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# Synthetic approaches to bis-adenosine derivatives as potential bisubstrates of RNA methyltransferases†

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The development of bisubstrate molecules mimicking the transition state of RNA methylation offers a promising approach for modulating post-transcriptional processes. In this study, five SAM–adenosine conjugates were synthesized, each incorporating a SAM cofactor analog linked to the *N*1 position of adenosine *via* triazole- and amide-based connectors. Cellular assays demonstrate that these compounds were not cytotoxic at 10 μM on SW620 and MCF-7 human cancer cell lines. Notably, one conjugate significantly affected several mRNA methylation processes in colorectal SW620 cells at this concentration. Furthermore, four compounds inhibited sphere formation in both cancer cell lines, underscoring their potential as tools to modulate RNA methylation in oncogenic contexts and guide the design of new therapeutic agents.

## Introduction

The bisubstrate strategy is used to provide molecular tools for the study of RNA methyltransferases (MTases) or potent inhibitors that mimic the transition state of RNA methylation by simultaneously targeting both MTase substrate binding sites (SAM and RNA sites) (Scheme 1A).[1] We were the first to develop SAM–RNA conjugates as $m^6A$ bisubstrates analogues for structural studies of bacterial and human $m^6A$ MTases.[2–7] The use of such compounds has also enabled the inhibition of coronavirus RNA N7 MTase with submicromolar $IC_{50}$ values.[8–15]

In this study, we aim to synthesize new RNA bisubstrate molecules, the design of such compounds being inspired by the transition state of the $m^1A$ catalytic process (Scheme 1B and C).

m1-Methyladenosine ($m^1A$) is one of the most frequent post-transcriptional modifications found in RNA.[16] Identified for the first time in 1963 by Dunn *et al.*,[17] this covalent mark is abundant in tRNA,[18–21] rRNA,[22,23] mitochondrial RNAs,[24–26] and was more recently observed at a low level in mRNA.[21,25,27,28] In addition to the introduction of the methyl group on the Watson–Crick edge, this modification also provides a positive charge to the nucleotide at physiological conditions, both conferring numerous and various biological functions to $m^1A$.[29] For example, m1A58 in tRNA is essential in stabilizing tRNA and translation initiation and has an impact on reverse transcription and protein translation by blocking Watson–Crick base pairing in mRNA.[29]

The introduction of the methyl group is performed by the writers $m^1A$ methyltransferases ($m^1A$ MTases), that use *S*-adenosyl-L-methionine (SAM) as a cofactor leading to the release of *S*-adenosyl-L-homocysteine (SAH) (Scheme 1B). This process is dynamic and some demethylases belonging to the ALKB family, also known to remove $m^6A$, have been identified as $m^1A$ erasers.[28,30] Finally, the $m^1A$ are recognized by members of the YTH domain protein family to regulate the function of the modified RNAs.[31]

Importantly, the dysregulation of the $m^1A$ process is involved in pathological disorders such as cancers,[32–34] HIV infection,[35] bacterial antibiotic resistance,[36–38] or Alzheimer's disease.[39] Most of the research work aimed at understanding the impact of $m^1A$ dysregulation in cancer has focused primarily on tRNA methylation.[32] More recently, a study has shown that decreased $m^1A$ levels mediated by ALKBH3 resulted in increased mRNA transcript levels of colony stimulating factor 1 (CSF1), thereby promoting cell invasion without altering cell proliferation or migration in ovarian and breast cancer cells.[40] This result suggests a potential pathological consequence of $m^1A$ dysregulation in mRNA molecules as well. In conclusion,

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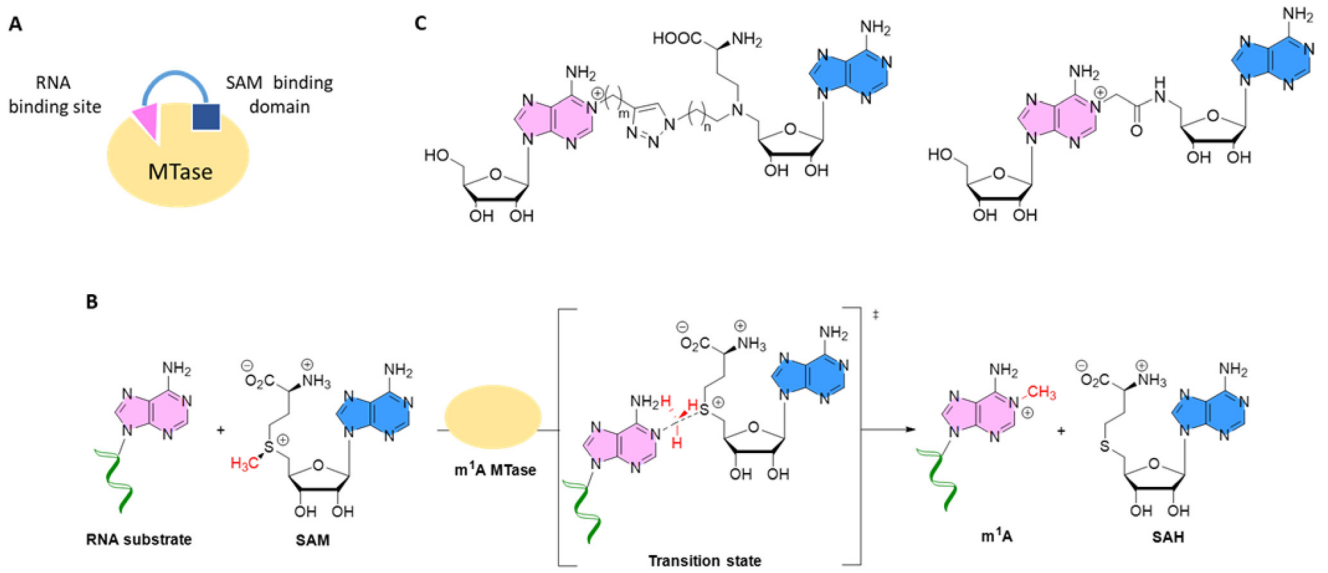


**Scheme 1** A) Bisubstrate strategy applied to RNA MTases. (B) $m^1A$ methylation of RNA catalyzed by $m^1A$ MTases. (C) General structure of bisubstrate analogues mimicking the transition state between *N*1 position of adenosine and SAM cofactor.

$m^1A$ is a highly significant modification. However, the effects of the $m^1A$ modification are still to be clarified, and there is limited information available regarding its frequency and its occurrence in cytosolic mRNA. For all these reasons, there is a need for molecules that affect $m^1A$ methylation in order to study this process at the molecular level and its biological effects.

The synthesis of bisubstrates for $m^1A$ MTases containing a complete analogue of the SAM moiety linked to a surrogate of the RNA substrate (such as adenosine) has never been achieved. An essential step in the chemical process to obtain $m^1A$ bisubstrate molecules is the use of a *N*1-selective alkylation reaction. In the literature, only a few examples report the synthesis of 1-*N*-alkylated adenosine derivatives. This may be due to the fact that 1-*N*-substituted adenosines are mainly used as intermediates to access the corresponding *N*6-functionalized adenosines by Dimroth rearrangement.[41] 1-*N*-Alkylated adenosine derivatives are mainly prepared by treating adenosine with the corresponding alkyl halide.[42–44] Following this methodology, the alkylation proceeds with high yield in the presence of iodomethane but reveals less efficient with other alkyl halides in which the methylene group adjacent to the halide has to be activated thanks to unsaturated carbon chains or aromatic substituents. Thus, this approach provides access to a limited number of derivatives containing methyl, ethyl, allyl or benzyl substituents. More recently, the use of barium carbonate to avoid acidification and iodide salts such as NaI or KI allowed for increased yields.[45,46] Notably, in 2016, the stereoselective synthesis of 1-tuberculosinyl adenosine, a virulence factor of *Mycobacterium tuberculosis*, was achieved in 76% yield, by reacting adenosine with an allyl chloride in the presence of sodium iodide.[47] Using phase transfer catalysis, Aritomo *et al.* observed the 1-*N*-alkylation of 6-*N*-benzoyl-2′,3′,5′-tris-*O*-(*tert*-butyldimethylsilyl) adenosine as a co-product.[48] This approach was also exploited by Oslovsky *et al.* for the alkylation of 6-*N*-acetyl-2′,3′,5′-tris-*O*-acetyladenosine at the *N*1 position.[46] 1-*N*-Alkyladenosine derivatives were also synthesized by Terrazas *et al.* by reacting activated 1-(2,4-dinitrobenzenesulfonyl)inosines with primary amines.[49] Finally, a different route allowed for the building of the 1-*N*-adenosine ring by cyclisation between an imidazole nucleoside and ribosylamines for the synthesis of stable analogues of cyclic ADP-ribose analogues.[50] Starting from adenosine, we recently introduced in a single step an alkyne group on the *N*1 position of adenosine which led us to obtain by copper(I)-catalyzed alkyne–azide cycloaddition[51,52] (CuAAC) the conjugate **I** as the first bisubstrate analogue of $m^1A$ MTases (Scheme 2A).[4] However, compound **I** does not contain the amino acid chain of the SAM cofactor which may be crucial for the recognition by the enzymes. Since our chemical route doesn't allow the introduction of structural variations on the conjugate, we decided to go forward to obtain more complex SAM–adenosine conjugates. In this new approach (Scheme 2B), an azido-ethyl or azido-propyl group is introduced on the *N*1 position of adenosine in a single step affording the partners mimicking the RNA substrate. The second partner, the SAM analogue, is obtained by a reductive amination followed by a nucleophilic substitution reaction and the key step to connect the two partners is achieved using CuAAC. This strategy affords conjugates bearing the amino acid chain, making these compounds more relevant for the study of $m^1A$ MTases (Scheme 2B). In addition, we prepared a SAM–adenosine conjugate containing an amide function to link the two units.

As the ultimate goal of this project is to target MTases in living cells, we evaluated the effect of these conjugates on two human cancer cell lines, namely SW620 (colorectal cancer)

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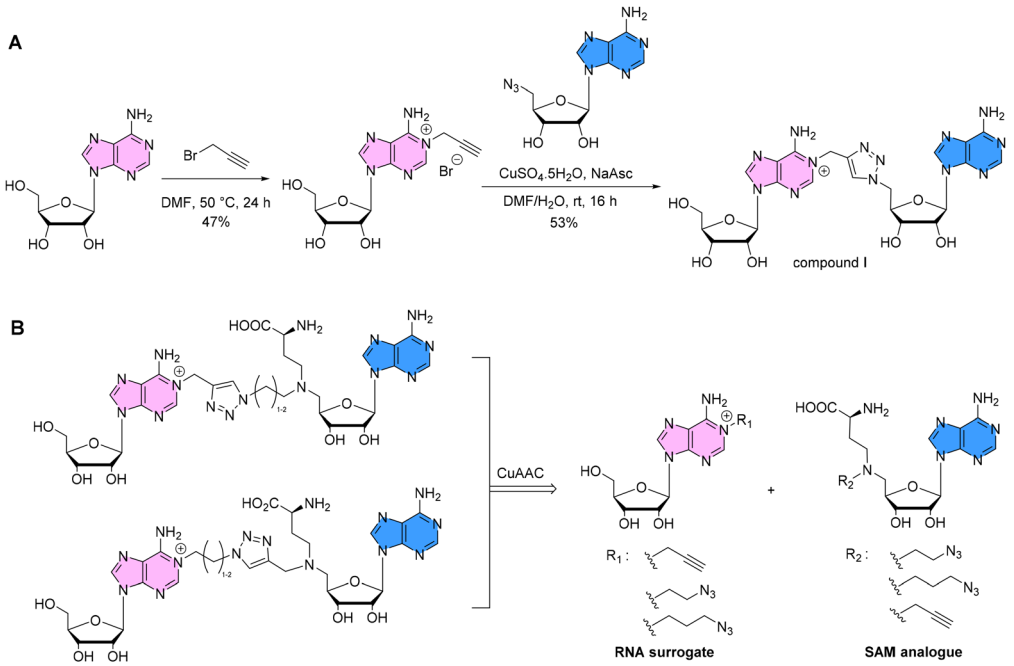


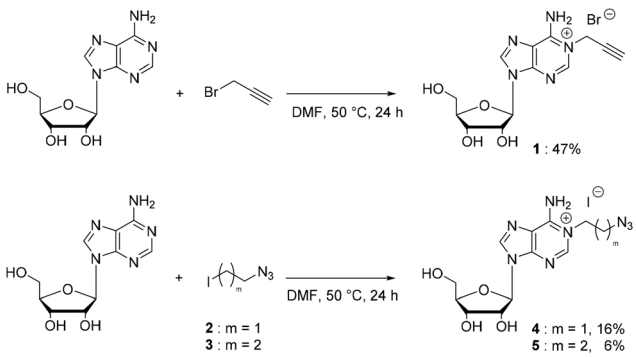

**Scheme 2** A) Synthesis of compound I. (B) Retrosynthetic route to access the bisubstrate analogues prepared in this work.

and MCF7 (breast cancer). To this end, we studied their impact on individual RNA species (tRNA and mRNA) to assess their selectivity in a cellular context. Finally, we examined the consequences on key biological features associated with cancer aggressiveness.

## Results and discussion

### Chemistry

To access the triazole-based bisubstrates **14–17**, adenosine was first treated with 5 equivalents of 2-azido-iodoethane **2** or 3-azido-iodopropane **3**,[53] using our previously reported conditions for the synthesis of 1-*N*-propargyl-adenosine **1**,[4] to afford the corresponding 1-*N*-alkylated adenosines **4** and **5** in 16 and 6% yield, respectively (Scheme 3). The low yields observed for the formation of compounds **4** and **5** can be attributed to the lower electrophilicity of the iodoalkanes compared to propargyl bromide. It is also important to note that this reaction is carried out with unprotected nucleosides. This approach reduces the number of synthetic steps by eliminating the need for protection and deprotection of reactive groups. However, purification must be performed by reversed-phase HPLC, which results in some loss of product contributing to the low yield of this reaction. The site of alkylation was confirmed by 2D NMR experiments. Indeed, the 2D HMBC experiment carried out with compound **4** shows two correlations between the protons (Hα) of the methylene and the carbons C2 and C6 of adenosine (Fig. 1). Despite low yields, the alkylation reaction provided sufficient quantities of the two adenosine derivatives **4** and **5** to carry out the CuAAC reactions.




**Scheme 3** Synthesis of the 1-*N*-alkylated adenosine derivatives **1**, **4** and **5**.

Two approaches were considered for the synthesis of the SAM analogues **11** and **12**. In a first intent, we used the classic strategy to access SAM analogues with a nitrogen atom instead of the sulfur,[3] by engaging the protected adenosine **6**[54] in a reductive amination reaction (path A). In this aim, compound **6** was reacted with 3-azidopropanal[55] providing the azido compound **8** in 50% yield. Unfortunately, we were not able to prepare azidoacetaldehyde following the reported synthesis[55] to get compound **7**. As an alternative, nucleophilic substitution reactions were carried out with the mesylate derivatives **9** and **10**[56] in the presence of $K_2CO_3$ and KI in refluxing acetonitrile for 72 hours (path B). In these conditions, derivatives **7** and **8** were obtained in 16 and 20% yield, due to the low nucleophilicity of the secondary amine. Subsequent removal of the protecting groups using aqueous TFA solution provided the azides **11** and **12** in 54 and 55% yield, respectively (Scheme 4).

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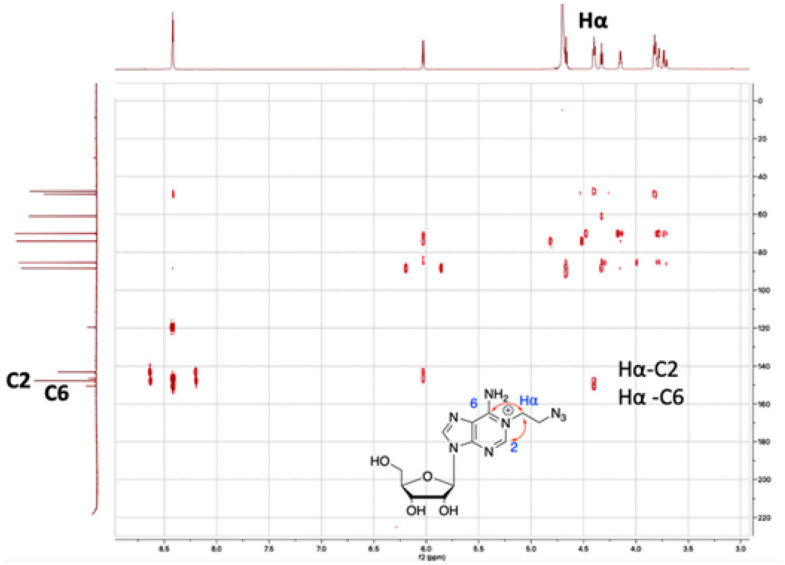
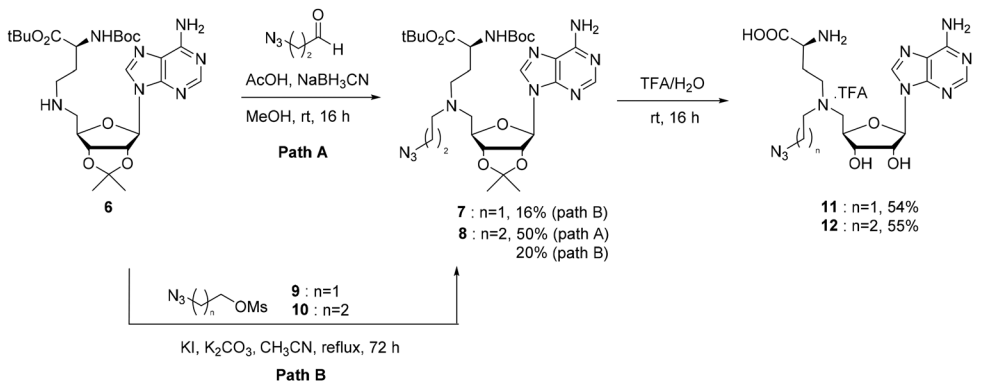



**Fig. 1** 2D HMBC spectrum (500 MHz, $D_2O$) of compound **4**.




**Scheme 4** Synthesis of the azido SAM analogues **11** and **12**.

CuAAC reactions were conducted in classic conditions between the alkynes modified nucleosides (compounds **1** and **13**[54]) and the azido-nucleosides **4**, **5**, **11** and **12**, in the presence of copper sulfate and sodium ascorbate. The SAM–adenosine conjugates **14–17** were obtained with good yields calculated by UV ($\lambda$ = 260 nm, $\varepsilon$ = 27 300 $M^{-1}$ $cm^{-1}$) between 49 and 64%, after HPLC purification (Scheme 5).

The synthesis of compound **21** was achieved in 3 steps starting from 5′-azidoadenosine **18**[5] (Scheme 6). The azide function of **18** was first reduced by hydrogenolysis in presence of Pd/C, leading to 5′-aminoadenosine **19** in 88% yield. The amide bond was then obtained by coupling amine **19** with 2-iodoacetic acid using DCC to afford the key intermediate **20** in 49% yield. Finally, adenosine was engaged in a reaction of alkylation with 1 equivalent of amide **20** to afford the expected compound **21** in 5% yield after HPLC purification. This low yield can be explained by the use of only 1 equivalent of the electrophile **20** to avoid its own alkylation.

## Biological evaluation

Most epitranscriptomic modifications play a key role in cell fate and adaptation in the context of health and disease.[57–59] In the context of cancer, the cells most prone to adaptation are termed Cancer Stem Cells (CSCs).[60] CSCs represent a minor fraction of tumor cells exhibiting stem-like characteristics, as well as enormous chemoresistance and tumor-initiating potential.[61] Growing cancer cells in suspension culture promote the CSC phenotype and the formation of microtumor-like spheroids derived from a single cancer progenitor cell.[62,63] As such, sphere-forming ability (SFA) is often considered as a mean to evaluate the tumorigenic potential of solid tumors. Recently, $m^1A$ has been identified, along with other RNA marks, as a potential adapting factor for colorectal cancer cells in suspension culture.[64] Further, we have recently highlighted the role of m1A58 modification, deposited by the SAM-dependent $m^1A$ MTase TRM6/TRM61 complex, in tumor aggressiveness and increased resistance to drug therapy, both hallmarks of

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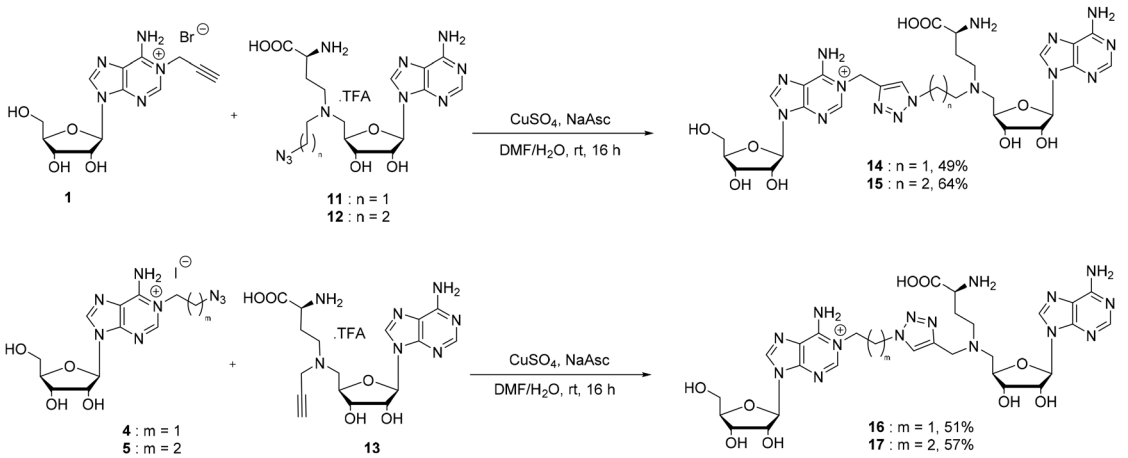
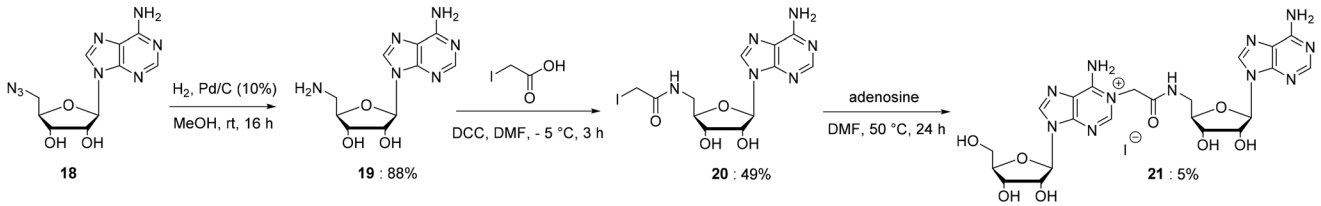



**Scheme 5** Synthesis of the SAM–adenosine conjugates **14–17**.




**Scheme 6** Synthesis of SAM–adenosine conjugate **21**.

CSCs.[32] In addition, the role of another modification, the *N*6-methyladenosine ($m^6A$) in colon cancer and glioma was highlighted, reinforcing the relevance of targeting the epitranscriptome in cancer. Based on this result, we decided to test the compounds to assess their impact to prevent cancer spread and recurrence while minimizing toxic effects that could be detrimental on healthy tissue.

The cytotoxicity of the bisubstrate analogues **14–17** and **21** was first assessed against two human cancer cell lines SW620 (a colorectal cancer cell line) and MCF7 (a breast cancer cell line), using a sulforhodamine B assay (SRB). The compounds were tested at concentrations between 1 and 10 μM for 72 h. As shown in Fig. 2, the SAM–adenosine conjugates **14–17** and **21** are not cytotoxic at these concentrations on the two cell lines. Therefore, we decided to use a concentration of 10 μM for further evaluation.

To identify RNA modifications that could be altered by the compounds, we employed liquid chromatography followed by tandem MS (LC-MS/MS). Briefly, colorectal cell line SW620 was exposed for 24 hours to a concentration of 10 μM of compounds **14–17** and **21**. mRNA and tRNA were extracted from treated cells, hydrolyzed and dephosphorylated, and nucleosides were analyzed by LC-MS/MS. For this evaluation, modifications located either on the base or on the ribose were examined. Mass spectrometry analysis revealed that all compounds have an impact on mRNA modifications when compared to control (Fig. 3). Particularly, compounds **14** and **15** containing the triazole ring close to the adenosine have similar activity

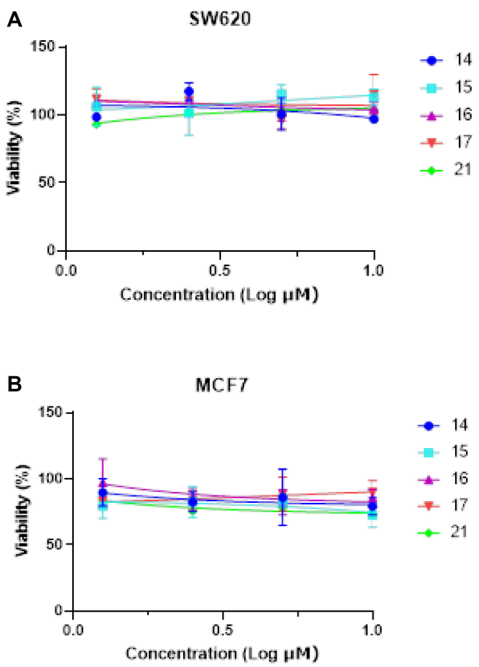



**Fig. 2** Evaluation of the cytotoxicity of the bisubstrate analogues **14–17** and **21**. SW620 (A) and MCF7 (B) were treated with each compound at concentrations between 1 and 10 μM for 72 h. Results are expressed in fold change compared to control (untreated cells) and are mean ± SEM of three distinct experiments.

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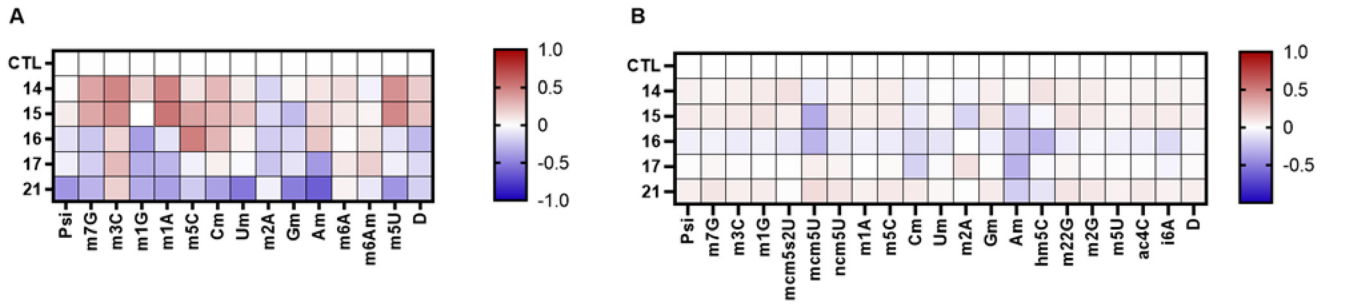


**Fig. 3** Multiple quantification of mRNA (A) and tRNA (B) modifications from colorectal cell line SW620. Heatmap representing log2 (fold change) of modified nucleoside level from mRNA (A) and tRNA (B) in the dose of 10 μM ($n$ = 3) after a 24 h exposure to the compounds **14–17** and **21**. A gradual change in the color from blue to red indicates a change in level of methylation from low to high.

profile on the modifications by increasing slightly the level of $m^7G$, $m^3C$, $m^1A$ and $m^5U$. In parallel, compound **16** and **17** with the triazole ring next to the SAM moiety show also a comparable behaviour, notably by decreasing $m^1G$ with the exception of their impact on $m^5C$ and Am modifications. Conjugate **21** affects most of the mRNA methylations with a slight decrease of $m^1A$ and Nm (2′-*O*-methylations) level (Fig. 3). Interestingly, the compounds do not affect $m^6A$ and $m^6Am$. Regarding tRNA modifications, the effects are less pronounced: conjugates **15–17** and **21** slightly reduce Am levels, while a small decrease in $mcm^5U$ levels is observed when cells are exposed to compounds **15** and **16**. Given the number of modifications altered upon exposure to the conjugates, these results suggest that the compounds can cross the cell membrane.

To assess the impact of the bisubstrate analogues on sphere formation ability, we performed this assay on the two previous cancer cell lines (SW620, MCF7). Briefly, cells were incubated at low density in a serum deprived medium in the presence of the conjugates **14–17** and **21** at a concentration of 10 μM for 7 days. The results show that compounds **14–17** decrease by 50% the ability of the two cancer cell lines to form spheres compared to control at the concentration of 10 μM (Fig. 4A and B). As shown in Fig. 2, this effect is not an indirect consequence of compounds toxicity. By contrast, compound **21** does not affect sphere formation, though its capabilities to reduce the level of several RNA methylations.

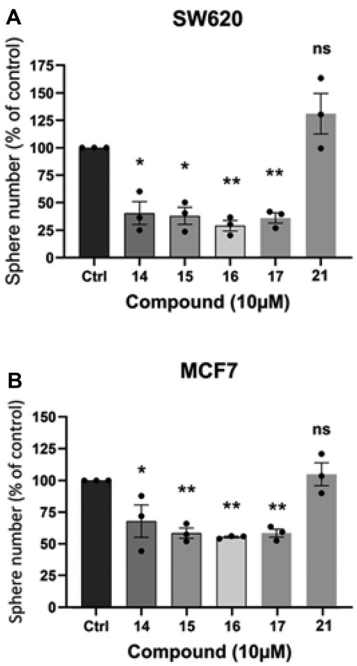


**Fig. 4** Quantification of the relative percentage of sphere formation at a concentration of 10 μM of compounds **14–17** and **21** on (A) SW620 and (B) MCF7. Results are expressed in fold change compared to control. $n$ = 3 biological replicates. Mean ± SEM. *$p$-Value < 0.05 **$p$-value < 0.01. One way Anova followed by multiple comparisons.

## Conclusions

In summary, we have reported the synthesis of five SAM–adenosine conjugates. These compounds contain either a triazole- or an amide-based linker to connect the SAM analogue and the adenosine that mimicks RNA substrate. These compounds were synthesized using CuAAC to obtain the triazole linker or by *N*1 alkylation of adenosine. Our chemical approach did not require a multi-step synthesis as we avoided the use of protecting groups for the functionalization of the adenosine moiety, though introduction of the azide function provides *N*1 alkylated adenosine in low yields.

Preliminary biological assays were conducted to evaluate the impact of these compounds on cancer cells. At a concentration of 10 μM, none of the five compounds exhibited cytotoxicity against the two human cancer cell lines SW620 and MCF-7. Then, the quantification of mRNA and tRNA modifications was carried out using mass spectrometry analysis. Only exposure of SW620 colorectal cancer cell to compound **17** and **21** leads to a slight decrease of $m^1A$ level of mRNA in the SW620 human cancer cell line but, also affects modifications such as Um, Gm, and Am. It has been previously reported in the literature that bisubstrate molecules, designed to interact with a specific methylation process (such as $m^1A$ RNA methylation here) can also affect other methylations. For instance,

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compounds designed to inhibit DNA MTases have shown activity on protein MTases,[65] and inhibitors targeting the 2′-*O*-methylation of viral MTases have been found to act on viral N7G MTases[8] and *vice versa*.[66]

Regardless, compounds exhibit a biological effect on sphere-forming ability. This effect may result from the inhibition of multiple RNA marks.[67] The next step will be to carry out further functional tests to determine whether other "stem-like" properties, such as chemotherapy resistance or tumor initiation capacity, are affected.

## Author contributions

M.E.Q. and E.B. conceived the idea and guided the project. J.D., B.F., D.C., and L.I. conducted the experiments and analysed the experimental data. F.M., A.D., M.E.Q., and E.B. co-wrote the paper with inputs from all the other authors. All authors have approved the final version of the manuscript.

## Data availability

The data supporting this article have been included in the ESI.†

## Conflicts of interest

There are no conflicts to declare.

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