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RAPID COMMUNICATION

Development of novel 5-FU modified siRNA against BCL-2 with enhanced efficacy and vehicle-free cellular uptake

One of the major obstacles for nucleic acid-based medicine is cellular uptake due to the concern of efficiency and potential toxicity of the vehicle. In this work, we developed a novel approach to eliminate the need for a vehicle to allow for the cellular uptake of siRNA-based therapy with a unique modification strategy that also further enhances therapeutic efficacy. This discovery may be of great importance for the development of siRNA-based therapeutics as cellular uptake remains a critical challenge. In this study, we have tested the potential of this modification strategy for siRNA using a siRNA that targets B-cell lymphoma-2 (BCL-2). BCL-2 is an important anti-apoptotic gene and is overexpressed in many different cancers.[1] As a result, venetoclax,[2] a BCL-2 inhibitor, has been developed and is currently used either alone or in combination with obinutuzumab (Gazyva) or rituximab (Rituxan) to treat certain types of chronic lymphocytic leukemia (CLL) or certain types of small lymphocytic lymphoma (SLL). It is also used in combination with either azacitidine (Vidaza), decitabine (Dacogen), or cytarabine as a treatment for acute myeloid leukemia (AML). In this study, we generated a modified siRNA against BCL-2 in which all uracil residues in the sense and antisense strands are replaced with 5-fluorouracil (5-FU). 5-FU is a uracil analog that blocks *de novo* pyrimidine biosynthesis by forming a suicide complex with its target protein thymidylate synthase (TYMS, TS) and its metabolite, fluorodeoxyuridine monophosphate (FdUMP).[3] Based on this, 5-FU is one of the major chemotherapeutic agents used to treat various tumor types.

To demonstrate the potential of 5-FU-modified siRNAs as novel anticancer therapeutics, we selected the well-characterized BCL-2 as our target. We generated a modified siRNA against BCL-2 in which all uracil residues in both the sense and antisense strands have been replaced with 5-FU (Fig. 1A). There are no other modifications on siBCL-2. The rationale behind this approach is to combine the therapeutic power of 5-FU and siBCL-2 into one entity to further enhance the therapeutic efficacy of siBCL-2, as the siRNA will eventually break down to release 5-FU intracellularly. Because the only difference between uracil and 5-FU is the fluorine at the 5-position, it should not alter the Watson-Crick base-pairing with its target mRNA and its incorporation into the RNA silencing complex (RISC). In addition, we reasoned that the fluorine group will enhance the lipophilicity of the siRNA molecule, facilitating its cellular uptake across the lipid bilayer of the cellular membrane without the need for a vehicle.

In order to test if 5-FU-siBCL-2 retains the ability to inhibit BCL-2 and be delivered into cancer cells without a transfection vehicle, we transfected HCT 116 human colon cancer cells with 50 nM of either control siRNA, unmodified siBCL-2, or 5-FU-siBCL-2 with or without a transfection vehicle. We used qRT-PCR to assess the expression of BCL-2 after transfection. Our results show that with the transfection vehicle, oligofectamine, both unmodified siBCL-2 and 5-FU-siBCL2 reduced the mRNA expression level of BCL-2 (left panel, Fig. 1B). In contrast, without a vehicle, unmodified siBCL-2 did not affect the level of BCL-2 mRNA as it cannot be internalized by the cells, while 5-FU-siBCL-2 decreased the level of BCL-2 mRNA (left panel, Fig. 1B). In addition to the mRNA expression level of the target BCL-2, we also assessed the effects of these siRNAs on BCL-2 protein expression levels. The protein expression levels of BCL-2 were consistent with the mRNA transcript level (right panel, Fig. 1B). With a transfection vehicle, both unmodified siBCL-2 and 5-FU-siBCL-2 inhibited BCL-2 protein expression. Without a transfection vehicle, only modified 5-FU-siBCL-2 was able to inhibit BCL-2 expression, and the unmodified siBCL-2 had no effect (right panel, Fig. 1B). These results suggest that without a vehicle, 5-FU-siBCL-2 was able to cross the lipid bilayer and be internalized by

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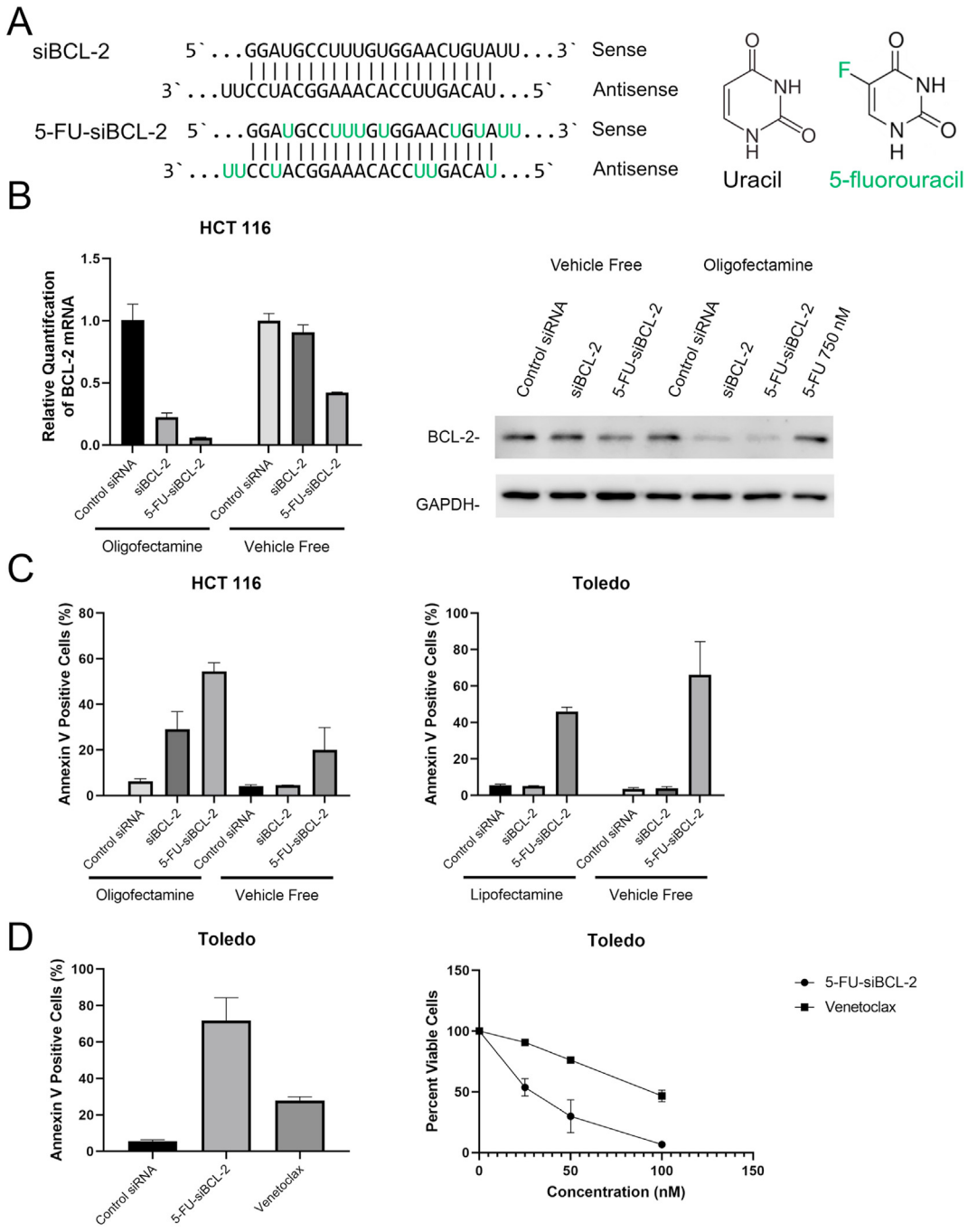

Figure 1 5-FU-siBCL-2 incorporates 5-FU into siBCL-2 and enhances the therapeutic potential of siBCL-2. **(A)** 5-FU-siBCL-2 was made by replacing all uracil residues in siBCL-2 with 5-fluorouracil. **(B)** 5-FU-siBCL-2 maintains its ability to inhibit BCL-2 expression with or without a transfection vehicle while unmodified siBCL-2 requires a vehicle to have this effect, at the mRNA level measured by qRT-PCR (ΔΔCt) (left) as well as at the protein level measured by Western blot (right). **(C)** 5-FU-siBCL-2 (50 nM) can induce apoptosis with or without a transfection vehicle in HCT 116 colon cancer cells (left) and Toledo large B cell lymphoma cells (right) while unmodified siBCL-2 requires a transfection vehicle to induce apoptosis in these cells. **(D)** 5-FU-siBCL-2 is more effective at inducing apoptosis in Toledo cells than venetoclax (left) and 5-FU-siBCL-2 (IC_{50} = 25 nM) inhibits Toledo cell viability at a lower dose than venetoclax (IC_{50} = 95 nM) (right).

the cancer cells due to its enhanced lipophilicity, a result of the fluorine molecule. This increase in lipophilicity by fluorine may allow for the negatively charged siRNA to cross the cell membrane.[4] We also treated cells with a molar concentration of 5-FU (750 nM) equal to the amount of 5-FU that would be released assuming that 50 nM of 5-FU-siBCL-2 is completely broken down to show that 5-FU alone does not cause the reduction of BCL-2 expression (right panel, Fig. 1B). In addition, we demonstrated that once 5-FU-siBCL-2 is broken down, it would release 5-FU to form a

suicide protein complex with its target protein, thymidylate synthase (TS), as seen by an upward shift in the TS protein band via Western blot analysis (Fig. S1).

To determine the impact of 5-FU-siBCL-2 on apoptosis, we measured the effect of both unmodified siBCL-2 and 5-FU-siBCL-2 (50 nM) in both colon cancer HCT 116 and large B cell lymphoma Toledo cells with or without a vehicle. Unmodified siBCL-2 and 5-FU-siBCL-2 were both able to increase apoptosis with a transfection vehicle in both cell lines (Fig. 1C). Without a vehicle, there was a significant increase in apoptosis with 5-FU-siBCL-2 treated HCT 116 cells (over 20%) (left panel, Fig. 1C) and Toledo cells (over 40%) (right panel, Fig. 1C). Without a vehicle, the unmodified siBCL-2 did not trigger apoptosis in these cells. These results along with the qRT-PCR and Western blot data suggest 5-FU-siBCL-2 was capable of inducing apoptosis by blocking BCL-2 expression.

We also compared the ability of 5-FU-siBCL-2 to induce apoptosis with venetoclax in Toledo cells. Our results show that 5-FU-siBCL-2 at 50 nM was able to increase apoptosis by over 60%, while 50 nM of venetoclax only increased apoptosis by over 20% (left panel, Fig. 1D). To directly compare the therapeutic efficacy of 5-FU-siBCL-2 with venetoclax, we determined the IC_{50} value using Toledo cells via cell proliferation assay. Our results show that without a vehicle, 5-FU-siBCL-2 (IC_{50} = 25 nM) was more potent than venetoclax (IC_{50} = 95 nM) in inhibiting lymphoma cell growth (right panel, Fig. 1D). These results indicate that 5-FU-siBCL-2 may have a therapeutic advantage compared to venetoclax, as 5-FU-siBCL-2 both inhibits BCL-2 and exhibits 5-FU function. As a result, in patients resistant to venetoclax,[5] 5-FU-siBCL-2 may offer a unique advantage.

In summary, our results suggest that 5-FU incorporation into siBCL-2 does not alter target specificity with a novel feature of vehicle-free cellular uptake. This vehicle-free cellular uptake may offer potential advancement for siRNA-based therapeutic development. Considering the fact that 5-FU-siBCL-2 is more potent than venetoclax, it may have potential as a next-generation BCL-2 inhibitor. In addition, once broken down, the incorporation of 5-FU provides an additional multi-target ability and as a result, has the potential to overcome resistance to cancer therapeutics. Future studies are clearly needed to demonstrate the therapeutic efficacy of 5-FU-siBCL-2 *in vivo*. Based on this proof of concept, this unique strategy of 5-FU modification of siRNA may be useful as a platform for drug development technology for cancer treatment and other human diseases.

Conflict of interests

A.F. and J.J. have filed a patent for 5-FU-modified siRNA mimetics. J.J. is a scientific co-founder of Curamir Therapeutics. The remaining authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.11.020.

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