1H NMR (300 MHz, DMF- d_7): δ 12.77 (s, 2H), 9.60 (s, 1H), 8.89 (s, 2H), 8.80–8.70 (m, 5H), 7.79
17 (d, $J = 6.0$ Hz, 4H); ^{13}C NMR (75 MHz, DMF- d_7): δ 159.7 (C_q), 159.1 (C_q), 158.0 (CH), 150.9 (CH), 148.8
18 (CH), 141.9 (C_q), 121.6 (CH), 116.8 (CH); MS (ESI⁺): $m/z = 375.2$ [$M + \text{H}$]⁺, 188.1 [$M + 2\text{H}$]²⁺.

19 *N*⁴,*N*⁶-Bis(quinolin-4-ylmethylene)pyrimidine-4,6-dicarbohydrazide (**7b**): Yield 99%; pale-yellow solid,
20 m.p. (decomp.) 282 °C; ^1H NMR (300 MHz, DMF- d_7): δ 12.89 (s, 2H), 9.68 (d, $J = 10.9$ Hz, 3H), 9.10 (d, J
21 = 4.4 Hz, 2H), 8.93–8.79 (m, 3H), 8.18 (d, $J = 8.2$ Hz, 2H), 8.02 (s, 2H), 7.96–7.86 (m, 2H), 7.86–7.76 (m,
22 2H); ^{13}C NMR (75 MHz, DMF- d_7): δ 160.0 (C_q), 157.7 (CH), 150.4 (CH), 149.5 (C_q), 148.4 (CH), 137.9 (C_q),
23 130.3 (CH), 129.5 (CH), 127.6 (CH), 125.6 (C_q), 124.6 (CH), 123.4 (C_q), 120.3 (CH), 116.8 (CH); MS (ESI⁺):
24 $m/z = 475.3$ [$M + \text{H}$]⁺, 238.2 [$M + 2\text{H}$]²⁺.

25 *N*⁴,*N*⁶-Bis(quinolin-6-ylmethylene)pyrimidine-4,6-dicarbohydrazide (**7c**): Yield 99%; pale-yellow solid,
26 m.p. > 290 °C; ^1H NMR (300 MHz, DMSO- d_6): δ 12.68 (s, 2H), 9.60 (s, 1H), 9.03–8.87 (m, 4H), 8.65 (s,
27 1H), 8.51 (d, $J = 8.0$ Hz, 2H), 8.32–8.20 (m, 4H), 8.10 (d, $J = 8.4$ Hz, 2H), 7.66–7.55 (m, 2H); ^{13}C NMR (75
28 MHz, CD₃CO₂D): δ 160.2 (C_q), 158.6 (C_q), 158.0 (CH), 150.5 (CH), 147.1 (CH), 145.2 (CH), 141.5 (C_q),
29 134.7 (C_q), 131.0 (CH), 130.6 (CH), 129.0 (C_q), 123.7 (CH), 123.0 (CH), 116.9 (CH); MS (ESI⁺): $m/z = 475.3$
30 [$M + \text{H}$]⁺, 238.2 [$M + 2\text{H}$]²⁺.

31 *N*²,*N*⁷-Bis(pyridin-4-ylmethylene)-1,8-naphthyridine-2,7-dicarbohydrazide (**8a**): Yield 78%; white solid,
32 m.p. > 290 °C; ^1H NMR (300 MHz, DMSO- d_6): δ 12.63 (s, 2H), 8.89 (d, $J = 8.3$ Hz, 2H), 8.81–8.58 (m, 6H),

1 8.42 (d, $J = 8.3$ Hz, 2H), 7.71 (d, $J = 4.6$ Hz, 4H); ^{13}C NMR (75 MHz, $\text{CD}_3\text{CO}_2\text{D}$): δ 162.7 (C_q), 153.5 (C_q),
2 149.3 (C_q), 147.1 (CH), 145.3 (CH), 141.5 (CH), 127.7 (C_q), 124.8 (CH), 123.1 (CH); MS (ESI $^+$): $m/z = 425.3$
3 $[\text{M} + \text{H}]^+$.

4 *N*²,*N*⁷-Bis(quinolin-4-ylmethylene)-1,8-naphthyridine-2,7-dicarbohydrazide (**8b**): Yield 80%; white
5 solid, m.p. > 290 °C; ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 12.72 (s, 2H), 9.51 (s, 2H), 9.05 (d, $J = 4.5$ Hz, 2H),
6 8.93 (d, $J = 8.3$ Hz, 2H), 8.78 (d, $J = 8.3$ Hz, 2H), 8.48 (d, $J = 8.3$ Hz, 2H), 8.14 (d, $J = 8.2$ Hz, 2H), 7.95 (d,
7 $J = 4.4$ Hz, 2H), 7.91–7.84 (m, 2H), 7.84–7.77 (m, 2H); ^1H NMR (300 MHz, $\text{CD}_3\text{CO}_2\text{D}/\text{D}_2\text{O}$ 1:1 v/v): δ 9.36
8 (s, 2H), 9.02 (d, $J = 5.5$ Hz, 2H), 8.67–8.55 (m, 4H), 8.36 (dd, $J = 13.8, 7.0$ Hz, 4H), 8.11 (d, $J = 8.4$ Hz, 2H),
9 7.99–7.90 (m, 2H), 7.90–7.80 (m, 2H); ^{13}C NMR (75 MHz, $\text{CD}_3\text{CO}_2\text{D}/\text{D}_2\text{O}$ 1:1 v/v): δ 162.0 (C_q), 152.7
10 (C_q), 145.6 (CH), 144.5 (CH), 141.2 (CH), 141.0 (C_q), 134.5 (CH), 130.8 (CH), 127.1 (C_q), 126.4 (C_q), 124.9
11 (CH), 123.6 (CH), 122.6 (CH), 119.7 (CH); MS (ESI $^+$): $m/z = 525.2$ $[\text{M} + \text{H}]^+$, 263.2 $[\text{M} + 2\text{H}]^{2+}$.

12 *N*²,*N*⁷-Bis(quinolin-6-ylmethylene)-1,8-naphthyridine-2,7-dicarbohydrazide (**8c**): Yield 80%; white
13 solid, m.p. > 290 °C; ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 12.56 (s, 2H), 9.07–8.74 (m, 6H), 8.52 (d, $J = 8.3$
14 Hz, 2H), 8.44 (d, $J = 8.2$ Hz, 2H), 8.33–8.25 (m, 4H), 8.12 (d, $J = 8.9$ Hz, 2H), 7.62 (dd, $J = 7.6, 4.0$ Hz, 2H);
15 ^{13}C NMR spectrum could not be obtained due to insufficient solubility; MS (ESI $^+$): $m/z = 263.1$ $[\text{M} +$
16 $2\text{H}]^{2+}$.

17 *N*²,*N*⁹-Bis(pyridin-4-ylmethylene)-1,10-phenanthroline-2,9-dicarbohydrazide (**9a**): Yield 77%; pale
18 yellow solid, m.p. > 290 °C; ^1H NMR (300 MHz, $\text{DMF}-d_7$): δ 12.87 (s, 2H), 9.11 (s, 2H), 8.92 (d, $J = 8.3$ Hz,
19 2H), 8.81 (d, $J = 5.8$ Hz, 4H), 8.67 (d, $J = 8.3$ Hz, 2H), 8.34 (s, 2H), 7.85 (d, $J = 5.8$ Hz, 4H); NMR (75 MHz,
20 $\text{DMF}-d_7$): δ 161.1 (C_q), 150.9 (CH), 149.8 (C_q), 147.9 (CH), 144.6 (C_q), 142.4 (C_q), 139.0 (CH), 131.5 (C_q),
21 128.8 (CH), 122.1 (CH), 121.6 (CH); MS (ESI $^+$): $m/z = 475.3$ $[\text{M} + \text{H}]^+$.

22 *N*²,*N*⁹-Bis(quinolin-4-ylmethylene)-1,10-phenanthroline-2,9-dicarbohydrazide (**9b**): Yield 55%; pale
23 yellow solid, m.p. > 290 °C; ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 12.97 (s, 2H), 9.62 (s, 2H), 8.86–8.83 (m,
24 4H), 8.65 (d, $J = 8.3$ Hz, 2H), 8.61 (d, $J = 8.2$ Hz, 2H), 8.26 (s, 2H), 7.99 (d, $J = 8.3$ Hz, 2H), 7.81 (d, $J = 4.5$
25 Hz, 2H), 7.61–7.53 (m, 2H), 7.10–7.02 (m, 2H); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 160.4 (C_q), 150.1 (CH),
26 149.0 (C_q), 148.1 (C_q), 147.3 (CH), 138.8 (CH), 138.1 (CH), 137.8 (C_q), 130.9 (C_q), 129.4 (CH), 129.4 (CH),
27 128.6 (CH), 126.9 (CH), 124.9 (CH), 124.2 (C_q), 122.0 (CH), 119.6 (CH); MS (ESI $^+$): $m/z = 575.3$ $[\text{M} + \text{H}]^+$,
28 288.2 $[\text{M} + 2\text{H}]^{2+}$.

29 *N*²,*N*⁹-Bis(quinolin-6-ylmethylene)-1,10-phenanthroline-2,9-dicarbohydrazide (**9c**): Yield 59%; pale
30 yellow solid, m.p. > 290 °C; ^1H NMR (300 MHz, $\text{DMF}-d_7$): δ 12.88 (s, 2H), 9.37 (s, 2H), 9.01 (dd, $J = 4.2,$
31 1.5 Hz, 2H), 8.93 (d, $J = 8.3$ Hz, 2H), 8.69 (d, $J = 8.3$ Hz, 2H), 8.48 (dd, $J = 8.8, 1.6$ Hz, 2H), 8.34 (d, $J = 7.4$
32 Hz, 4H), 8.19 (d, $J = 8.8$ Hz, 2H), 8.11 (d, $J = 7.9$ Hz, 2H), 7.53 (dd, $J = 8.3, 4.2$ Hz, 2H); ^{13}C NMR (75 MHz,
33 $\text{DMF}-d_7$): δ 160.8 (C_q), 151.8 (CH), 150.0 (C_q), 149.5 (C_q), 149.5 (CH), 144.5 (C_q), 138.9 (CH), 136.6 (CH),

1 133.6 (C_q), 131.4 (C_q), 130.4 (CH), 129.3 (CH), 128.7 (CH), 128.6 (C_q), 127.0 (CH), 122.5 (CH), 122.0 (CH);
2 MS (ESI⁺): $m/z = 575.3 [M + H]^+$, $288.2 [M + 2H]^{2+}$.

3

4 **4-Formyl-1-methylpyridinium iodide (10a)**:^[47] The mixture of pyridine-4-carboxaldehyde (5.4 mL,
5 6.10 g, 56.9 mmol) and methyl iodide (7.0 mL, 15.95 g, 112.4 mmol) in DCM (20 mL) was stirred for 72
6 h at room temperature under argon atmosphere. The precipitated solid was filtered and washed twice
7 with DCM, to give **10a** (13.3 g, 94%) as an orange solid. ¹H NMR (300 MHz, D₂O): δ 8.80 (d, $J = 6.5$ Hz,
8 2H), 8.12 (d, $J = 6.2$ Hz, 2H), 6.21 (s, 1H), 4.39 (s, 3H); ¹³C NMR (75 MHz, D₂O): δ 160.1 (C_q), 146.0 (CH),
9 125.7 (CH), 88.2 (CH), 48.5 (CH₃); MS (ESI⁺): $m/z (\%) = 140.1 (100) [M + H_2O]^+$.

10

11 **General procedure for the synthesis of quaternized heterocyclic aldehydes (11a–b, 12a–b)**: The
12 solution of aldehyde (10 mmol) and alkylating agent (100 mmol) in acetone (18 mL) was stirred at 60
13 °C for 18 h and then cooled to room temperature. The precipitated solid was filtered, washed twice
14 with acetone, once with ether, and dried.

15 **4-Formyl-1-methylquinolinium iodide (11a)**: Obtained from quinoline-4-aldehyde and methyl iodide in
16 a 77% yield. Red solid, ¹H NMR (300 MHz, D₂O): δ 9.25 (d, $J = 6.1$ Hz, 1H), 8.63 (d, $J = 8.4$ Hz, 1H), 8.42
17 (d, $J = 9.0$ Hz, 1H), 8.24 (m, 2H), 8.04 (m, 1H), 6.87 (s, 1H), 4.66 (s, 3H); ¹³C NMR (75 MHz, D₂O): δ 157.8
18 (C_q), 150.1 (CH), 139.5 (C_q), 135.9 (CH), 130.8 (CH), 127.4 (C_q), 127.1 (CH), 119.5 (CH), 118.5 (CH), 86.8
19 (CH), 46.3 (CH₃); MS (ESI⁺): $m/z (\%) = 190.3 (100) [M + H_2O]^+$, $172.1 (25) [M]^+$.

20 **4-Formyl-1-benzylquinolinium bromide (11b)**: Obtained from quinoline-4-aldehyde and benzyl
21 bromide in an 81% yield. ¹H NMR (300 MHz, D₂O): δ 9.39 (d, $J = 6.1$ Hz, 1H), 8.63 (d, $J = 7.9$ Hz, 1H),
22 8.45–8.25 (m, 2H), 8.15–8.04 (m, 1H), 7.97 (m, 1H), 7.42 (s, 3H), 7.32 (s, 2H), 6.89 (s, 1H), 6.28 (s, 2H);
23 ¹³C NMR (75 MHz, D₂O): δ 158.7 (C_q), 149.9 (CH), 139.0 (C_q), 136.1 (CH), 133.4 (C_q), 130.8 (CH), 130.1
24 (CH), 129.9 (CH), 128.1 (C_q), 128.0 (CH), 127.4 (CH), 120.0 (CH), 118.7 (CH), 86.9 (CH), 61.8 (CH₂); MS
25 (ESI⁺): $m/z (\%) = 266.2 (100) [M + H_2O]^+$, $248.2 (6) [M]^+$.

26 **6-Formyl-1-ethylquinolinium iodide (12a)**: Obtained from quinoline-6-aldehyde and ethyl iodide in a
27 73% yield. ¹H NMR (300 MHz, DMSO-*d*₆): δ 10.31 (s, 1H), 9.68 (d, $J = 4.7$ Hz, 1H), 9.48 (d, $J = 8.3$ Hz, 1H),
28 9.08 (d, $J = 1.7$ Hz, 1H), 8.79 (d, $J = 9.2$ Hz, 1H), 8.60 (dd, $J = 9.2, 1.7$ Hz, 1H), 8.31 (dd, $J = 8.4, 5.8$ Hz,
29 1H), 5.13 (q, $J = 7.2$ Hz, 2H), 1.62 (t, $J = 7.2$ Hz, 3H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 191.9 (C_q), 151.5
30 (CH), 148.7 (CH), 139.7 (C_q), 135.6 (C_q), 134.4 (CH), 132.5 (CH), 129.6 (C_q), 123.5 (CH), 120.3 (CH), 53.5
31 (CH₂), 15.2 (CH₃); MS (ESI⁺): $m/z (\%) = 204.2 (8) [M + H_2O]^+$, $186.1 (100) [M]^+$.

1 *6-Formyl-1-benzylquinolinium iodide (12b)*: Obtained from quinoline-6-aldehyde and benzyl bromide
2 in an 85% yield. ¹H NMR (300 MHz, DMSO-*d*₆): δ 10.26 (s, 1H), 9.85 (d, *J* = 5.8 Hz, 1H), 9.58 (d, *J* = 8.3
3 Hz, 1H), 9.10 (d, *J* = 1.5 Hz, 1H), 8.67 (d, *J* = 9.2 Hz, 1H), 8.54 (dd, *J* = 9.2, 1.7 Hz, 1H), 8.41 (dd, *J* = 8.4,
4 5.8 Hz, 1H), 7.40 (s, 5H), 6.42 (s, 2H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 191.7 (C_q), 152.5 (CH), 149.6 (CH),
5 139.9 (C_q), 135.7 (C_q), 134.3 (CH), 133.6 (C_q), 132.8 (CH), 129.9 (CH), 129.1 (C_q), 128.8 (CH), 127.4 (CH),
6 123.7 (CH), 120.6 (C_q), 60.2 (CH₂); MS (ESI⁺): *m/z* (%) = 266.3 (10) [*M* + H₂O]⁺, 248.2 (100) [*M*]⁺.

7

8 **Synthesis of cationic bis(acylhydrazones) (PyDH1, PyDH2). Method A:** A mixture of bis(acylhydrazone)
9 precursor **6a** or **6b** (0.5 mmol), alkyl halogenide (75 mmol) and DMF (3 mL) was sealed in a tube and
10 heated at 40 °C during 18 h. After cooling to room temperature, the precipitate was filtered and
11 washed with DMF and diethyl ether, and dried in vacuum. The crude product was purified by
12 recrystallization from boiling MeCN–H₂O. *Note: this method gave sufficiently pure products PyDH1 and*
13 *PyDH2; however, in the case PymDH1, PymDH2, and NaphDH2, the mono-alkylated by-product could*
14 *not be removed by recrystallization. Method B (below) was therefore preferred for the synthesis of all*
15 *bis(acylhydrazones).*

16 *N*²,*N*⁶-Bis[(1-methylpyridinium-4-yl)methylene]pyridine-2,6-dicarbohydrazide iodide (**PyDH1**): Yield
17 279 mg (85%). Orange powder; ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.93 (s, 2H), 9.03 (d, *J* = 5.9 Hz, 4H),
18 8.94 (s, 2H), 8.54–8.31 (m, 7H), 4.37 (s, 6H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 160.3 (C_q), 148.8 (C_q), 147.6
19 (C_q), 146.1 (CH), 143.8 (CH), 140.6 (CH), 126.6 (CH), 124.3 (CH), 47.7 (CH₃); MS (ESI⁺): *m/z* (%) = 402.3
20 (100) [*M* – H]⁺, 201.7 (41) [*M*]²⁺; purity (LC) 100%.

21 *N*²,*N*⁶-Bis[(1-methylquinolinium-4-yl)methylene]pyridine-2,6-dicarbohydrazide iodide (**PyDH2**): Yield
22 360 mg (95%). Orange powder; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.03 (s, 2H), 9.74 (s, 2H), 9.54 (d, *J* =
23 6.3 Hz, 2H), 9.07 (d, *J* = 8.5 Hz, 2H), 8.66–8.56 (m, 4H), 8.52 (d, *J* = 7.0 Hz, 2H), 8.48–8.43 (m, 1H), 8.42–
24 8.33 (m, 2H), 8.27–8.18 (m, 2H), 4.68 (s, 6H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 160.1 (C_q), 149.4 (CH),
25 147.6 (C_q), 147.0 (C_q), 143.4 (CH), 140.7 (CH), 139.1 (C_q), 135.3 (CH), 130.5 (CH), 126.7 (CH), 126.3 (C_q),
26 126.0 (CH), 120.0 (CH), 119.0 (CH), 45.7 (CH₃); MS (ESI⁺): *m/z* (%) = 502.3 (100) [*M* – H]⁺, 616.3 (9) [*M* +
27 CF₃COO]⁺; purity (LC) 90%.

28

29 **Synthesis of cationic bis(acylhydrazones). Method B:** The mixture of dicarbohydrazide **1–4** (0.5 mmol)
30 and quaternized aldehyde **10a**, **11a–b**, or **12a–b** (1.1 mmol) in DMF (3 mL) was heated at 100 °C (80 °C
31 for NaphDH5 and PhenDH5) for 2 h and then cooled to room temperature. The precipitate was
32 collected by filtration, washed three times with MeCN, once with ether, dried and then additionally

1 recrystallized from MeCN/H₂O. The yields are indicated for ¹H-NMR spectroscopically pure material
2 prior to the final recrystallization step.

3 *N*²,*N*⁶-Bis[(1-methylpyridinium-4-yl)methylene]pyridine-2,6-dicarbohydrazide iodide (**PyDH1**): Yield
4 90%. The appearance and spectroscopic properties were identical with those described above; purity
5 (LC): 100%; anal. calcd. for C₂₁H₂₁I₂N₇O₂ × 2 H₂O (693.3): C 36.38, H 3.63, N 14.14; found: C 36.03, H
6 3.31, N 14.05.

7 *N*²,*N*⁶-Bis[(1-methylquinolinium-4-yl)methylene]pyridine-2,6-dicarbohydrazide iodide (**PyDH2**): Yield
8 76%. The appearance and spectroscopic properties were identical with those described above; purity
9 (LC): 100%; anal. calcd. for C₂₉H₂₅I₂N₇O₂ × 3.5 H₂O (820.4): C 42.46, H 3.93, N 11.95; found: C 42.46, H
10 3.59, N 11.60.

11 *N*²,*N*⁶-Bis[(1-benzylquinolinium-4-yl)methylene]pyridine-2,6-dicarbohydrazide bromide (**PyDH3**): Yield
12 93%. Yellow-orange powder; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.34 (s, 2H), 10.29 (s, 2H), 9.74 (d, *J* =
13 6.2 Hz, 2H), 9.06 (d, *J* = 8.5 Hz, 2H), 8.66 (d, *J* = 6.2 Hz, 2H), 8.62–8.50 (m, 4H), 8.49–8.38 (m, 1H), 8.31–
14 8.23 (m, 2H), 8.19–8.11 (m, 2H), 7.41 (s, 10H), 6.42 (s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 160.0 (C_q),
15 149.4 (CH), 148.3 (C_q), 147.8 (C_q), 143.1 (CH), 140.4 (CH), 138.2 (C_q), 135.5 (CH), 134.0 (C_q), 130.2 (C_q),
16 129.2 (CH), 128.9 (CH), 127.4 (CH), 127.2 (C_q), 127.0 (CH), 126.3 (CH), 120.0 (CH), 118.4 (CH), 59.9 (CH₂);
17 MS (ESI⁺): *m/z* (%) = 654.4 (34) [*M* – H]⁺, 327.8 (100) [*M*]²⁺; purity (LC): 100%; anal. calcd. for
18 C₄₁H₃₃Br₂N₈O₂ × 1.5 H₂O (842.6): C 58.44, H 4.31, N 11.64; found: C 58.28, H 4.28, N 11.62.

19 *N*²,*N*⁶-Bis[(1-ethylquinolinium-6-yl)methylene]pyridine-2,6-dicarbohydrazide iodide (**PyDH4**): Yield
20 92%. Pale brown solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.67 (s, 2H), 9.59 (d, *J* = 5.4 Hz, 2H), 9.38 (d, *J*
21 = 8.3 Hz, 2H), 9.07 (s, 2H), 8.85 (s, 2H), 8.74 (q, *J* = 9.6 Hz, 4H), 8.45 (d, *J* = 7.1 Hz, 2H), 8.41–8.32 (m,
22 1H), 8.32–8.16 (m, 2H), 5.14 (q, *J* = 7.0 Hz, 4H), 1.66 (t, *J* = 7.0 Hz, 6H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ
23 160.0 (C_q), 149.7 (CH), 148.0 (C_q), 147.4 (CH), 147.1 (CH), 140.3 (CH), 138.1 (C_q), 135.2 (CH), 132.3 (CH),
24 130.1 (C_q), 129.9 (CH), 126.0 (CH), 123.2 (CH), 120.0 (CH), 53.3 (CH₂), 15.3 (CH₃); MS (ESI⁺): *m/z* (%) =
25 644.3 (32) [*M* + CF₃COO]⁺, 265.8 (100) [*M*]²⁺; purity (LC): 100%; anal. calcd. for C₃₁H₂₉I₂N₇O₂ × 1.5 H₂O
26 (821.5): C 45.33, H 4.05, N 11.94; found: C 45.44, H 4.11, N 11.96.

27 *N*²,*N*⁶-Bis[(1-benzylquinolinium-6-yl)methylene]pyridine-2,6-dicarbohydrazide bromide (**PyDH5**): Yield
28 55%. Pale yellow solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.72 (s, 2H), 9.76 (d, *J* = 5.0 Hz, 2H), 9.48 (d, *J*
29 = 8.3 Hz, 2H), 9.12 (s, 2H), 8.85 (s, 2H), 8.73–8.58 (m, 4H), 8.46–8.30 (m, 5H), 7.49–7.34 (m, 10H), 6.41
30 (s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 159.9 (C_q), 150.6 (CH), 148.3 (CH), 148.1 (C_q), 146.9 (CH), 140.2
31 (CH), 138.3 (C_q), 135.4 (C_q), 133.8 (C_q), 132.6 (CH), 130.4 (C_q), 129.7 (CH), 129.1 (CH), 128.9 (CH), 127.5
32 (CH), 126.1 (CH), 123.3 (CH), 120.3 (CH), 60.0 (CH₂); MS (ESI⁺): *m/z* (%) = 768.3 (59) [*M* + CF₃COO]⁺,

1 327.8 (100) $[M]^{2+}$; purity (LC): 99%; anal. calcd. for $C_{41}H_{33}Br_2N_7O_2 \times 1.5 H_2O$ (842.6): C 58.44, H 4.31, N
2 11.64; found: C 58.74, H 4.42, N 11.56.

3 *N⁴,N⁶-Bis[(1-methylpyridinium-4-yl)methylene]pyrimidine-4,6-dicarbohydrazide iodide (PymDH1):*
4 Yield 97%. Yellow-orange solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.31 (s, 2H), 9.67 (s, 1H), 9.01 (d, *J* =
5 6.5 Hz, 4H), 8.86 (s, 2H), 8.64 (s, 1H), 8.38 (d, *J* = 6.4 Hz, 4H), 4.35 (s, 6H); ¹³C NMR (75 MHz, DMSO-*d*₆):
6 δ 159.9 (C_q), 158.2 (C_q), 157.7 (CH), 148.6 (C_q), 146.0 (CH), 144.9 (CH), 124.5 (CH), 119.3 (CH), 47.7
7 (CH₃); MS (ESI⁺): *m/z* (%) = 403.3 (35) $[M - H]^+$, 202.2 (100) $[M]^{2+}$; purity (LC): 100%; anal. calcd. for
8 C₂₀H₂₀I₂N₈O₂ × H₂O (676.3): C 35.52, H 3.28, N 16.57; found: C 35.13, H 3.33, N 16.29.

9 *N⁴,N⁶-Bis[(1-methylquinolinium-4-yl)methylene]pyrimidine-4,6-dicarbohydrazide iodide (PymDH2):*
10 Yield 92%. Orange solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.28 (s, 2H), 9.75 (s, 1H), 9.70 (s, 2H), 9.51
11 (d, *J* = 6.2 Hz, 2H), 8.86 (d, *J* = 8.4 Hz, 2H), 8.73 (s, 1H), 8.60 (d, *J* = 8.7 Hz, 2H), 8.52 (d, *J* = 6.1 Hz, 2H),
12 8.40–8.32 (m, 2H), 8.25–8.16 (m, 2H), 4.67 (s, 6H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 159.6 (C_q), 158.0
13 (C_q), 157.8 (CH), 149.4 (CH), 146.7 (C_q), 144.3 (CH), 139.0 (C_q), 135.3 (CH), 130.6 (CH), 126.3 (C_q), 125.6
14 (CH), 120.0 (CH), 118.8 (CH), 117.0 (CH), 45.7 (CH₃); MS (ESI⁺): *m/z* (%) = 503.3 (12) $[M - H]^+$, 252.3 (100)
15 $[M]^{2+}$; purity (LC): 100%; anal. calcd. for C₂₈H₂₄I₂N₈O₂ × 2.2 H₂O (798.0): C 42.18, H 3.58, N 14.05; found:
16 C 42.55, H 3.39, N 13.66.

17 *N²,N⁶-Bis[(1-benzylquinolinium-4-yl)methylene]pyridine-2,6-dicarbohydrazide bromide (PymDH3):*
18 Yield 93%. Brown crystals. ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.31 (s, 2H), 9.74 (d, *J* = 5.3 Hz, 5H), 8.85
19 (d, *J* = 8.7 Hz, 2H), 8.74 (s, 1H), 8.60 (dd, *J* = 17.3, 7.6 Hz, 4H), 8.33–8.22 (m, 2H), 8.20–8.12 (m, 2H),
20 7.40 (s, 10H), 6.42 (s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 159.7 (C_q), 158.1 (C_q), 157.8 (CH), 149.6 (CH),
21 147.9 (C_q), 144.3 (CH), 138.2 (C_q), 135.5 (CH), 134.0 (C_q), 130.5 (CH), 129.1 (CH), 128.8 (CH), 127.2 (CH),
22 127.2 (C_q), 126.1 (CH), 120.1 (CH), 119.2 (CH), 117.1 (CH), 60.0 (CH₂); MS (ESI⁺): *m/z* (%) = 655.5 (12)
23 $[M - H]^+$, 328.4 (100) $[M]^{2+}$; purity (LC): 100%; anal. calcd. for C₄₀H₃₂Br₂N₈O₂ × H₂O (834.6): C 57.57, H
24 4.11, N 13.43; found: C 57.43, H 4.25, N 13.62.

25 *N²,N⁶-Bis[(1-ethylquinolinium-6-yl)methylene]pyridine-2,6-dicarbohydrazide iodide (PymDH4):* Yield
26 96%. Orange solid. ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.93 (s, 2H), 9.65 (s, 1H), 9.57 (d, *J* = 5.7 Hz, 2H),
27 9.37 (d, *J* = 8.3 Hz, 2H), 9.00 (s, 2H), 8.80–8.61 (m, 7H), 8.25 (dd, *J* = 8.3, 5.9 Hz, 2H), 5.12 (q, *J* = 7.2 Hz,
28 4H), 1.64 (t, *J* = 7.2 Hz, 6H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 159.4 (C_q), 158.6 (CH), 149.7 (CH), 148.0
29 (CH), 147.5 (CH), 138.2 (C_q), 138.1 (C_q), 135.1 (C_q), 132.3 (CH), 130.1 (CH), 130.1 (C_q), 123.2 (CH), 119.9
30 (CH), 116.8 (CH), 53.2 (CH₂), 15.3 (CH₃); MS (ESI⁺): *m/z* (%) = 266.3 (100) $[M]^{2+}$; purity (LC): 100%; anal.
31 calcd. for C₃₀H₂₈I₂N₈O₂ × 3.2 H₂O (844.1): C 42.69, H 4.11, N 13.28; found: C 42.34, H 3.84, N 13.25.

32 *N²,N⁶-Bis[(1-benzylquinolinium-6-yl)methylene]pyridine-2,6-dicarbohydrazide bromide (PymDH5):*
33 Yield 97%. Pale-yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.91 (s, 2H), 9.74 (d, *J* = 5.4 Hz, 2H), 9.62

1 (d, $J = 1.2$ Hz, 1H), 9.46 (d, $J = 8.3$ Hz, 2H), 8.96 (s, 2H), 8.80 (s, 2H), 8.67 – 8.55 (m, 5H), 8.34 (dd, $J =$
2 8.3, 5.9 Hz, 2H), 7.47–7.37 (m, 10H), 6.39 (s, 4H); ^{13}C NMR spectrum could not be obtained due to
3 insufficient solubility; MS (ESI⁺): m/z (%) = 769.5 (7) [$M + \text{CF}_3\text{COO}$]⁺, 328.3 (100) [M]²⁺; purity (LC): 99%;
4 anal. calcd. for $\text{C}_{40}\text{H}_{32}\text{Br}_2\text{N}_8\text{O}_2 \times 2 \text{H}_2\text{O}$ (852.6): C 56.35, H 4.26, N 13.14; found: C 56.09, H 4.20, N 13.21.

5 *N*²,*N*⁷-Bis[(1-methylpyridinium-4-yl)methylene]-1,8-naphthyridine-2,7-dicarbohydrazide iodide
6 (**NaphDH1**): Yield 76%. Yellow solid; ^1H NMR (300 MHz, DMSO-*d*₆): δ 13.14 (s, 2H), 9.01 (d, $J = 6.0$ Hz,
7 4H), 8.94 (d, $J = 8.3$ Hz, 2H), 8.83 (s, 2H), 8.45 (d, $J = 8.3$ Hz, 2H), 8.39 (d, $J = 5.9$ Hz, 4H), 4.36 (s, 6H); ^{13}C
8 NMR (75 MHz, DMSO-*d*₆): δ 161.6 (C_q), 153.5 (C_q), 152.5 (C_q), 148.9 (C_q), 146.0 (CH), 143.7 (CH), 140.4
9 (CH), 139.1 (C_q), 124.4 (CH), 122.1 (CH), 47.7 (CH₃); MS (ESI⁺): m/z (%) = 453.3 (78) [$M - \text{H}$]⁺, 227.3 (92)
10 [M]²⁺; purity (LC): 100%; anal. calcd. for $\text{C}_{24}\text{H}_{22}\text{I}_2\text{N}_8\text{O}_2 \times 0.5 \text{H}_2\text{O}$ (717.3): C 40.19, H 3.23, N 15.62; found:
11 C 40.29, H 3.47, N 15.56.

12 *N*²,*N*⁷-Bis[(1-methylquinolinium-4-yl)methylene]-1,8-naphthyridine-2,7-dicarbohydrazide iodide
13 (**NaphDH2**): This compound was obtained in an analytically pure form through a reaction of **3** with **11a**
14 performed in DMSO instead of DMF. Yield 81%. Red solid; ^1H NMR (300 MHz, DMSO-*d*₆): δ 13.14 (s,
15 2H), 9.72 (s, 2H), 9.52 (d, $J = 6.2$ Hz, 2H), 8.99 (d, $J = 8.3$ Hz, 2H), 8.90 (d, $J = 8.4$ Hz, 2H), 8.68–8.47 (m,
16 6H), 8.39–8.32 (m, 2H), 8.25–8.15 (m, 2H), 4.67 (s, 6H); ^{13}C NMR spectrum could not be obtained due
17 to insufficient solubility; MS (ESI⁺): m/z (%) = 553.2 (63) [$M - \text{H}$]⁺, 277.3 (100) [M]²⁺; purity (LC): 100%;
18 anal. calcd. for $\text{C}_{32}\text{H}_{26}\text{I}_2\text{N}_8\text{O}_2 \times 0.6 \text{DMSO}$ (855.3): C 46.62, H 3.49, N 13.10; found: C 46.77, H 3.23, N
19 13.45.

20 *N*²,*N*⁷-Bis[(1-benzylquinolinium-4-yl)methylene]-1,8-naphthyridine-2,7-dicarbohydrazide bromide
21 (**NaphDH3**): Yield 90%. Yellow solid; ^1H NMR (300 MHz, DMSO-*d*₆): δ 13.19 (s, 2H), 9.77 (s, 4H), 9.00 (d,
22 $J = 8.3$ Hz, 2H), 8.90 (d, $J = 8.1$ Hz, 2H), 8.66 (d, $J = 6.0$ Hz, 2H), 8.56 (dd, $J = 16.8, 8.7$ Hz, 4H), 8.32–8.22
23 (m, 2H), 8.21–8.12 (m, 2H), 7.41 (s, 10H), 6.44 (s, 4H); ^{13}C NMR (75 MHz, DMSO-*d*₆): δ 161.3 (C_q), 153.2
24 (C_q), 152.4 (C_q), 149.6 (CH), 148.2 (C_q), 143.1 (CH), 140.6 (CH), 138.2 (C_q), 135.5 (CH), 134.1 (C_q), 130.5
25 (CH), 129.1 (CH), 128.8 (CH), 127.3 (CH), 127.1 (C_q), 126.1 (CH), 122.0 (CH), 120.2 (CH), 119.0 (CH), 60.0
26 (CH₂); MS (ESI⁺): m/z (%) = 705.4 (50) [$M - \text{H}$]⁺, 353.4 (100) [M]²⁺; purity (LC): 97%; anal. calcd. for
27 $\text{C}_{44}\text{H}_{34}\text{Br}_2\text{N}_8\text{O}_2 \times 3 \text{H}_2\text{O}$ (920.7): C 57.40, H 4.38, N 12.17; found: C 57.28, H 4.21, N 12.11.

28 *N*²,*N*⁷-Bis[(1-ethylquinolinium-6-yl)methylene]-1,8-naphthyridine-2,7-dicarbohydrazide iodide
29 (**NaphDH4**): Yield 68%. Pale brown solid; ^1H NMR (300 MHz, DMSO-*d*₆): δ 12.79 (s, 2H), 9.58 (d, $J = 5.9$
30 Hz, 2H), 9.38 (d, $J = 8.6$ Hz, 2H), 8.99 (s, 2H), 8.93 (d, $J = 8.4$ Hz, 2H), 8.72 (dd, $J = 20.4, 11.1$ Hz, 6H),
31 8.45 (d, $J = 8.3$ Hz, 2H), 8.26 (dd, $J = 8.3, 5.8$ Hz, 2H), 5.13 (q, $J = 7.2$ Hz, 4H), 1.65 (t, $J = 7.1$ Hz, 6H); ^{13}C
32 NMR (75 MHz, DMSO-*d*₆): δ 161.1 (C_q), 153.8 (C_q), 152.5 (C_q), 149.6 (CH), 148.5 (C_q), 147.4 (CH), 146.8
33 (CH), 140.3 (CH), 138.0 (C_q), 135.3 (C_q), 132.4 (CH), 130.1 (C_q), 129.9 (CH), 123.2 (CH), 121.8 (CH), 119.9

1 (CH), 53.3 (CH₂), 15.3 (CH₃); MS (ESI⁺): *m/z* (%) = 695.4 (15) [*M* + CF₃COO]⁺, 291.3 (100) [*M*]²⁺; purity
2 (LC): 100%; anal. calcd. for C₃₄H₃₀I₂N₈O₂ × 1.5 H₂O (863.5): C 47.29, H 3.85, N 12.98; found: C 47.44, H
3 4.11, N 12.95.

4 *N*²,*N*⁷-Bis[(1-benzylquinolinium-6-yl)methylene]-1,8-naphthyridine-2,7-dicarbohydrazide bromide
5 (**NaphDH5**): Yield 80%. Pale brown solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 12.76 (s, 2H), 9.75 (d, *J* = 5.6
6 Hz, 2H), 9.48 (d, *J* = 8.3 Hz, 2H), 8.98–8.85 (m, 4H), 8.80 (s, 2H), 8.63 (m, 4H), 8.43 (d, *J* = 8.3 Hz, 2H),
7 8.35 (dd, *J* = 8.2, 5.9 Hz, 2H), 7.41 (d, *J* = 6.6 Hz, 10H), 6.40 (s, 4H); ¹³C NMR (75 MHz, DMSO-*d*₆): δ 161.1
8 (C_q), 153.8 (C_q), 152.6 (C_q), 150.6 (CH), 148.3 (CH), 146.7 (CH), 140.3 (CH), 138.3 (C_q), 135.5 (C_q), 133.8
9 (C_q), 132.7 (CH), 130.6 (C_q), 129.8 (CH), 129.2 (C_q), 128.9 (CH), 127.5 (CH), 123.3 (CH), 121.8 (CH), 120.3
10 (CH), 60.1 (CH₂); MS (ESI⁺): *m/z* (%) = 705.4 (5) [*M* + CF₃COO]⁺, 353.2 (100) [*M*]²⁺; purity (LC): 100%;
11 anal. calcd. for C₄₄H₃₄Br₂N₈O₂ × H₂O (884.6): C 59.74, H 4.10, N 12.67; found: C 59.55, H 4.44, N 12.56.

12 *N*²,*N*⁹-Bis[(1-methylpyridinium-4-yl)methylene]-1,10-phenanthroline-2,9-dicarbohydrazide iodide
13 (**PhenDH1**): Yield 50%. Yellow solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.38 (s, 2H), 9.04 (s, 6H), 8.89 (d,
14 *J* = 8.1 Hz, 2H), 8.64 (d, *J* = 8.0 Hz, 2H), 8.41 (d, *J* = 5.9 Hz, 4H), 8.32 (s, 2H), 4.41 (s, 6H), ¹³C NMR (75
15 MHz, DMSO-*d*₆): δ 161.1 (C_q), 149.3 (C_q), 148.6 (C_q), 146.0 (CH), 144.3 (CH), 143.7 (C_q), 138.9 (CH), 131.1
16 (C_q), 128.8 (CH), 124.4 (CH), 122.2 (CH), 47.7 (CH₃); MS (ESI⁺): *m/z* (%) = 617.3 (20) [*M* + CF₃COO]⁺, 503.3
17 (90) [*M* – H]⁺, 252.2 (100%) [*M*]²⁺; purity (LC): 100%; anal. calcd. for C₂₈H₂₄I₂N₈O₂ × 4.6 H₂O (841.2): C
18 39.98, H 3.98, N 13.32; found: C 40.36, H 4.20, N 12.95.

19 *N*²,*N*⁹-Bis[(1-methylquinolinium-4-yl)methylene]-1,10-phenanthroline-2,9-dicarbohydrazide iodide
20 (**PhenDH2**): Yield 78%. Dark-red solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.28 (s, 2H), 9.86 (s, 2H), 9.45
21 (d, *J* = 6.3 Hz, 2H), 8.95 (d, *J* = 8.3 Hz, 2H), 8.71 (d, *J* = 8.3 Hz, 2H), 8.56 (d, *J* = 6.1 Hz, 2H), 8.48–8.34 (m,
22 4H), 8.27 (d, *J* = 9.1 Hz, 2H), 7.88–7.80 (m, 2H), 7.15–7.08 (m, 2H), 4.62 (s, 6H); ¹³C NMR (75 MHz,
23 DMSO-*d*₆): δ 160.6 (C_q), 149.3 (C_q), 148.3 (C_q), 147.3 (C_q), 143.6 (C_q), 143.5 (CH), 139.1 (CH), 138.2 (C_q),
24 134.6 (CH), 131.2 (C_q), 129.2 (CH), 128.8 (CH), 126.1 (C_q), 125.1 (CH), 122.1 (CH), 119.2 (CH), 118.0 (CH),
25 45.5 (CH₃); MS (ESI⁺): *m/z* (%) = 717.4 (14) [*M* + CF₃COO]⁺, 603.4 (83) [*M* – H]⁺, 302.3 (100) [*M*]²⁺; purity
26 (LC): 100%; anal. calcd. for C₃₆H₂₈I₂N₈O₂ × 5.7 H₂O (961.2): C 44.99, H 4.13, N 11.66; found: C 45.23, H
27 4.52, N 11.63.

28 *N*²,*N*⁹-Bis[(1-benzylquinolinium-4-yl)methylene]-1,10-phenanthroline-2,9-dicarbohydrazide bromide
29 (**PhenDH3**): Yield 87%. Pale brown solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.34 (s, 2H), 9.94 (s, 2H),
30 9.76 (d, *J* = 6.0 Hz, 2H), 8.96 (d, *J* = 8.2 Hz, 2H), 8.73 (d, *J* = 7.7 Hz, 4H), 8.37 (d, *J* = 7.9 Hz, 4H), 8.14 (d,
31 *J* = 9.2 Hz, 2H), 7.65–7.30 (m, 10H), 7.09–7.00 (m, 2H), 6.84–6.79 (m, 2H), 6.35 (s, 4H); ¹³C NMR (75
32 MHz, DMSO-*d*₆): δ 160.7 (C_q), 149.6 (CH), 148.4 (C_q), 148.2 (C_q), 143.7 (C_q), 143.2 (CH), 139.1 (CH), 137.5
33 (C_q), 134.3 (CH), 134.2 (C_q), 131.2 (C_q), 129.2 (CH), 128.9 (CH), 128.8 (CH), 127.3 (C_q), 126.9 (C_q), 125.7

1 (CH), 122.2 (CH), 119.4 (CH), 118.3 (CH), 118.3 (CH), 59.8 (CH₂); MS (ESI⁺): *m/z* (%) = 755.4 (20) [*M* –
2 H]⁺, 378.4 (100) [*M*]²⁺; purity (LC): 100%; anal. calcd. for C₄₈H₃₆Br₂N₈O₂ × 3.5 H₂O (979.7): C 58.85, H
3 4.42, N 11.44; found: C 58.84, H 4.30, N 11.47.

4 *N*²,*N*⁹-Bis[(1-ethylquinolinium-6-yl)methylene]-1,10-phenanthroline-2,9-dicarbohydrazide iodide
5 (**PhenDH4**): Yield 72%. Pale brown solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.10 (s, 2H), 9.60 (d, *J* = 5.6
6 Hz, 2H), 9.25 (s, 2H), 9.17 (d, *J* = 8.2 Hz, 2H), 8.89 (d, *J* = 8.3 Hz, 2H), 8.76 (d, *J* = 2.6 Hz, 6H), 8.64 (d, *J* =
7 8.3 Hz, 2H), 8.32 (s, 2H), 8.22 (dd, *J* = 8.4, 5.9 Hz, 2H), 5.16 (q, *J* = 7.0 Hz, 4H), 1.67 (t, *J* = 7.1 Hz, 6H); ¹³C
8 NMR (75 MHz, DMSO-*d*₆): δ 160.7 (C_q), 149.6 (CH), 149.0 (C_q), 147.3 (C_q), 147.2 (CH), 143.7 (C_q),
9 138.7(CH), 138.0 (C_q), 135.7 (C_q), 132.8 (CH), 130.9 (C_q), 130.1 (C_q), 129.3 (CH), 128.5 (CH), 123.2 (CH),
10 122.0 (CH), 120.0 (CH), 53.3 (CH₂), 15.4 (CH₃); MS (ESI⁺): *m/z* (%) = 745.4 (28) [*M* + CF₃COO]⁺, 316.2
11 (100) [*M*]²⁺; purity (LC): 98%; anal. calcd. for C₃₈H₃₂I₂N₈O₂ × 1.5 H₂O (904.5): C 50.46, H 3.79, N 12.39;
12 found: C 50.13, H 3.90, N 12.41.

13 *N*²,*N*⁹-Bis[(1-benzylquinolinium-6-yl)methylene]-1,10-phenanthroline-2,9-dicarbohydrazide bromide
14 (**PhenDH5**): Yield 91%. Pale brown solid; ¹H NMR (300 MHz, DMSO-*d*₆): δ 13.10 (s, 2H), 9.79 (d, *J* = 5.7
15 Hz, 2H), 9.30 (d, *J* = 8.3 Hz, 2H), 9.21 (s, 2H), 8.87 (d, *J* = 8.3 Hz, 2H), 8.81 (s, 2H), 8.76–8.65 (m, 4H),
16 8.63 (d, *J* = 8.3 Hz, 2H), 8.39–8.28 (m, 4H), 7.53–7.37 (m, 10H), 6.46 (s, 4H); ¹³C NMR (75 MHz, DMSO-
17 *d*₆): δ 160.7 (C_q), 150.5 (CH), 149.0 (C_q), 148.1 (CH), 147.0 (CH), 143.8 (C_q), 138.7 (CH), 138.3 (C_q), 135.9
18 (C_q), 133.8 (C_q), 133.0 (CH), 130.9 (C_q), 130.4 (C_q), 129.5 (CH), 129.2 (CH), 128.9 (CH), 128.6 (CH), 127.6
19 (CH), 123.4 (CH), 122.0 (CH), 120.3 (CH), 60.1 (CH₂); MS (ESI⁺): *m/z* (%) = 755.4 (5) [*M* – H]⁺, 378.4 (100)
20 [*M*]²⁺; purity (LC): 100%; anal. calcd. for C₄₈H₃₆Br₂N₈O₂ × 2 H₂O (952.7): C 60.51, H 4.23, N 11.76; found:
21 C 60.76, H 4.39, N 11.77.

22 5.2. Single-crystal X-ray diffraction analysis

23 X-ray diffraction data for **PyDH1** (2I⁻) were collected using a VENTURE PHOTON100 CMOS Bruker
24 diffractometer with Micro-focus IuS source Mo *K*_α radiation. X-ray diffraction data for **PymDH1** (2I⁻)
25 were collected using a X8 APEXII CCD Bruker diffractometer with graphite-monochromated Mo *K*_α
26 radiation. X-ray diffraction data for **PhenDH1** (2I⁻) were collected using a VENTURE PHOTON100 CMOS
27 Bruker diffractometer with Micro-focus IuS source Cu *K*_α radiation. All crystals were mounted on a
28 CryoLoop (Hampton Research) with Paratone-N (Hampton Research) as cryoprotectant and then flash-
29 frozen in a nitrogen gas stream at 100 K. For compounds, the temperature of the crystal was
30 maintained at the selected value by means of a 700 series Cryostream (for X8) or N-Helix (for VENTURE)
31 cooling device within an accuracy of ±1 K. The data were corrected for Lorentz polarization, and
32 absorption effects. The structures were solved by direct methods using SHELXS-97 [48] and refined
33 against *F*² by full-matrix least-squares techniques using SHELXL-2018 [49] with anisotropic

1 displacement parameters for all non-hydrogen atoms. All calculations were performed using the
2 Crystal Structure crystallographic software package WINGX [50]. The crystal data collection and
3 refinement parameters are given in Table S3. CCDC 1881844–1881846 contain the supplementary
4 crystallographic data for this paper. These data can be obtained free of charge from the Cambridge
5 Crystallographic Data Centre via <https://www.ccdc.cam.ac.uk/structures/>.

6 **5.3. Biophysical assays**

7 *5.3.1. Fluorescence melting assay*

8 The assay was performed with a double-labeled oligoribonucleotide (g4-EBNA1, 5'-FAM-
9 r(GGGGCAGGAGCAGGAGGA)-TAMRA-3'), which was annealed prior to experiments (95 °C, 5 min) in K-
10 10 buffer (10 mM LiAsO₂Me₂, 10 mM KCl, 90 mM LiCl, pH 7.3) at a concentration of 10 μM. Double-
11 stranded DNA competitor (ds26, 5'-CAATCGGATCGAATTCGATCCGATTG-3') was annealed in the same
12 buffer at a strand concentration of 200 μM. Thermal denaturation runs were performed in 96-well
13 plates with a real-time PCR apparatus (7900HT Fast Real-Time PCR, Applied Biosystems) using a heating
14 ramp of 0.5 °C per minute from 25 to 95 °C; the fluorescence intensity was monitored in the FAM
15 channel. Each well contained 0.2 μM of double-labeled g4-EBNA1, 1 μM of tested compound, and/or
16 0, 3 or 10 μM of ds26 competitor, in a total volume of 25 μL of K10 buffer. The denaturation
17 temperatures (T_m) were determined from the maxima of first-derivative plots of FAM emission
18 intensity vs. temperature, and ligand-induced T_m shifts (ΔT_m) were calculated as a difference of mean
19 denaturation temperatures in the presence and in the absence of ligands ($T_m^0 = 58.6$ °C), from three
20 independent experiments. Analogous experiments were performed with G4-forming DNA
21 oligonucleotides F-myc22-T: 5'-FAM-d(TGAGGGTGGGTAGGGTGGGTAA)-TAMRA-3' and F-25TAG-T: 5'-
22 FAM-d(TAGGGTTAGGGTTAGGGTTAGGGAA)-TAMRA-3', in K-1 (10 mM LiAsO₂Me₂, 1 mM KCl, 99 mM
23 LiCl, pH 7.3) and K-100 (10 mM LiAsO₂Me₂, 100 mM KCl, pH 7.3) buffers, respectively.

24 *5.3.2. TO displacement assay*

25 This assay is performed in a 96-well microplate format as described [39]. The non-labeled g4-EBNA1
26 oligoribonucleotide was annealed (95 °C, 5 min) in K100 buffer (10 mM KAsO₂Me₂, 100 mM KCl, 1 mM
27 EDTA, 1% v/v DMSO) at a concentration of 5 μM and, after cooling, supplemented with TO (10 μM).
28 Every row of a black-bottom, 96-well microplate was filled with K100 buffer (q.s.p. 200 μL per well),
29 pre-folded g4-EBNA1 + TO solution (final concentrations: 0.25 and 0.5 μM, respectively), and an
30 extemporaneously prepared ligand solution (5 μM in the same buffer; final ligand concentration: 0 to
31 2.5 μM). After 5 min of orbital shaking, fluorescence intensity was measured with a Fluostar Omega
32 microplate reader (BMG Labtech) using the following parameters: 20 flashes per well, emission /
33 excitation filters: 485 / 520 nm, gain adjusted at 80% of the fluorescence from the most fluorescent

1 well. The experiments were performed in duplicate. The percentage of TO displacement was calculated
2 from the fluorescence intensity (F) as $\%(\text{TO displacement}) = 100 \times (1 - F / F_0)$, where F_0 is the
3 fluorescence intensity of TO–RNA complex in the absence of ligands. The percentage of displacement
4 was plotted against the concentration of added ligand, and ligand affinity was characterized by the
5 concentration required to decrease the fluorescence of the probe by 50% (DC_{50}) after interpolation of
6 the displacement curve. A control experiment was performed at identical conditions with double-
7 stranded DNA substrate ds26.

8 **5.4. Biological assays**

9 *5.4.1. EBNA1 expression assay*

10 The human lung carcinoma cell line H1299 was cultured in RPMI-1640 medium supplemented with
11 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin.
12 All cells were cultured at 37 °C with 5% CO₂. Transient transfections were performed using Genejuice
13 reagent (Merck Bioscience) according to the manufacturer's protocol. For cell treatments with the
14 indicated G4 ligands or **PhenDC3**, cells were incubated with 10 µM of drug for 40 h after transfection.
15 Stock solutions of drugs were prepared in DMSO (Euromedex). Whole-cell lysates were prepared 48 h
16 post-transfection, and protein concentration was determined using a Bradford assay. Samples were
17 electrophoretically separated in NuPAGE® Bis-Tris gels 10% (Invitrogen), transferred onto 0.45 µm
18 nitrocellulose membranes (GE) and blotted under standard conditions using the following antibodies:
19 anti-GAPDH (Sigma, 1:5,000), anti-EBNA1 mouse monoclonal antibody (OT1X, 1:2,000) or anti-OVA
20 rabbit polyclonal antibody (C6534 Sigma, 1:2,500). The membranes were then washed with fresh PBS,
21 1X 0.1% Igepal and incubated for 45 min with swine anti-rabbit or goat anti-mouse secondary
22 antibodies (Dako) conjugated to horseradish peroxidase at a 1:3,000 dilution, and analyzed by
23 enhanced chemiluminescence (ECL, GE Healthcare) using a Chemistart 5000 imager (Vilber–Lourmat).
24 All experiments were repeated at least three times. Relative protein levels for each sample were
25 normalized to GAPDH or Actin levels as indicated, using Fusion-Capt Advance software.

26 *5.4.2. Antigen presentation assay*

27 Naive OVA_{257–264} specific CD8⁺ T cells were isolated by negative selection from peripheral and
28 mesenteric lymph nodes of a 12-week-old female OT-I mice using the CD8⁺ T cell isolation kit (Miltenyi
29 Biotec, Germany). Afterwards, CD8⁺ T cells were co-cultured with H1299 cells co-transfected with
30 mouse Kb expression vector and the indicated constructs. For all assays, 10⁵ H1299 cells were
31 harvested 40 h after treatment and co-incubated with 4 × 10⁵ CD8⁺ T cells at 37 °C in humidified air/CO₂
32 atmosphere in RPMI medium containing 10% FBS, 4 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 µg
33 mL⁻¹ streptomycin, 5 mM HEPES and 0.05 mM 2-mercaptoethanol. After 3 days of co-incubation,

1 supernatants were collected and IL-2 levels were measured employing the IL-2 ELISA MAX™ Standard
2 kit (Biolegend, USA) according to the manufacturer's instructions.

3 5.4.3. MTT toxicity assay

4 About 30,000 Mutu-1 cells (derived from an EBV-positive Burkitt's lymphoma biopsy specimen from a
5 Kenyan patient) were plated in 0.1 mL per well in 96-well, flat-bottom plates and exposed to the
6 indicated compounds at the indicated concentrations or DMSO (vehicle). After indicated time (24 or
7 48 h), 10 µL of MTT solution (5 mg mL⁻¹, CT01-5, Merck Millipore) in PBS (pH 7.4) was added to each
8 well and incubated for 4 h. A mixture of isopropanol / 0.1 N HCl / 10% Triton X-100 (0.1 mL) was added
9 to each well to dissolve the formazan crystals, and the absorbance was then measured at 540 nm.

10 5.4.4. PLA assay

11 H1299 cells were transfected with an EBNA1 construct and, 7 h post-transfection, treated with the
12 tested compounds during 40 h. In the experiments with Mutu-1 cells expressing endogenous EBNA1
13 mRNA, drug treatment was carried out during 36 h. Following compounds administration, cells were
14 processed as previously described [42]. Briefly, paraformaldehyde-fixed samples were incubated in
15 70% (v/v) ethanol at 4 °C overnight, rehydrated in PBS for 30 min, and permeabilized in PBS 0.4%
16 Triton X-100, 0.05% CHAPS for 10 min at room temperature. Before the *in situ* hybridization step,
17 samples were pre-treated with hybridization buffer (10% formamide, 2X SSC buffer, 0.2 mg mL⁻¹ *E. coli*
18 522 tRNAs, 0.2 mg mL⁻¹ sheared salmon sperm DNA, and 2 mg mL⁻¹ BSA) and then incubated overnight
19 with 50 ng of an EBNA1-digoxigenin DNA probe (5'-CTTTCAAACCCCTCTTTTTTGGCGCT-
20 GCCTCCATCAAAA-digoxigenin-3') in a humidified chamber at 37 °C. To avoid secondary structure
21 formation, the probe was diluted in 5 µL of water, denatured at 80 °C for 5 min, chilled on ice for 5
22 min, and then re-suspended in 35 µL of hybridization buffer. After hybridization, samples were serially
23 washed for 20 min with 2X SSC buffer, 10% formamide, hybridization buffer (twice), 2X SSC buffer, and
24 PBS. Samples were then saturated with PBS 3% BSA for 30 min and incubated for 2 h at room
25 temperature with a mix of primary antibodies containing the mouse anti-digoxigenin (clone DI-22,
26 Sigma, 1:200) and the rabbit anti-nucleolin (ab22758, Abcam, 1:1,000). Subsequently, PLA was carried
27 out using the anti-rabbit plus and anti-mouse minus Duolink probes (Sigma) and the Duolink FarRed
28 PLA *in situ* kit (Sigma) following the manufacturer's protocol. Experiments were performed in duplicate
29 and 100 cells per sample and per replicate were imaged and analyzed.

30 5.4.5. RNA pull-down assay

31 These experiments were performed as previously described [22,51]. Briefly, recombinant NCL (kind ly
32 provided by Prof. Weinhold, Aachen, Germany) was used for pulldown assays with the following RNA

1 oligonucleotides: g4-EBNA1 (5'-GGGGCAGGAGCAGGAGGA-3'-TEG-Biotin) or GM (5'-GAGGCAGUAG-
2 CAGUAGAA-3'-TEG-Biotin, not able to form G4 according to the GQRS-H software). To allow the
3 formation of the G4, the oligonucleotides were resuspended in the folding buffer (10 mM Tris-HCl pH
4 7.5, 100 mM KCl, 0.1 mM EDTA;), heated at 95 °C for 5 min, and then slowly and progressively cooled
5 down to 4 °C (cooling rate of 2 °C min⁻¹) using a Veriti thermal cycler (Applied BioSystems). To avoid
6 unspecific binding, high-affinity streptavidin sepharose beads (GE Healthcare) were incubated in 1 mL
7 blocking buffer containing (10 mM Tris-HCl pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Triton
8 X-100, 0.1% BSA, 0.02% *S. cerevisiae* tRNAs) for 1 h at 4 °C on a rotating wheel. A 10-pg amount of
9 folded biotinylated RNA oligonucleotide was incubated with 50 µL of suspension containing the
10 streptavidin-coupled sepharose beads for 90 min at 4 °C on a rotating wheel. Then, 200 ng of
11 recombinant NCL was incubated with the RNA oligonucleotides bound to the beads during 90 min at
12 room temperature. Beads were washed with increasing KCl concentration (200 to 800 mM). Protein
13 still bound to beads after the washes were eluted using 2X SDS loading buffer and analyzed by western
14 blotting against NCL, as previously described. In the input lane of the western blots was loaded a
15 quantity of recombinant NCL protein which corresponds to half of the quantity that was incubated
16 with the beads for each condition.

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26 **ABBREVIATIONS**

27 BOILED, Brain Or Intestinal Estimated permeation; EBNA1, EBV-encoded nuclear antigen-1; EBV,
28 Epstein–Barr virus; FID, fluorescent indicator displacement; G4, G-quadruplex; GAr, glycine–alanine
29 repeat; IL-2, Interleukin-2; MHC, major histocompatibility complex; OVA, ovalbumin; PLA, proximity
30 ligation assay; TO, Thiazole Orange.

31 **SUPPLEMENTARY DATA**

32 Supplementary data to this article can be found online.

1 **DECLARATION OF INTERESTS**

2 None.

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