**Background** Claudin-6 (CLDN6), one of the over 20 known human CLDN family members, is upregulated in many solid tumors but shows minimal or no expression in healthy adult tissues. This makes it an ideal target for cancer therapies. Although several approaches using various therapeutic modalities targeting CLDN6, including T cell engagers (TCE), CAR-T cells, and antibody drug conjugates, are currently under investigation, a more potent therapeutic option is still desirable.

**Methods** To achieve successful clinical outcomes by enhancing immune activation, we generated SAIL66, a next generation TCE, named Dual-Ig®, which targets CLDN6. SAIL66 is a trispecific monoclonal antibody designed to bind to CLDN6 on cancer cells with one Fab arm and to both CD3 and CD137 with the other Fab arm. This boosts immune activation through signal 1 and 2 in T cells more strongly than a conventional TCE which only binds to CD3 and activates signal 1. The preclinical characterization of SAIL66 was performed in a series of in vitro and in vivo studies, including direct comparisons to a conventional TCE targeting CLDN6 and CD3.

**Results** SAIL66 revealed a high specificity for CLDN6, despite its high similarity to CLDN3, CLDN4, and CLDN9, suggesting that SAIL66 has no undesirable off-target toxicity in patients. Using a Jurkat cell system harboring NFAT or NF-kB reporter genes, we confirmed that SAIL66 exerts CD3 and CD137 signal induction activity depending on CLDN6 expression. SAIL66 induced activation of T cells, release of cytokines, and lysis of CLDN6-positive cancer cells. In vivo studies with immune competent syngeneic mouse models, engineered to express either human CD3 alone or both hCD3 and CD137, showed that SAIL66 exhibited better anti-tumor efficacy than a conventional TCE by inducing the activation of both CD3 and CD137. Notably, the increase in the number of intratumoral T cells and suppression of exhausted T cell populations were more remarkable in mice treated with SAIL66 than in those treated with a conventional TCE. Although SAIL66 monotherapy was effective in the immune checkpoint inhibitor (ICI) resistant non-immunogenic tumor, the anti-tumor efficacy was further improved by combining SAIL66 with ICI.

**Conclusions** SAIL66 offers a novel therapeutic option by incorporating CD137 co-stimulatory signaling in addition to CD3 signaling for the treatment of patients with cancer expressing CLDN6. Clinical studies of SAIL66 are currently underway for patients with solid cancer (NCT05735366).

**Ethics Approval** All in vivo procedures and the experiments with human materials were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) and research ethics committee at Chugai Pharmaceutical.