Supplementary Information

Translational Repression of Thymidylate Synthase by Targeting its mRNA

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SUPPLEMENTARY FIGURES

Fig S1. The two docking poses, A and B, generated for the HT-TSMC complex and used as starting structures for molecular dynamics simulations. TSMC is represented as a cartoon colored by the same scheme as in Fig. 5, whereas HT is shown in stick representation colored by atom type (C - cyan, N - blue, O - red).
Fig. S2. Protocol used to minimize, heat and equilibrate the systems in molecular dynamics simulations.

Fig. S3. Upper panel: Intracellular TS protein content measured after incubating the 2008 human ovarian cancer cell line with increasing concentrations of N1, N12,-bis(ethyl)spermine (BESpm) for 72 hours. The expression of vinculin protein was used as an internal reference. Lower panel: The corresponding Western blot.
Fig S4. Superposition of 2D $^1$H TOCSY spectra of TSMC (A); TSGC (B); and 2D $^1$H NOESY spectra of TSMC (C) and TSGC (D) recorded in $^2$H$_2$O, indicating chemical shift changes in the aromatic-anomeric region upon addition of HT. In the TOCSY spectra, HT/RNA ratios of 0:1, 0.5:1, 1:1 and 1.5:1 are marked in black, blue, red and green respectively, while in the NOESY spectra, HT/RNA ratios of 0:1 and 1.5:1, are marked in black and green respectively. In (B), the H5/H6 resonances for C7, C8 and C20 are assigned tentatively (indicated by brackets) based on their position in the TOCSY spectrum for TSMC. In the NOESY spectra, the assignments of inter-residue cross-peaks as well as the intra-residue H8/H1' adenine cross-peaks for the GNRA tetraloop are indicated. In (D), the H6/H5 signals for the stem cytosine residues are boxed.
Fig. S5. Superposition of 2D $^1$H NOESY of TSMC recorded in H$_2$O. Chemical shift changes in the imino-amino region, upon addition of HT with HT/RNA ratios of 0:1, 0.5:1, 1:1, 1.5:1 are marked in black, blue, red and green respectively. The base-pairs with a large degree of chemical shift change are colored in cyan on the TSMC RNA sequence on the right.
Fig S6. Absorption (left) and fluorescence emission (right) evolutions of HT titrated with AT-DNA. Insets: absorbances at 340 nm (left) and relative emission band areas (right) (both corrected for dilution) as functions of the AT-DNA/HT mole ratio. $\lambda_{\text{exc}} = 330$ nm. The areas of the emission spectra were not corrected for the change in absorbance at this wavelength.

Fig S7. Absorption (left) and fluorescence emission (right) evolutions of HT titrated with GC-DNA. Insets: absorbance at 340 nm and relative emission band areas (both corrected for dilution) as functions of the GC-DNA/HT mole ratio. $\lambda_{\text{exc}} = 330$ nm. The areas of the emission spectra were not corrected for the change in absorbance at this wavelength.
Fig S8. Absorption (left) and fluorescence emission evolutions of HT titrated with TSGC-RNA. Insets: absorbance at 340 nm and relative emission band areas (both corrected for dilution) as functions of the TSGC-RNA/HT mole ratio. $\lambda_{\text{exc}} = 330$ nm. The areas of the emission spectra were not corrected for the change in absorbance at this wavelength.

Fig S9. Absorption (left) and fluorescence emission evolutions of HT titrated with TS1-RNA. Insets: absorbance at 340 nm and relative emission band areas (both corrected for dilution) as functions of the TS1C-RNA/HT mole ratio. $\lambda_{\text{exc}} = 330$ nm. The areas of the emission spectra were not corrected for the change in absorbance at this wavelength.
Fig S10. All atom root-mean-square fluctuations of TSMC in uncomplexed form (green) and in complex with HT. The fluctuations during 0-9 ns of the simulation of the HT-TSMC complex, i.e. before initiation of intercalation are in blue, whereas those for 15-24 ns, i.e. after the intercalation complex had stabilized, are in red.

Fig S11. The solvent exposed surface area of the benzimidazole fragment of HT, i.e rings R2 and R3, during the molecular dynamics simulations of A) free HT and B) HT bound to TSMC.
Fig S12. The torsion angle $\beta$ between rings R2 and R3 of HT during the molecular dynamics simulations of A) free HT and B) HT bound to TSMC.
Fig S13. (A) 1D $^1$H spectra of free TSMC (black), of a sample with a HT/TSMC molar ratio of 0:50:1 (blue) and free HT (red). In the 1D spectra of free HT, and of HT/RNA ratio 0.50:1, the signal for the HT methyl group of methylpiperazine ring (R1) is marked with *. (B) Superposition of 2D $^1$H TOCSY spectra recorded in $^2$H$_2$O for samples with HT:TSMC RNA molar ratios of 1:0 (black) and 1:0.25 (purple). The assignments for the proton pairs of R4 as well R3 and R2 are shown. The protons from the methyl group in R1 at 2.8 ppm is the only signal observed for HT after addition of RNA, whereas the signals for aromatic protons between 6.5-7.5 ppm were completely bleached.
SUPPLEMENTARY DISCUSSION

Intercalation is the major binding mode of HT to RNA: UV-Vis and fluorescence results

Reference for HT groove binding - AT-DNA. Titration of HT with AT-DNA caused a decrease of the absorption (maximum extinction ratio, 0.6) and a slight red shift with the first additions (DNA/HT mole ratio lower than 1:10, Fig S5). Subsequent DNA additions resulted in a gradual absorption increase, with a final maximum extinction ratio of ca. 1 with respect to HT alone, and a maximum at 352 nm (Table 1 and Fig. S5).

On the other hand, starting from the 510 nm maximum of HT alone, an immediate blue-shift of the fluorescence emission and a regular, marked increase in the emission band area were observed upon addition of AT-DNA. The final maximum wavelength and area ratio were respectively, 445 nm and ca. 60. For HT alone, the fluorescence excitation spectrum is red-shifted (+9 nm) relative to the absorption spectrum (Fig. 3B), an indication of the presence of a significant amount of hypsochromically absorbing, non-fluorescent species (the nature of this species was not investigated further). However, at the end of the titration with AT-DNA the absorption and excitation spectra were almost superimposable (Table 1 and Fig. 3B). Also, for both HT alone and its complex with AT-DNA, the emission and excitation spectra were independent of the excitation and emission wavelengths, respectively. Therefore, a single fluorescent species, which was the only absorbing one for the HT/AT-DNA complex, was observed. This conclusion was supported by time-correlated single-photon counting experiments, where, apart from a minor additional contribution of 0.3 ns due to uncomplexed HT, fluorescence time decays at the end of the titration with AT-DNA were well described as single exponentials with a lifetime of 2.8 ns. These observations were consistent with the available literature (8, S1-S2).

The sudden blue-shift and the regular intensity increase of fluorescence observed with the first additions of AT-DNA, as opposed to the steep initial absorption decrease, suggest that the main species formed at low AT-DNA/H ratios do not significantly emit. We identify these species as H-type (S3) HT aggregates bound to DNA molecules (26). Consistent with this assignment, the increase of AT-DNA/HT ratio along the titration, i.e. increased availability of binding sites for HT, resulted in progressive disruption of the non-emitting aggregates, which completed at an AT-DNA/HT mole ratio around 3 (insets in Fig. S5). The features observed at titration end (Table 1) could be considered reliable markers of the classical minor groove binding of HT with AT-rich DNAs. The absorption red-shift was attributed to a decrease in polarity of the environment around HT in the DNA minor groove relative to the water solution. The marked emission blue-shift and intensity increase are usually attributed to a cage effect in the DNA minor groove, with the HT chromophoric unit loosing torsional freedom. In fact, torsional relaxation of uncomplexed HT is believed to give rise to a very large Stokes shift and a fast radiationless excited-state deactivation, resulting in the low emission quantum yield (S2). This reduced torsional freedom would also account for about a ten-fold increase in lifetime.
Reference for partial intercalation - GC-DNA. For the complexation of HT with GC-rich DNA, partial intercalation, characterized by a lifetime component around 1.5 ns (8), ‘stacking or adsorption’ resulting in high exposure to water (lifetime component ca. 500 ps) (1), and binding as a dimer in the major groove (26) have been proposed. Upon addition of GC-DNA, we observed an initial pronounced decrease in absorption (maximum extinction ratio, 0.43) that remained stable up to rather high DNA/H ratios (around 10), but essentially no maximum shift (Table 1 and Fig. S6). Further additions of GC-DNA resulted in a recovery of the absorption that was not complete at the largest GC-DNA/HT ratio achieved (ca. 30). The emission intensity first decreased, in correspondence with the fast absorption decrease and then increased very regularly with no sign of saturation. The emission maximum shifted hypsochromically, but both the relative intensity and the maximum blue-shift remained much lower than the corresponding properties of the complex with AT-DNA (Fig. S5 and Table 1). The excitation maximum for the largest GC-DNA/HT mole ratio, measured at 361 nm, was about 20 nm to the red of the absorption maximum; indicating that complexation with GC-DNA corresponded to formation of several different species. However, based on the single exponential fluorescence decay and the independence of the emission and excitation spectra from the excitation and emission wavelengths, respectively, it was concluded that a single emitting form with the properties reported in Table 1 was obtained. However, other hypsochromically absorbing, non-emitting species were present. The difference between the absorption and the excitation bands indicates that no significant excitation transfer occurred between these species and the emitting one. The decrease in absorption, its relatively blue-shifted maximum and the low emission quantum yield suggest that GC-DNA addition initially promoted formation of solvent exposed aggregates, likely at the major groove, which were progressively disrupted with increasing DNA/HT ratio. However, the process required very large GC-DNA additions, as no sign of saturation was visible at a GC-DNA/H mole ratio as large as 30:1 (Fig. S6). The fluorescent complex had a lifetime of 4.1 ns (Table 1), longer than that of the AT-DNA/HT complex, 2.8 ns. This, together with the red-shift of its excitation spectrum relative to those of free HT and of its complex with AT-DNA, suggest that, at sufficiently high GC-DNA/H ratios, HT was bound to GC-DNA in a rotationally hindered and relatively little exposed conformation, properties that are consistent with an intercalative, rather than a major-groove, binding mode.

Second binding site at the loop

In the simulation B1, after the HT was released into the solvent, it diffused back to make stable van der Waals contacts with the sugar moieties in the GNRA tetraloop. Such surface contacts were probably driven by the favorable desolvation of aromatic rings of HT. The binding at the loop would be consistent with the chemical shift perturbations of the aromatic-anomeric correlations for the loop residues, upon ligand binding (Fig S3C, D). However, since the loop does not have any biological relevance, this was not pursued further.
SUPPLEMENTARY REFERENCES

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