RESEARCH ARTICLE

Creating MHC-Restricted Neoantigens with Covalent Inhibitors That Can Be Targeted by Immune Therapy

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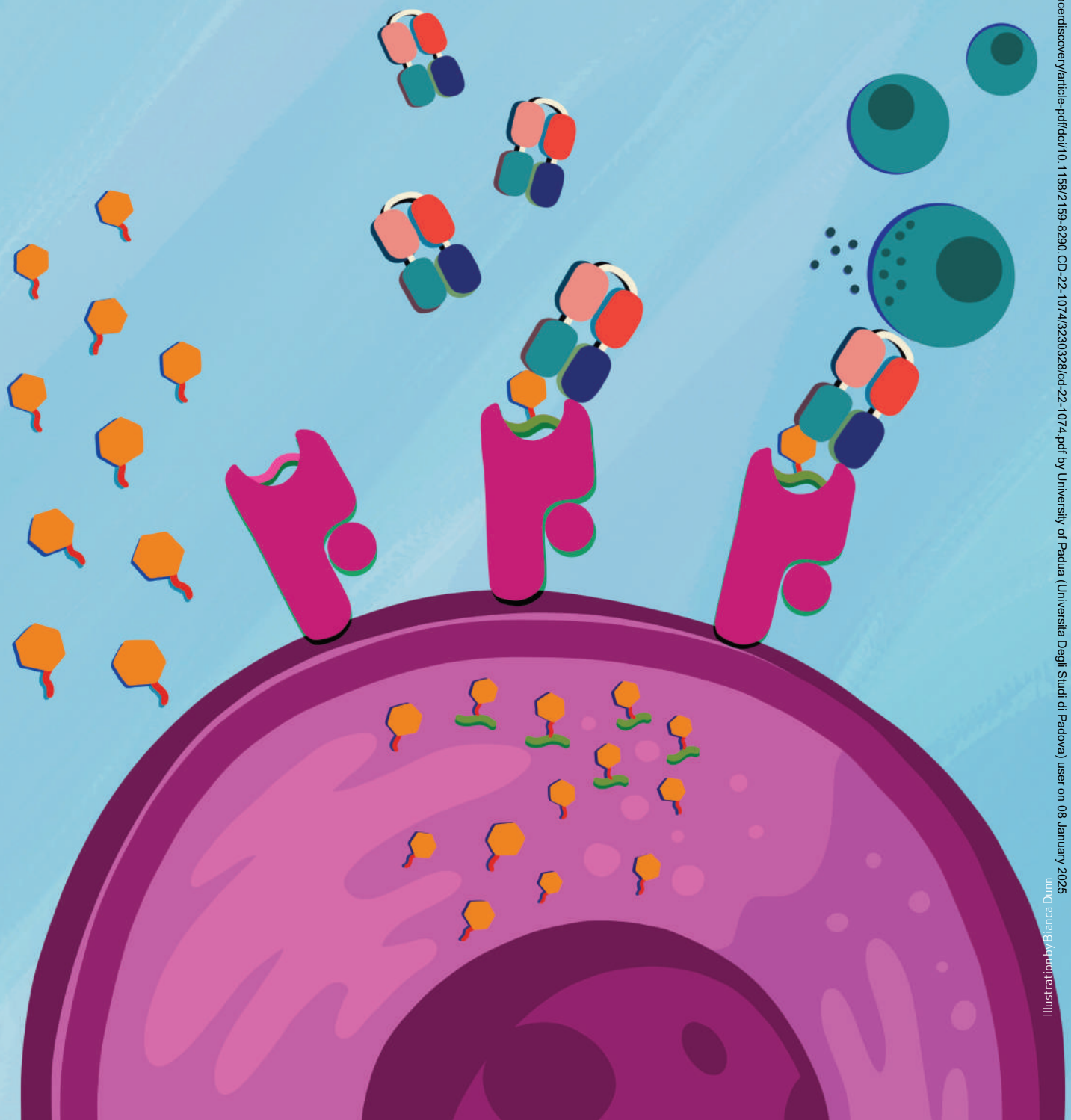

Illustration by Bianca Dunn

ABSTRACT Intracellular oncoproteins can be inhibited with targeted therapy, but responses are not durable. Immune therapies can be curative, but most oncogene-driven tumors are unresponsive to these agents. Fragments of intracellular oncoproteins can act as neoantigens presented by the major histocompatibility complex (MHC), but recognizing minimal differences between oncoproteins and their normal counterparts is challenging. We have established a platform technology that exploits hapten–peptide conjugates generated by covalent inhibitors to create distinct neoantigens that selectively mark cancer cells. Using the FDA-approved covalent inhibitors sotorasib and osimertinib, we developed "HapImmune" antibodies that bind to drug–peptide conjugate/MHC complexes but not to the free drugs. A HapImmune-based bispecific T-cell engager selectively and potently kills sotorasib-resistant lung cancer cells upon sotorasib treatment. Notably, it is effective against $KRAS^{G12C}$-mutant cells with different HLA supertypes, HLA-A*02 and A*03/11, suggesting loosening of MHC restriction. Our strategy creates targetable neoantigens by design, unifying targeted and immune therapies.

SIGNIFICANCE: Targeted therapies against oncoproteins often have dramatic initial efficacy but lack durability. Immunotherapies can be curative, yet most tumors fail to respond. We developed a generalizable technology platform that exploits hapten–peptides generated by covalent inhibitors as neoantigens presented on MHC to enable engineered antibodies to selectively kill drug-resistant cancer cells.

INTRODUCTION

The past 20 years have witnessed a revolution in cancer therapeutics along two major fronts. First, "targeted therapies" (e.g., small-molecule signal transduction inhibitors, antibodies against receptor tyrosine kinases) have been developed against specific mutant oncogenes or components of their downstream signal transduction cascades (1, 2). Even KRAS, long viewed as "undruggable," has now been targeted in tumors bearing the specific mutant allele $KRAS^{G12C}$ (3–5). Targeted therapies can cause remarkable regressions, but unfortunately, some mutant cells are able to resist the initial drug onslaught via "adaptive resistance" (6–10) or as drug-tolerant persisters (11–16). Such cells can serve as reservoirs for the eventual development of stable resistance, which leads to disease recurrence and, ultimately, patient demise. In parallel, "immune therapies" emerged [e.g., immune-checkpoint blockade, adoptive T-cell transfer, chimeric antigen receptor (CAR) T cells, and CAR-natural killer (NK) cells]. These modalities, unlike targeted therapies, can sometimes induce durable remissions (and likely cures), but most patients, including those with oncogene-driven tumors, fail to respond (17–25). Therefore, achieving durable responses and ultimately cures for metastatic cancers driven by intracellular oncogenes remains a major unmet medical need.

Conceivably, targeted therapies fail because they are unable to evoke a sustained antitumor immune response. Thus, a key question is how we can effectively combine the benefits of targeted therapies as debulking agents with the durability of immune therapies. In principle, aberrant intracellular oncoproteins could be recognized by the immune system. Specifically, mutant peptides derived from oncoproteins and presented on class I major histocompatibility complex (class I MHC, hereafter, MHC) molecules might be recognized by cytotoxic T cells with cognate T-cell receptors (TCR). That tumors are present, presumably due to immune escape, indicates that such T cells must be few in number, exhausted, senescent, or otherwise dysfunctional. Targeting mutant peptide/MHC complexes (hereafter p/MHC; e.g., KRAS mutants) with TCRs or antibodies is conceptually feasible and has been demonstrated in some cases (26, 27). Recognizing the typically minimal differences between the mutant and wild-type peptides in the context of the p/MHC complex makes this approach quite challenging (28).

To address these challenges, we developed a technology platform, "HapImmune," that capitalizes on covalent targeted therapies to create drug–peptide conjugates as cancer neoantigens (Fig. 1A). The bulky chemical moiety of the conjugated inhibitor substantially alters the surface topography and chemistry with respect to unconjugated peptides. Thus, inhibitor-p/MHC should be a distinctly different and unique antigen, which could be more readily recognized by antibodies (or TCRs), leading to high selectivity. We utilized antibody-engineering technologies to develop human antibodies that recognize such neoantigens on MHC and

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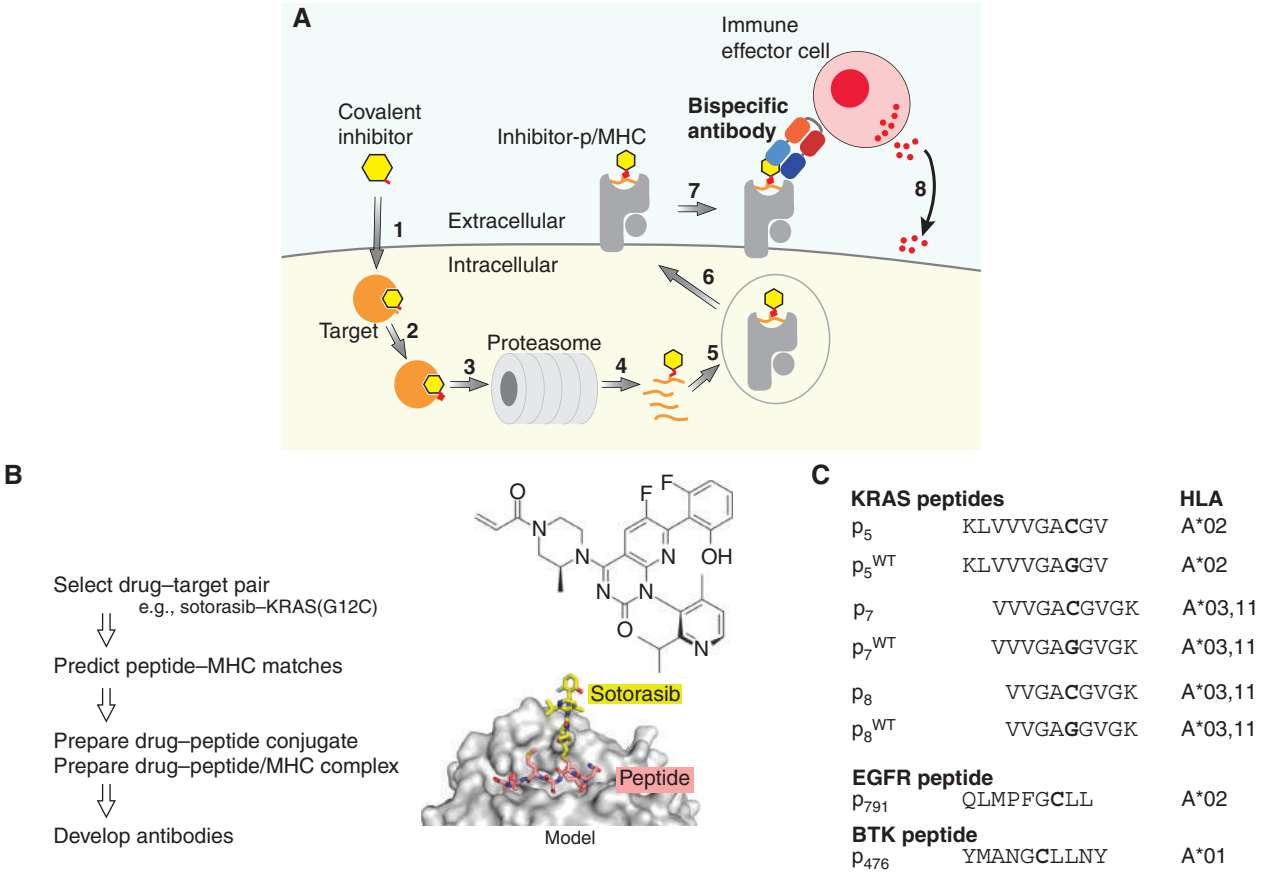

Figure 1. The HapImmune concept. **A,** A covalent inhibitor enters the cell (step 1) and binds and forms a covalent bond with its target (step 2). As a part of natural protein turnover, the target–drug conjugate is degraded, and peptides with the conjugated drug are produced (steps 3 and 4). A drug–peptide conjugate is incorporated into a compatible MHC molecule (step 5). The drug–peptide/MHC complex translocates to the cell surface (step 6). A HapImmune antibody binds the complex (step 7) and recruits an immune effector cell, which initiates cell killing (step 8). Alternatively, the HapImmune antibody can serve as the recognition element for antibody conjugates or cellular therapies. **B,** Overview of antibody development strategy. The molecular model was based on Protein Data Bank ID 3RL1 (67). **C,** Peptides used in this study and their predicted HLA matches.

are minimally inhibited by the free inhibitor or inhibitor-p in the absence of MHC, a prerequisite for coadministration with the inhibitor. Such antibodies could kill tumor cells by engaging immune cells (e.g., T lymphocytes, NK cells, tumoricidal macrophages) or delivering toxic cargos (24, 29, 30). Importantly, the small-molecule drug need not act as an inhibitor of cancer cell growth or even as an inhibitor of the target protein, so long as it forms a stable covalent bond with the target protein and the inhibitor-p/MHC is presented on the surface of cancer cells. We present proof-of-concept data by developing highly specific human antibodies that specifically recognize complexes of inhibitor–peptide conjugates and their matched MHCs generated by two FDA-approved covalent drugs, sotorasib, which targets KRAS(G12C), and osimertinib, which targets activated EGFR. We also present initial data showing similar reagents can be generated for a third FDA-approved agent, ibrutinib, conjugated to a fragment of its target, BTK. Our concept enables the development and targeting of any drug–peptide conjugate capable of presentation on MHC and could substantially enhance the effectiveness of both targeted therapy and biologics against cancer.

RESULTS

Antigen Design for Inhibitor–Peptide Conjugates Originating from Sotorasib and KRAS(G12C)

We chose KRAS(G12C) as an initial target for testing the HapImmune concept. *RAS* mutations at codon 12 are among the most common oncogenic drivers, and these and other RAS-mutant proteins had long been viewed as challenging, if not "undruggable," targets. Recent breakthroughs led to the development of multiple covalent inhibitors for KRAS(G12C), hereafter termed G12Cis. Sotorasib (AMG510) is the first FDA-approved G12Ci, and it evokes therapeutic responses and extends progression-free survival in a significant fraction of patients with non–small cell lung cancer whose tumors express the target oncoprotein (31). Unfortunately, as is the case for other targeted therapies, resistance to G12Ci develops quickly, and cures remain elusive (32–34).

We previously developed biologics (synthetic antibodies and monobodies) that directly target KRAS(G12C) and its covalent complex with ARS1620 (35, 36). Although these reagents are effective tools for mechanistic studies, their inability to enter cells made them ineffective as potential therapeutics.

Nevertheless, the relatively high abundance (~1 μmol/L) of KRAS(G12C) in cells (37), the effective target engagement by G12Ci, and the emerging mechanisms of sotorasib resistance all suggested that the sotorasib–KRAS(G12C) peptide conjugates might be amenable to the HapImmune approach.

Although no data explicitly demonstrate that sotorasib–peptide conjugates are presented on MHCs, much evidence suggested that this was likely. First, MHC presentation of RAS peptides that include residue 12 has been reported (27, 38). Cys12 in these p/MHC complexes should be located outside the anchor positions of the presented peptides that are crucial for MHC binding, suggesting that drug conjugation would minimally affect peptide presentation (Fig. 1B). Second, NetMHCpan (39) predicts that peptides containing Trp at the 12th position, mimicking the bulky side chain of sotorasib-conjugated Cys12, can be presented on HLA-A*03, HLA-A*11, and HLA-A*02, with the highest score for the 9-residue peptide corresponding to residues 8 to 16 (hereafter termed p_8, where the subscript number indicates the position of the N-terminal residue of the peptide within the full-length, parental protein) on HLA-A*03 (Fig. 1B and C). For brevity, we will use abbreviations to refer to an inhibitor–peptide conjugate in complex with an MHC molecule: for example, soto-p_8/A03 refers to the sotorasib–p_8 conjugate in complex with HLA-A*03.

We conjugated sotorasib to these peptides and produced their MHC complexes using a standard refolding procedure (40). Size-exclusion chromatography showed the formation of stable MHC complexes (Supplementary Fig. S1). As controls, we also prepared the corresponding complexes harboring the cognate wild-type peptides. Hereafter, peptides with the wild-type sequence are denoted as p^{WT} (see Fig. 1C for nomenclature of peptides used in this study).

Development of Antibodies Selective for Sotorasib–KRAS(G12C) Conjugates in Complex with MHC

We set out to develop antibodies that selectively recognize inhibitor–peptide conjugates in the context of MHCs. Using the soto-p_8/A03 complex as a target and the p_8^{WT}/A03 complex as an off-target control for negative selection, we performed selections on a human synthetic antibody phage-display library and identified a clone, R001, that preferentially bound to soto-p_8/A03 (Fig. 2A; Supplementary Fig. S2A). To facilitate the characterization and improvement of its properties, we transferred the phage-displayed Fab clone into a yeast display vector in the single-chain Fv (scFv) format. Consistent with its preliminary characterization as a phage-displayed Fab, R001 specifically bound to soto-p_8/A03 with an apparent dissociation constant ($K_{D,APP}$) of 2.7 nmol/L and showed no binding to p_8^{WT}/A03 or p_7^{WT}/A03 (Supplementary Fig. S2A). This antibody was highly selective to soto-p_8/A03. We detected significant but weak binding to soto-p_7/A03, the p/MHC complex with a longer, 10mer peptide ($K_{D,APP}$ >100 nmol/L), but no binding to soto-p_7/A11 and soto-p_8/A11 complexes (Supplementary Fig S2A). R001 also was highly selective to sotorasib, showing no cross-reactivity to two other G12Ci-p8/A03 complexes, ARS1620-p_8/03 or MRTX849 (adagrasib)-p_8/03 (Supplementary Fig. S2B).

To improve upon the affinity of R001 and to explore whether it is possible to expand its recognition spectrum to the related sotorasib-conjugated peptides on HLA-A*11 while maintaining selectivity for soto-p/MHC complexes, we performed rounds of affinity maturation (Fig. 2A; Supplementary Fig. S3). Following mutagenesis and library sorting of CDR residues, we developed clone R011, which showed increased affinity toward soto-p_7/03 and weak but detectable binding to the soto-p_8 and soto-p_7 conjugates presented by HLA-A*11 (Fig. 2A; Supplementary Fig. S2A). We then performed deep mutational scanning (DMS; ref. 41) of the CDR-L3 and CDR-H3 residues of clone R011 to identify permissible substitutions (Fig. 2A; Supplementary Fig. S3). This step allowed us to define the sequence landscape of antibodies toward different soto-p/MHC antigens. Based on these data, we designed a tailored library that combined permissible residues in CDR-L3 and CDR-H3, and identified three clones, named R021, R022, and R023, that bound with low nanomolar affinity to all four targets: soto-p_8/A03, soto-p_7/A03, soto-p_8/A11, and soto-p_7/A11; Supplementary Fig. S2A). We chose clone R023 for further characterization and produced it in the Fab format for biophysical characterization. Biolayer interferometry (BLI) experiments using purified Fab confirmed its high affinity to all four soto-p/MHCs, with K_D values ranging from 110 pmol/L to 1.8 nmol/L (Fig. 2B), and lack of detectable binding to p^{WT}/MHCs (Fig. 2B, black traces). Intriguingly, BLI experiments also revealed that R023 bound, though with lower affinity, to sotorasib conjugated with a distinct peptide, p_5, presented on a different HLA supertype, HLA-A*02 (Fig. 2C).

These antibodies bound only minimally to the free sotorasib-conjugated peptide in the absence of MHC or to free sotorasib. Binding to the free conjugate was observed only at very high concentrations (free soto-p $K_{D,APP}$ >1 μmol/L; Fig. 2D; Supplementary Fig. S2C). Furthermore, antibody binding to soto-p/MHCs was inhibited only marginally by free sotorasib [the half maximal inhibition concentration (IC_{50}) of 7–12 μmol/L, Fig. 2E and Supplementary Fig. S2D]. Remarkably, despite the ability of these antibodies to bind the sotorasib-conjugated peptides in a manner not restricted to a single HLA, their specificity toward the inhibitor–peptide conjugates in complex with MHCs, over free sotorasib, was maintained. Taken together, these data establish the feasibility of developing potent and selective antibodies to the complex of an inhibitor–peptide conjugate and its matched HLA that are minimally inhibited by the free inhibitor. These data also demonstrate the potential to expand the patient population that could be treated with this approach (see Discussion).

T-cell Engaging Bispecific Antibodies Selectively Kill Cells Presenting Drug–Peptide Conjugates as MHC Complexes

Direct detection of specific p/MHC complexes on the cell surface using standard immunochemical methods such as flow cytometry is extremely challenging because of their low copy number (38). Likewise, low copy numbers are expected for sotorasib–KRAS(G12C) conjugates presented by MHCs on the cell surface. Therefore, to detect these neoantigen complexes and maximize the efficacy of target cell killing by our antibodies, we utilized a T cell–engaging bispecific antibody platform. Specifically, we constructed a single-chain diabody

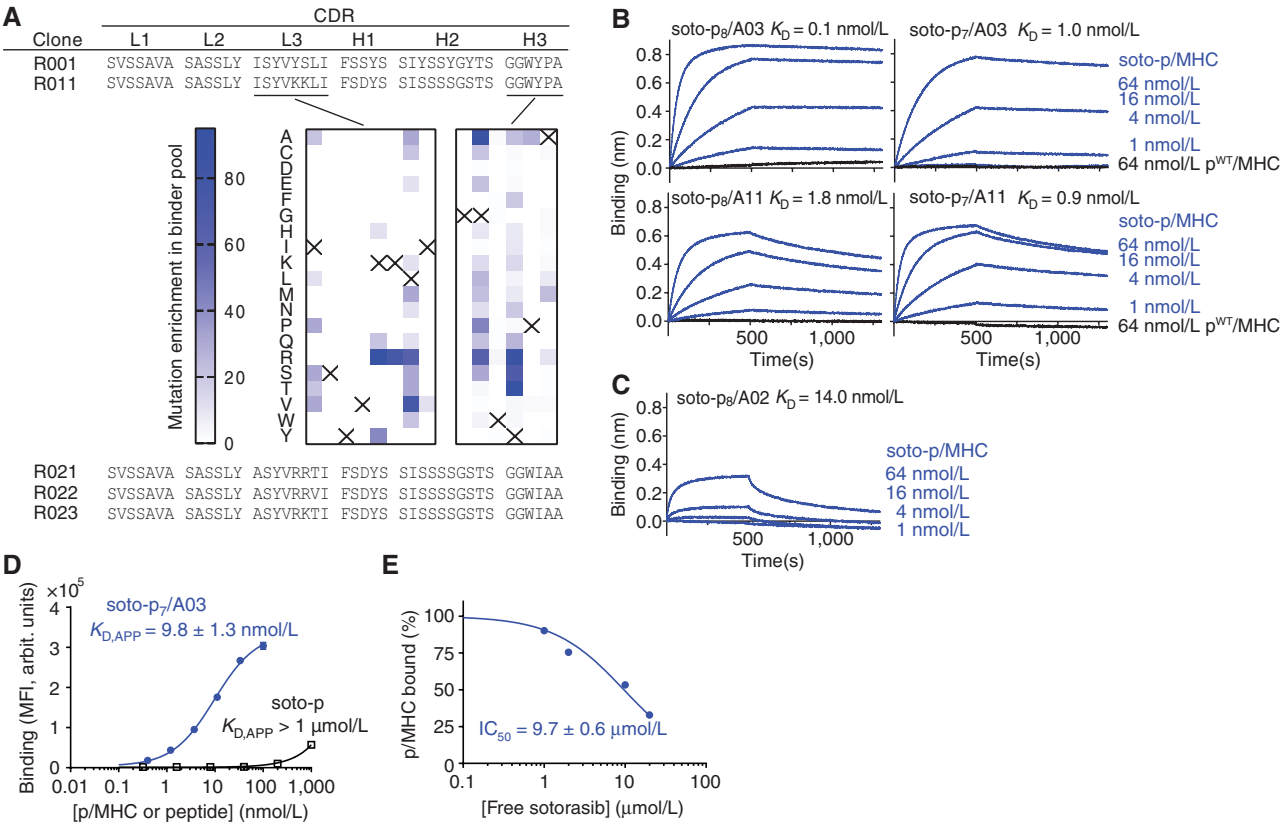

Figure 2. Development and binding properties of the R023 antibody. **A,** CDR sequences of R023 and its precursors and related clones. The middle images show the results of DMS of clone R011. The numbers indicate the total numbers of sequencing reads for each mutation, divided by the total number of reads for all mutations at the position, multiplied by 1,000. The crosses show the wild-type residue. **B,** BLI sensorgrams of the interaction between R023 Fab and the indicated MHC complexes. Biotinylated R023 Fab was immobilized, and binding of soluble p/MHC samples was measured. K_D values from global fitting are shown. **C,** BLI sensorgrams of the interaction between R023 Fab and the soto-p_5/A02 complex. **D,** Binding titration of scFv R023 displayed on the yeast cell surface to soto-p_7/A03 (blue) and the soto-p_7 conjugate in the absence of an MHC (open squares). arbit., arbitrary; MFI, median fluorescence intensity. **E,** Inhibition by free sotorasib of the interaction between soto-p_7/A03 (10 nmol/L) and scFv R023 displayed on the yeast cell surface. The binding signal intensity was normalized using the value without sotorasib (100%) and in the absence of soto-p_7/A03 (0%). IC_{50} values are reported ± standard error. In **B** and **C**, each data point shows the mean ($n = 3$; technical replicates) of the median fluorescence intensity. Error bars represent the standard deviation.

(scDb; ref. 42) comprising a HapImmune antibody for recognizing the target cell and the UCHT1 clone as the component that engages CD3ε on T lymphocytes (43), and used cell killing as a sensitive readout of p/MHC on the cell surface.

We used Raji cells harboring HLA-A*03 (Fig. 3A; Supplementary Fig. S4A) and pulsed with the soto-p_7 and soto-p_8 conjugates to ask whether the R023 scDb could have cytotoxic effects on cells displaying soto-p/A03 complexes. Raji cells express the transporter associated with antigen processing, which is required for the assembly of p/MHC complexes and their consequent transport to the cell surface (44). Consequently, MHC molecules on the Raji cell surface are already bound with endogenous peptides, and only a small fraction of HLA-A*03 on the surface of these cells can be loaded with exogenously added peptide–drug conjugates. When cocultured with T cells, the R023 scDb showed potent cytotoxic effects on cells pulsed with soto-p_7 or soto-p_8 (EC_{50} = 2.8 pmol/L and 5.2 pmol/L, respectively) but not with the p_7^{WT} or p_8^{WT} peptides, indicating selective killing (Fig. 3B). As predicted by our binding studies (Fig. 2B; Supplementary Fig. S2A), the cell killing efficacy of the R023 scDb was substantially higher than that of the original R001 clone in the scDb format, particularly for cells pulsed with soto-p_7. Importantly, the R023 scDb showed no cytotoxic effect on sotorasib-treated, unpulsed Raji cells, which do not express KRAS(G12C) (Fig. 3C), indicating that the killing depends on the presence of KRAS(G12C) peptides. Notably, the R023 scDb also killed OCI-AML3 cells (expressing HLA-A*02) pulsed with soto-p_5 but not cells pulsed with p_5^{WT}, although the efficacy was lower than Raji cells pulsed with soto-p_7 or soto-p_8 (Fig. 3D). This result is consistent with the weaker affinity of R023 for the soto-p_5/A02 complex than for soto-p_7/A03 (Fig. 2B and C). In concert, these data show that the R023 scDb can induce potent, highly selective killing of cells presenting sotorasib–KRAS(G12C) peptide conjugates bound to MHC complexes on the cell surface. They also provide further evidence that the range of actionable MHCs can be extended via the HapImmune approach.

Sotorasib-Treated Tumor Cells Can Be Killed Selectively by HapImmune Antibodies

We next asked whether the R023 scDb can target sotorasib-treated KRAS(G12C)-harboring tumor cells that are