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Antibody-Targeted Covalent Inhibitor Conjugate (Ab-TCI) Bridged by an Arsenic-Thiol Bond Enables "Double Insurance" Targeting of Cancer Cells and Kinase for Effective Cancer Treatment

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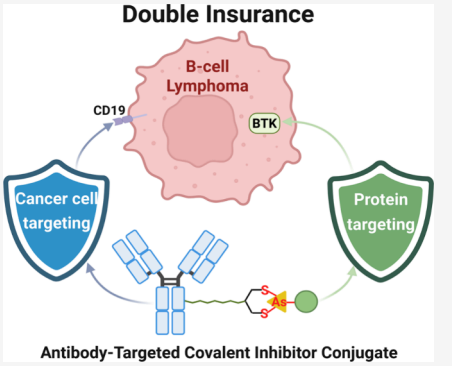
ABSTRACT: Targeted covalent inhibitors (TCIs), a class of pharmaceuticals that specifically recognize disease-related proteins and form covalent bonds for precise therapy, still suffer from side effects and diminished efficacy due to poor tumor cell specificity. To address this, we present a new cell/protein "Double Insurance" targeting strategy through constructing a novel antibody-TCI conjugate (Ab-TCI), which comprises a monoclonal antibody (Loncastuximab), a TCI (As-Ibt) for Bruton's tyrosine kinase (BTK), and between them an arsenic-thiol bond as a new cleavable linker. This Ab-TCI enables B-cell lymphoma cell recognition, internalization, and release of As-Ibt by intracellular GSH, efficiently inhibiting BTK, and effectively suppressing the growth of xenograft tumors. To our knowledge, this is the first Ab-TCI enabling simultaneous dual targeting capabilities for cancer cells and kinase, achieving a remarkable improvement in drug efficacy. New potent dual-targeting Ab-TCIs could be inspired by integrating different FDA approved TCIs and antibodies through our strategy for cancer treatment.



Targeted covalent inhibitors (TCIs) are an important class of drugs aiming to inhibit disease-associated proteins by target recognition and the formation of covalent bonds, thereby achieving precise therapeutic effects.[1,2] The mechanism of TCIs hinges on two independent steps. First, its high-affinity ligand reversibly binds to the target protein, bringing the electrophilic warhead close to the nucleophilic residues on the protein. Subsequently, the electrophilic warhead spontaneously forms a covalent bond with the residue, achieving potent protein inhibition.[3,4] The US Food and Drug Administration (FDA) has approved numerous TCIs for cancer treatment.[5] However, TCIs still face the challenge of poor cell specificity since they lack the capability to specifically recognize the targeted cancer cells, resulting side effects and diminished efficacy.[6] Therefore, endowing small molecule TCIs with a cellular targeting capability is expected to further enhance their pharmacological activity.

Monoclonal antibodies (mAbs) are increasingly crucial in tumor treatment due to their excellent cell-targeting properties and pharmacokinetic characteristics.[7,8] With the ability to specifically target tumor cells through unique antigens, mAbs offer outstanding cellular targeting capabilities and minimal side effects. However, most mAbs exhibit a limited cytotoxicity against tumor cells. Hence, they are frequently conjugated with high toxic small molecule payloads to develop antibody−drug conjugates (ADCs).[9,10] Although exhibiting a significant killing effect on cancer cells, risks still remain due to unwanted leakage of these high toxic payloads which lack specificity to the targeted protein and cells.[11,12]

To address this, we propose a novel tumor cell/protein dual-targeting strategy enabling "Double Insurance" to empower TCIs with cancer cell targeting ability and reduce the unwanted toxicity of traditional high toxic payloads, significantly improving drug efficacy and safety. On the basis of this strategy, we developed a novel antibody-targeted covalent inhibitor conjugate (Ab-TCI, **LTX-S_2-As-Ibt**) with dual targeting capabilities toward both tumor cells and kinases, comprising a monoclonal antibody (Loncastuximab, LTX[13]), a targeted covalent As inhibitor (As-Ibt, I-As-1[14]) for Bruton's tyrosine kinase (BTK). Additionally, taking advantage of the reversible covalent property of arsenic−sulfur bonds, we designed an arsenic-thiol-PEG linker as a new cleavable linker which is stable in the extracellular environment while cleaved inside cells by GSH through a nucleophilic substitution reaction (Figure 1A). As shown in Figure 1B, **LTX-S_2-As-Ibt** specifically recognizes the CD19[13] receptor present on the surface of B-cell lymphoma cells and is internalized into cells. Upon encountering intracellular GSH, the arsenic-thiol-PEG

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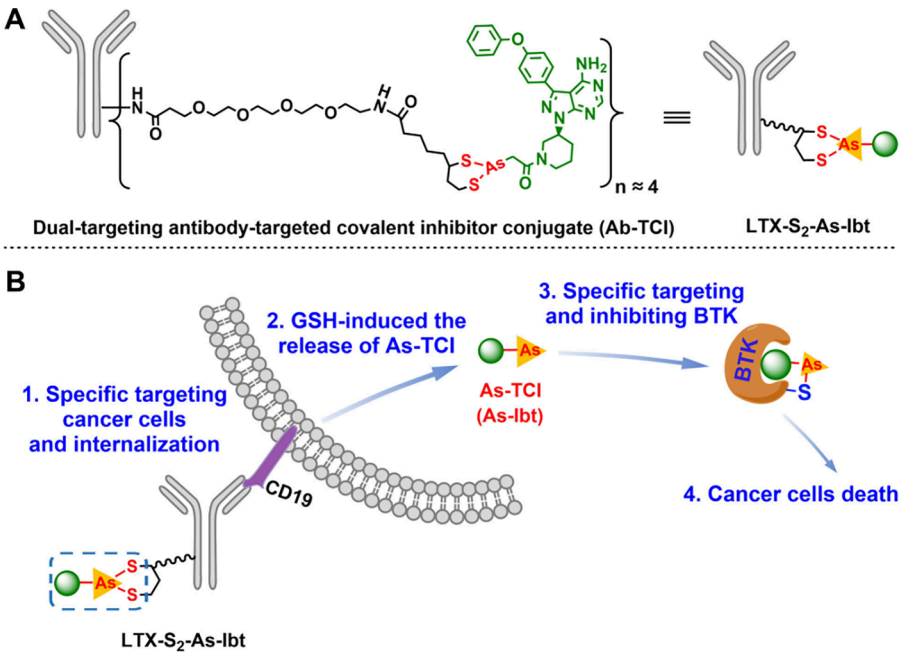

Figure 1. (A) The chemical structure of **LTX-S_2-As-Ibt**, designed through a reversible covalent arsenic−thiol bond linking antibody and TCI. (B) Schematic illustrating the mechanism of action of **LTX-S_2-As-Ibt**, demonstrating antibody mediated tumor cell targeting and subsequent intracellular release of TCI to specifically inhibit BTK.

linker of **LTX-S_2-As-Ibt** is cleaved, and then As-Ibt is released from the antibody. The targeting ligand of As-Ibt further specifically binds to BTK, bringing in close proximity to the thiol of Cys481, resulting in the formation of a covalent bond between As-Ibt and BTK.[14] In this way, specific recognition of cancer cells and targeted covalent inhibition of intracellular BTK are realized simultaneously.

B-cell malignancies are a heterogeneous group of cancers, including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and marginal zone lymphoma (MZL), ranking as the fifth most common cancer in adults. Ibrutinib[15,16] (also known as PCI-32765), the first TCI of Bruton's tyrosine kinase (BTK), was approved by the FDA in 2013 as a breakthrough therapeutic for B-cell malignancies. However, ibrutinib still suffers from side effects and drug resistance.[17,18] To address this, under the guidance of our "Double Insurance" strategy, we connect the mAbs and TCIs to develop a new Ab-TCI for the effective treatment of B-cell lymphoma. To specifically target the B-cell lymphoma cells, we introduce LTX, a cell internalizable monoclonal antibody that can specifically recognize the CD19 antigen highly expressed on the surface of targeted B-cell lymphoma cells. Meanwhile, rituximab (RTX),[19] an antibody to CD20[20] which also presents on the surface of B-cell lymphoma cells but cannot be internalized by cells, was used as a negative control of cell internalization. For targeted inhibition of the BTK strongly expressed in B-cell lymphoma,[21] we employ As-Ibt (I-As-1,[14] Figure S3) which comprises an arsenic electrophilic warhead and the guiding group of ibrutinib as the TCI toward BTK. Benefiting from the reversible covalent nature of the arsenic-thiol bond,[22,23] we exploit the arsenic-thiol bond as the cleavable linker to connecting LTX and As-Ibt. Hence, the reversible covalent arsenic-thiol bonds serve "three-in-one" functions: 1) an electrophilic warhead of TCI, 2) a bridge connecting TCI with mAbs, and 3) a cleavable linker induced by arsenic-thiol exchange reaction.

In order to conjugate As-Ibt to the antibody, we designed and synthesized NHS-PEG_4-S_2-As-Ibt (Scheme S1), which incorporates a targeting ligand of ibrutinib (green), an arsenic warhead (red) protected by the dithiol (S_2) of lipoic acid (LA), polyethylene glycol (PEG_4) as the linker to enhance water solubility, and a *N*-hydroxysuccinimide (NHS) ester group for conjugation to the amines of the antibody. The synthesis route of NHS-PEG_4-S_2-As-Ibt is shown in Scheme S1. Detailed synthetic steps are presented in the SI Experimental Section, and characterizations of the above compounds by ESIMS and NMR are provided in Figures S1, S2. We then prepared **Ab-TCI** through an amide bond formation reaction between the NHS ester group of NHS-PEG_4-S_2-As-Ibt and the amino groups of the antibodies (Scheme S2).

We first characterized **Ab-TCI (LTX-S_2-As-Ibt, RTX-S_2-As-Ibt)** using MALDI-MS, as illustrated in Figure S4A, and the LTX was detected, supported by the molecular weight (MW) of 147202 Da. The MW of 150708 Da of the highest peak was observed for **LTX-S_2-As-Ibt**, with an obvious move of the peak to higher MW, indicating the successful conjugation of the antibody with PEG_4-S_2-As-Ibt (MW = 938 Da). Furthermore, we used size-exclusion chromatography combined with inductively coupled plasma mass spectrometry (SEC-ICPMS)[24] to measure the average DAR of Ab-TCI and also to verify that the excess NHS-PEG_4-S_2-As-Ibt has been removed by ultrafiltration (Figure S5). Likewise, the RTX (MW = 147530 Da) and **RTX-S_2-As-Ibt** (MW = 151050 Da) were also characterized by MALDI-MS and SEC-ICPMS (Figure S4B, S5B). By measuring the protein concentration of **Ab-TCI** with the BCA assay and arsenic concentration with ICPMS, we calculated the average DAR of **Ab-TCI** (4.17 ± 0.31 for **LTX-S_2-As-Ibt** and 3.95 ± 0.26 for **RTX-S_2-As-Ibt**).

We then investigated the cell-targeting and internalization ability of **Ab-TCI** toward OCI-LY10 cells (a CD19+ human cell line derived from diffuse large B-cell lymphoma). Initially,

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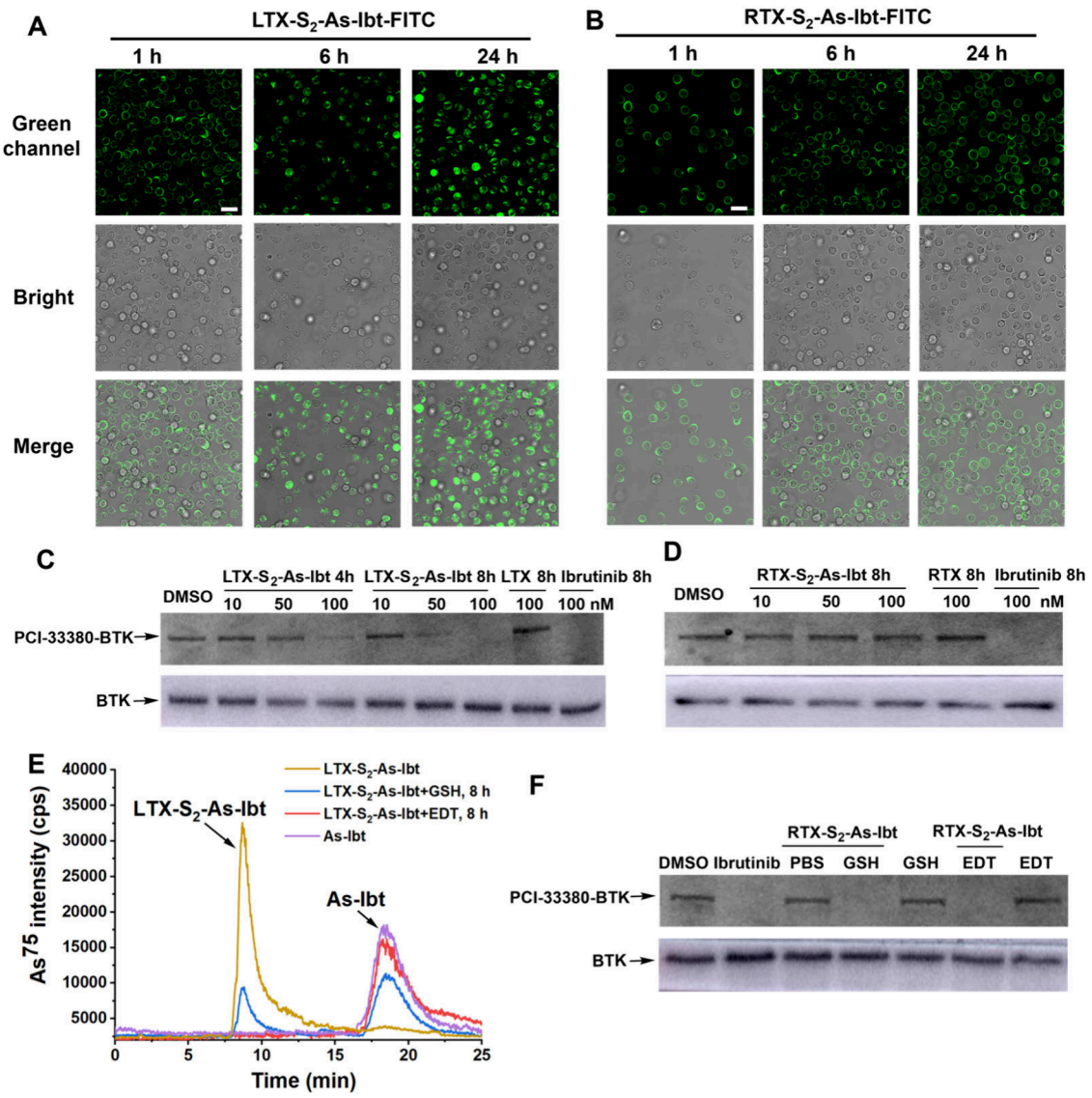

Figure 2. Confocal fluorescence image of (A) **LTX-S_2-As-Ibt** and (B) **RTX-S_2-As-Ibt** in the OCI-LY10 cells. Scale bars: 20 μm. (C, D) In situ profiling the concentration-dependent binding of Ab-TCIs to cellular BTK by competitive fluorescent labeling. (E) SEC-ICPMS analysis performed to detect the reaction products of **LTX-S_2-As-Ibt** with GSH or EDT. (F) In situ profiling of the concentration-dependent binding between cellular BTK and **RTX-S_2-As-Ibt** pretreating with GSH or EDT by competitive fluorescent labeling.

to evaluate their cell-targeting and internalization ability through confocal fluorescence imaging, we labeled the **Ab-TCI** with FITC to prepare LTX-S_2-As-Ibt-FITC/RTX-S_2-As-Ibt-FITC. As shown in Figure S6, after incubation with LTX-S_2-As-Ibt-FITC for 1 h, a green fluorescence emitted from FITC was observed on the cell membrane, indicating that LTX-S_2-As-Ibt-FITC can bind to the surface of the OCI-LY10 cells. However, OCI-LY10 cells pretreated with LTX and then incubated with LTX-S_2-As-Ibt-FITC did not exhibit fluorescence in the green channel. The results demonstrated the specific binding of LTX-S_2-As-Ibt-FITC to CD19 on OCI-LY10 cell surfaces and that the introduction of As-TCI to LTX has negligible interference to the cell-targeting ability of LTX.

As shown in Figures 2A and S7, with the incubation time increasing from 1 to 24 h, the green fluorescence in cytosol becomes stronger, indicating that LTX-S_2-As-Ibt-FITC gradually internalizes into the OCI-LY10 cells, with significant internalization observed after 6 h. In contrast, we found that HepG2 cells (CD19−) incubated with LTX-S_2-As-Ibt-FITC for 24 h did not exhibit fluorescence in the green channel (Figure S8). The results demonstrated that **LTX-S_2-As-Ibt** can specifically bind to CD19 and be internalized by target B-cell lymphoma cells. Similarly, as shown in Figure S9, RTX-S_2-As-Ibt-FITC can specifically bind to CD20, a noninternalization antigen on the surface of the OCI-LY10 cell (CD20+) surfaces. However, as the incubation time of cells increases, RTX-S_2-As-Ibt-FITC still cannot be internalized into OCI-LY10 cells even up to 24 h (Figures 2B and S10). These results indicated that **LTX-S_2-As-Ibt** had the ability to specifically target B-cell lymphoma cells and be internalized efficiently through CD19 on the cell surface.

We further investigated the in situ reactivity of **Ab-TCI** in OCI-LY10 cells. To demonstrate that the internalized **LTX-S_2-As-Ibt** can be cleaved by intracellular GSH through arsenic-thiol exchange reaction[22] and the released As-Ibt can further target and covalently bind to cellular BTK, a competitive binding assay (Figure S11A) was applied using PCI-33380[25,26] (an irreversible fluorescence probe of BTK) (Figure S3). PCI-

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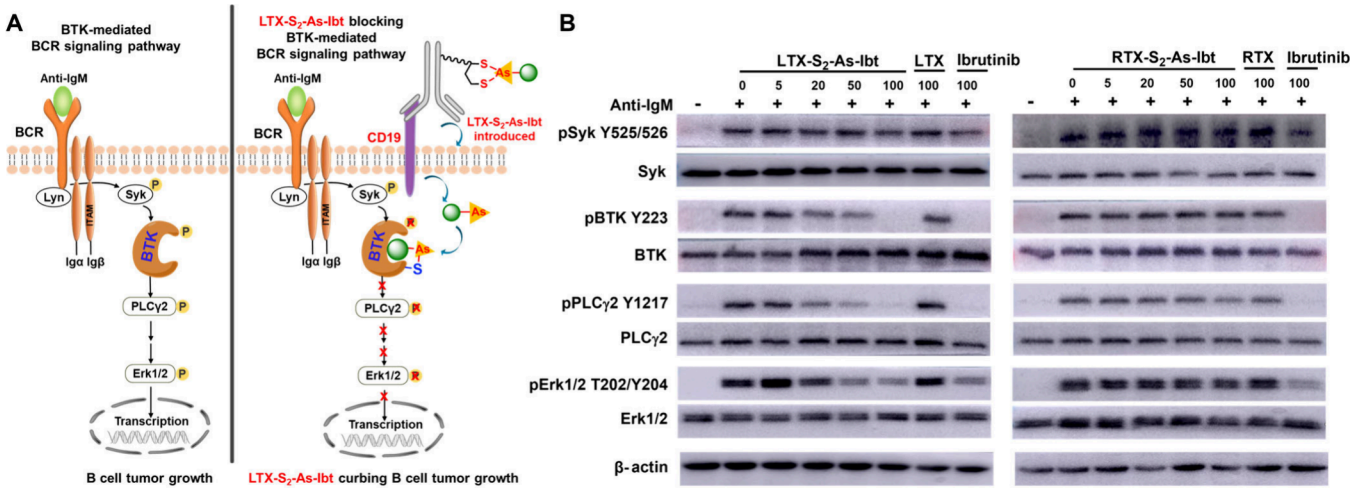

Figure 3. (A) Schematic illustrating the BTK-mediated BCR signaling pathway and its inhibition by **LTX-S_2-As-Ibt**. (B) Concentration-dependent inhibition of BCR signaling pathway stimulation-induced phosphorylation in OCI-LY10 cells by **LTX-S_2-As-Ibt**, determined by SDS-PAGE immunoblotting. Blots were probed with the indicated antibodies to detect the phosphorylation of kinases along the BCR signaling pathway.

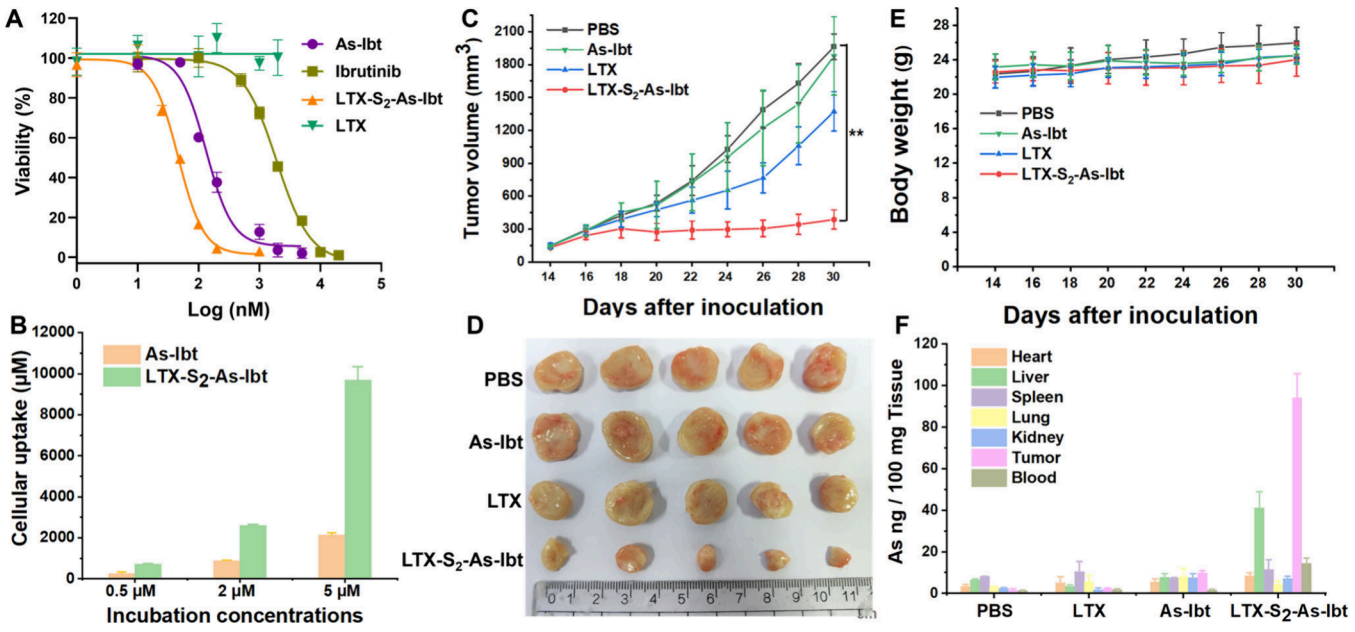

Figure 4. (A) Dose−response viability curves of inhibitors treating OCI-LY10 cells. (B) Concentration-dependent uptake of As-Ibt and LTX-S_2-As-Ibt by OCI-LY10 cells. Data are shown as the mean ± SD (n = 3). (C) Mean volume of xenograft tumor in SCID mice treated with PBS, **As-Ibt**, **LTX** and **LTX-S_2-As-Ibt**. SCID mice were injected subcutaneously with the OCI-LY10 cells. When xenograft tumor volumes reached approximately 100 mm^3, the mice were randomized into four groups (n = 5/group). They were then treated with PBS, 65 μg/kg (110 nM/kg) **As-Ibt**, 4 mg/kg (27 nM/kg) **LTX**, and 4 mg/kg (27 nM/kg) **LTX-S_2-As-Ibt** administered via the tail vein once every 4 days for a total of 4 doses. $^{**}P < 0.01$ indicates statistical significance of tumor growth inhibition. (D) Photographs of tumors excised from the mice after the completion of treatment with PBS, **As-Ibt**, **LTX** and **LTX-S_2-As-Ibt**. (E) Mice treated with PBS, **As-Ibt**, **LTX** and **LTX-S_2-As-Ibt** showed changes in body weight over time. (F) The biodistribution of arsenic in organs/tissues of PBS, **As-Ibt**, **LTX** and **LTX-S_2-As-Ibt** treated mice. After treatment, mice were sacrificed and the arsenic content in the tumor, heart, liver, spleen, lung, kidney, and serum were quantified by ICPMS.

33380 was found to form stable adducts with cellular BTK after 1 h of incubation (DMSO lane, PCI-33380-labeled BTK, Figure 2C, and D). Pretreatment with 100 nM ibrutinib for 8 h completely eliminated the PCI-33380-labeled BTK band, demonstrating its high potency. Pretreatment with 100 nM **LTX-S_2-As-Ibt** for 4 h and 50 nM for 8 h were observed, and both completely eliminated the PCI-33380-labeled BTK band in a concentration- and time-dependent manner (Figure 2C, S11B). In contrast, treatment with 100 nM **RTX-S_2-As-Ibt**, RTX or LTX alone for 8 h had no influence on the PCI-33380-labeled BTK band (Figure 2D, S11). These results indicated that **LTX-S_2-As-Ibt** can be internalized into cells and efficiently binds to BTK.

In order to further confirm that As-Ibt is released from **Ab-TCI** in the cellular environment, we analyzed the reaction products of **Ab-TCI** and GSH by SEC-ICPMS. As shown in Figures 2E and S12A, upon adding 10 mM GSH to **LTX-S_2-As-Ibt**/**RTX-S_2-As-Ibt** for 8 h, the peak of **LTX-S_2-As-Ibt**/**RTX-S_2-As-Ibt** attenuated and a new peak at 18.5 min was observed, whose retention time was the same as that of As-Ibt. We then investigated the reaction products **LTX-S_2-As-Ibt**/**RTX-S_2-As-Ibt** and 1,2-ethanedithiol (EDT). We observed

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that the peak of **Ab-TCI** completely disappeared and the peak of the released As-Ibt appeared after reacting with EDT for 8 h, indicating that the EDT facilitates arsenic thiol exchange reactions through the formation of a stable EDT-As-Ibt complex[14] (Figures 2E and S12A). Moreover, when preincubating OCI-LY10 cells with the reaction products of **RTX-S_2-As-Ibt** and GSH/EDT, the PCI-33380-labeled BTK band was completely eliminated (Figures 2F and S12B), while preincubating OCI-LY10 cells with **RTX-S_2-As-Ibt** in PBS had no influence on the PCI-33380-labeled BTK band. The above results indicate that **Ab-TCI** is stable outside the cells, and only the released As-Ibt through the arsenic-thiol exchange reaction with GSH can bind to intracellular BTK.

We further investigated the inhibitory effect of **LTX-S_2-As-Ibt** on the BTK-mediated BCR signaling pathway[27] in OCI-LY10 cells through SDS-PAGE immunoblotting. We utilized anti-IgM (a mimic of BCR-antigen) to stimulate the phosphorylation of BTK and its upstream (Syk) and downstream effectors (PLCγ2, Erk1/2) along the BCR signaling pathway[28] (Figures 3A). As shown in Figure 3B, 100 nM **LTX-S_2-As-Ibt** completely inhibits the phosphorylation of BTK and its downstream effectors (PLCγ2, Erk1/2) in a concentration-dependent manner without affecting the phosphorylation of its upstream kinase Syk. LTX or RTX alone and noninternalized **RTX-S_2-As-Ibt** had no effect on the BCR signaling pathway, even when their concentration reached 100 nM (Figures 3B). These results demonstrate that **LTX-S_2-As-Ibt** can be internalized into cells and cleaved by cellular GSH, and released As-Ibt efficiently inhibits BTK and the BCR signaling pathway in live B-cell lymphoma cells.

We further investigated the antiproliferative activity of **LTX-S_2-As-Ibt** against the OCI-LY10 cells. As shown in Figure 4A, **LTX-S_2-As-Ibt** showed an IC_{50} of 46.4 nM against OCI-LY10 cells while LTX exhibited no significant toxicity against OCI-LY10 cells within the tested concentration range (IC_{50} > 2000 nM). Compared to free As-Ibt (**I-As-1**,[14] IC_{50} = 133.9 nM) and ibrutinib (IC_{50} = 1899.7 nM), **LTX-S_2-As-Ibt** showed significantly enhanced cytotoxicity against OCI-LY10 cells, increased by 2.9 and 40.9 times, respectively. These results demonstrated that the antibody-directed cell/protein dual-targeting strategy by conjugating As-TCI with a cell internalized antibody effectively enhances the cytotoxicity to the target cancer cells. To explain the excellent cytotoxicity of **LTX-S_2-As-Ibt**, we further conducted a cellular uptake experiment through quantifying the arsenic element using ICPMS. As shown in Figure 4B, within the incubation concentrations ranging from 0.5 μM to 5.0 μM, **LTX-S_2-As-Ibt** exhibits 2.84- to 4.51-fold higher uptake compared to **As-Ibt**. The results indicate that **LTX-S_2-As-Ibt** enhanced the accumulation of As-TCI within cancer cells, explaining the possible reason for its superior antiproliferative activity over As-TCI.

The potent antiproliferative effects of **LTX-S_2-As-Ibt** prompted our investigation into its *in vivo* treatment in SCID mice bearing cancer cell xenograft tumors. As shown in Figure 4C, D, compared to the PBS group, **As-Ibt** (65 μg/kg, 110 nM/kg, about 4-fold concentration of antibody) was found to not have an inhibition effect on tumor growth. The tumor volume in mice treated with LTX (4 mg/kg, 27 nM/kg) decreased by 32%, whereas in those treated with **LTX-S_2-As-Ibt** (4 mg/kg, 27 nM/kg), the tumor volume significantly decreased by 81%. Tumor Suppression Rate (%) = [(Tumor Volume of PBS Group − Tumor Volume of **LTX-S_2-As-Ibt** Group)/Tumor Volume of PBS] × 100%. These results indicated that **LTX-S_2-As-Ibt** significantly enhances *in vivo* antitumor activity compared with the antibody and As-TCI through the antibody-directed cell/protein dual-targeting strategy. Furthermore, following 16 days of treatment, mice in the **LTX-S_2-As-Ibt** group exhibited slight weight gain (Figure 4E) and no poisoning symptom was observed, indicating that **LTX-S_2-As-Ibt** was well-tolerated by SCID mice. Upon completion of treatment, mice were euthanized and the *in vivo* distribution of **LTX-S_2-As-Ibt** was examined. As shown in Figure 4F, **LTX-S_2-As-Ibt** accumulates most prominently in the mouse tumor, with minimal distribution in organs other than the liver. In contrast, free **As-Ibt**, without the guidance of an antibody, was found widely present in normal organs, e.g., the lung, liver, and kidney, as shown in a previous report.[14] These results demonstrate that **LTX-S_2-As-Ibt** not only exhibits excellent tumor targeting and facilitates the accumulation of As-TCI at the tumor site, but also achieves highly efficient inhibition of xenograft tumors ultimately.

In summary, we have established a novel tumor cell/protein "Double Insurance" targeting strategy to empower TCIs with cancer cell targeting ability and reduce the unwanted toxicity of traditional high toxic payloads. We developed a new Ab-TCI, **LTX-S_2-As-Ibt**, by connecting the LTX with a BTK-targeted covalent ligand through arsenic-thiol linkers. We demonstrated that **LTX-S_2-As-Ibt** is stable outside the cells and able to specifically recognize cell surface antigens and be internalized into the B-cell lymphoma cells. As-Ibt is further released from the internalized **LTX-S_2-As-Ibt** by intracellular GSH and specifically inhibits BTK and the BCR signaling pathway at a nM level. **LTX-S_2-As-Ibt** achieves a specific accumulation of TCI in cancer cells, with a strong xenograft tumor growth suppressing rate up to 81%, demonstrating a significant improvement of drug efficacy and safety. New Ab-TCIs could be developed by the rational integration of FDA approved TCIs[29] and antibodies[30] through our "Double Insurance" strategy for effective treatment of different cancers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.6c00131.

Synthesis route and ^{1}H NMR, ^{13}C NMR, and HRMS spectra of the synthesized compounds, MALDI-TOF MS and SEC-ICP-MS characterization of Ab-TCI, Confocal fluorescence image of Ab-TCI, SEC-ICP-MS analysis, competitive binding assay of RTX-S_2-As-Ibt to GSH or EDT and Experimental Section (PDF)

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Author Contributions

#X.Z., Y.Z., Z.F. contributed equally. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

No unexpected or unusually high safety hazards were encountered.

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■ ABBREVIATIONS

TCIs, targeted covalent inhibitors; Ab-TCI, antibody-targeted covalent inhibitor conjugate; LTX, Loncastuximab; RTX, rituximab; BTK, Bruton's tyrosine kinase; GSH, glutathione; FDA, the US Food and Drug Administration; mAbs, monoclonal antibodies; ADCs, antibody−drug conjugates; PEG, polyethylene Glycol; CLL, lymphocytic leukemia; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; RTX, rituximab; LA, lipoic acid; NHS, *N*-hydroxysuccinimide; DAR, drug-to-antibody ratio; SEC-ICPMS, size-exclusion chromatography combined with inductively coupled plasma mass spectrometry; EDT, 1,2-ethanedithiol; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PLCγ2, phospholipase C gamma 2; Erk1/2, Extracellular Signal-Regulated Kinase 1/2; TFA, trifluoroacetic acid; EDCI, 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride.

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