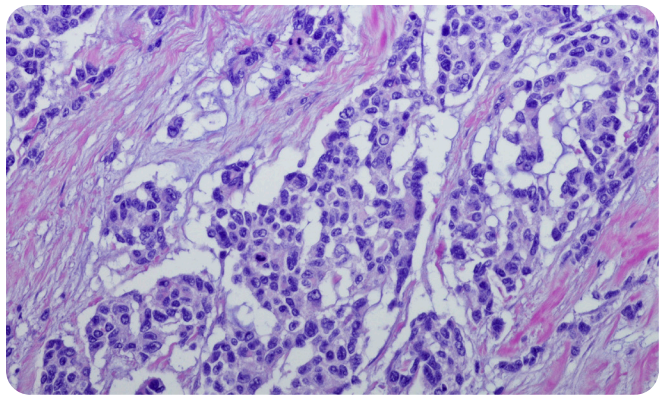
Overcoming challenges for treating solid tumors

Characterizing and developing new treatments to target solid cancers utilizing biochemical and cell based assays

Solid tumors are abnormal masses of malignant cells found in cancers of organs, muscle, and bone. These cancer types are classified into two main categories: carcinomas, which form in the epithelial lining of organs, skin, or glands; or sarcomas, which form in supportive tissue such as bone, muscle, tendons, and blood or lymph vessels. Carcinoma is the most common type of cancer, accounting for around 80-90% of all cancer cases.[1] By contrast, sarcomas are relatively rare in adults and make up approximately 1% of all adult cancer diagnoses;2 however, long-term survival still remains poor for these patients.

Compared with blood cancers, solid tumors present a unique set of challenges to researchers, including a lack of expression of tumor-specific antigen targets and a metabolically challenging and highly immunosuppressive tumor microenvironment. Some solid cancers can successfully be removed surgically, and there has been tremendous progress in the development of novel therapeutics to treat solid tumors, often leading to patient remission.

In this review, we highlight two examples of solid tumors – Non-Small Cell Lung Carcinoma (NSCLC) and soft Tissue Sarcoma (STS) – and the types of assays that can be utilized to characterize and develop new treatments targeting solid cancers.



Non-small cell lung carcinoma

In the US, lung cancer is the leading cause of cancer-related mortality among both men and women.[3] More than half of patients with lung cancer die within one year of diagnosis and the five-year survival rate is less than 20%. Among all lung cancers, NSCLC is the most common type, accounting for approximately 85% of cases.[3]

Research has shown that mutations in the genes encoding Epidermal Growth Factor Receptor (EGFR) and Anaplastic Lymphoma Kinase (ALK) play an important role in the progression and metastasis of NSCLC.[4] EGFR is a transmembrane protein that is activated by binding of an extracellular protein ligand, such as Epidermal Growth Factor (EGF) or Transforming Growth Factor α (TGFα). Once activated, EGFR undergoes a conformational change and phosphorylation of the intracellular domain occurs, leading to downstream signal transduction by various pathways, including protein kinase B (AKT/PKB) and mitogen activated protein kinases (MAPK), as illustrated in Figure 1. These pathways can regulate multiple cellular processes, including proliferation, invasion, metastasis, and inhibition of apoptosis.

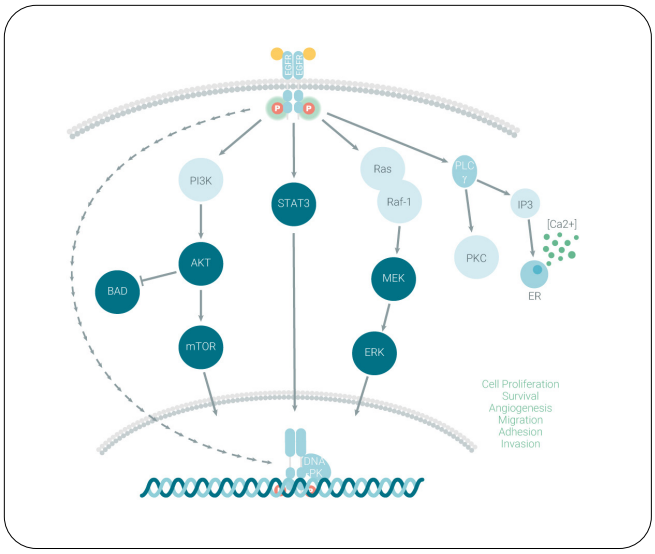


Figure 1: EGFR signaling pathways. Binding of EGFR ligands drive receptor homodimerization or heterodimerization leading to the activation of the EGFR tyrosine kinase domain and specific tyrosine residues. The phosphorylated tyrosine residues then provide docking sites for a variety of factors that induce downstream activation of several signal transduction cascades, including the Ras/Raf/MEK/ERK, PI3K/AKT, and JAK-STAT pathways.

EGFR is frequently overexpressed in NSCLC due to genetic mutations, resulting in the constitutive activation of downstream signal transduction pathways, regardless of the presence of an extracellular ligand.[5] The two most common EGFR mutations are small in-frame deletions in exon 19 (particularly E746-A750del) and amino acid substitution in exon 21 (L858R), which collectively account for over 90% of known activating EGFR mutations.[6]

Three generations of EGFR-TKIs

Tyrosine kinase inhibitors of EGFR (EGFR-TKIs) are a group of drugs that specifically target the EGFR mutation. First-generation EGFR-TKIs (gefitinib, erlotinib, and icotinib) reversibly bind EGFR and inhibit the binding of ATP to the TK domain. This subsequently prevents cell proliferation, ultimately leading to cell death. Although first-generation EGFR-TKIs have shown excellent therapeutic effect in patients with EGFR mutation-positive NSCLC, resistance to these agents often develops after 9–13 months. The T790M mutation in exon 20 of EGFR is the most common mechanism of acquired resistance, having been detected in up to 50% of patients treated with first-generation EGFR-TKIs.[6]

To overcome T790M-mediated acquired resistance, the second-generation EGFR-TKI afatinib was developed, which irreversibly inhibits the EGFR T790M-resistant mutation in animal models and NSCLC patients. However, concerns have been raised about side effects of afatinib at the clinical stage due to its poor selectivity toward wild-type (WT) EGFR.[7]

In 2015, the FDA granted accelerated approval to osimertinib, a third-generation EGFR-TKI, that irreversibly binds to EGFR kinase. This agent was shown to be highly selective against the T790M mutation over WT EGFR. Consequently, osimertinib was approved in March 2017 as a second-line treatment in the event of resistance to other EGFR-TKIs.

SH-1028: An irreversible third-generation EGFR-TKI

Another irreversible third-generation EGFR-TKI currently being explored for NSCLC is SH-1028. Like osimertinib, SH-1028 has a pyrimidine skeleton structure (a typical mutant-selective EGFR-TKI structure). However, SH-1028 is modified on the indole ring, resulting in a more stable 6,7,8,9-tetrahydro-pyrrolo [1, 2-a] indol structure compared with osimertinib.

In a recent study,[8] researchers from China Pharmaceutical University and Nanjing Sanhome Pharmaceutical Co., Ltd, utilized biochemical and cell-based assays to further explore the anti-tumor effect of SH-1028 in NSCLC. First, the team investigated the inhibitory effect of SH-1028 against human EGFR kinases (WT, L858R, d746-750, L861Q, T790M/L858R, and T90M/d746-750) using a homogenous time-resolved fluorescence (HTRF®) assay (Figure 2A). The enzyme reaction contained recombinant N-terminal GST-tagged human EGFR (T790M/L858R) that phosphorylates the HTRF biotinylated peptide substrate. The team also conducted a cell proliferation assay to compare the antiproliferative capabilities of SH-1028 and its primary metabolite, Imp3 (Figure 3A), with osimertinib using a number of tumor cell lines harboring either the WT or mutant forms of EGFR.

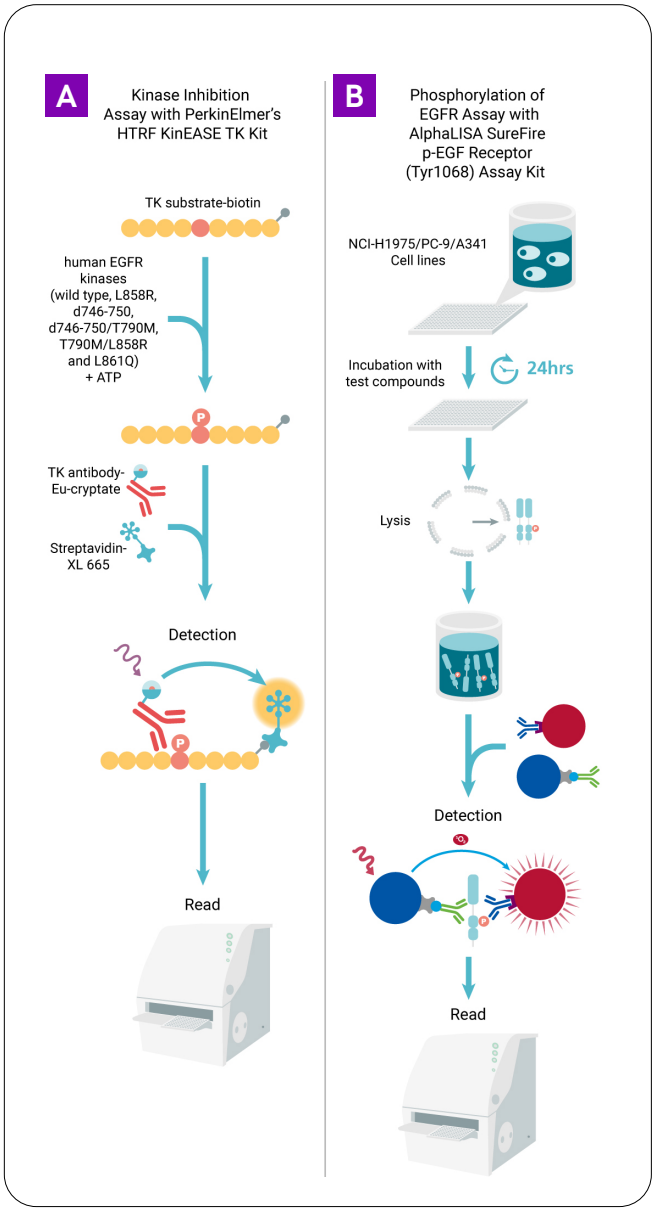


Figure 2: Experimental workflow of the kinase inhibition assay and phosphorylation assay. (A) Human EGFR kinase inhibition by test compounds was monitored using an HTRF kinase assay. Enzymes and compounds were incubated with a biotinylated Tyrosine Kinase (TK) substrate peptide. Detection of the phosphorylated peptide was measured using a TK-Ab cryptate and Strep-XL665 conjugates. HTRF signal was measured on an HTRF compatible plate reader. (B) Phosphorylation of EGFR in NCI-H1975, PC-9, and A341 cell lines were measured utilizing an AlphaLISA SureFire Ultra p-EGF Receptor (Tyr1068) detection kit. Cells were incubated with test compounds for 24 hours, lysed, and the amount of p-EGFR was quantified using the AlphaLISA detection reagents. The amount of AlphaLISA signal generated on an AlphaLISA-compatible plate reader is proportional to the amount of phosphorylated EGFR present in the sample.

Molecular docking indicated that SH-1028 could bind irreversibly to EGFR kinase by targeting cysteine-797 residue in the ATP binding site via covalent bond formation (Figure 3B). Results of the kinase inhibition assay (Figure 3C) revealed that SH-1028 exhibited nearly 80 times greater potency against T790M/L858R than WT EGFR. Furthermore, SH-1028 displayed a higher selectively toward WT EGFR compared with osimertinib. The inhibitory activities of Imp3 against the mutant EGFR kinases were slightly weaker than those of SH-1028; however, the metabolite was shown to maintain selectivity for WT EGFR kinase.

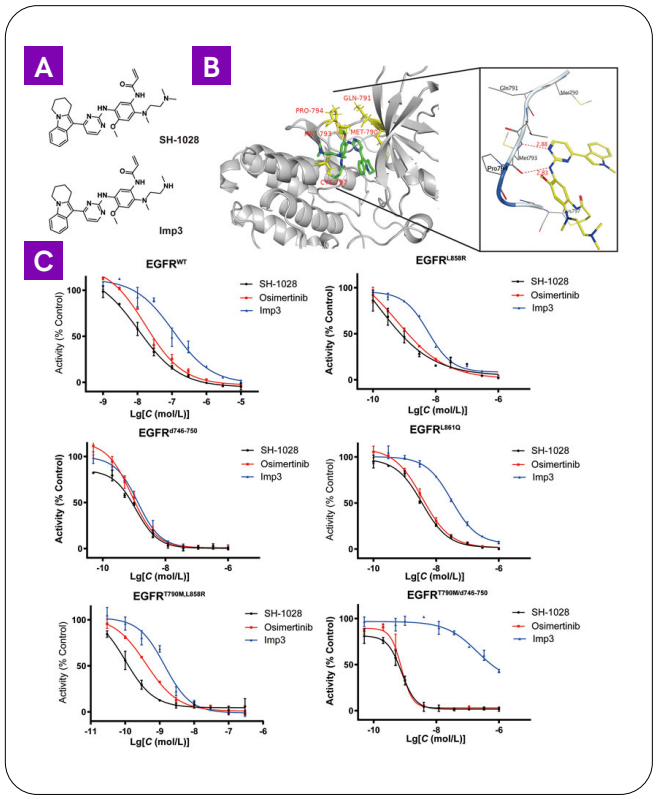


Figure 3: Preliminary efficacy of SH-1028. (A) Chemical structure of SH1028 and Imp3. (B) Docking structure of SH1028 to T790M EGFR. (C) kinase inhibition assay of SH-1028 in EGFR (WT, L858R, d746-750, L861Q, T790M/L858R, T90M/d746-750). Data were expressed as mean ± SD (n=3). (Image credit: Han L, Zhang X, Wang Z, Zhang X, Zhao L, Fu W et al.[8]).

In the cell proliferation assay, SH-1028 showed an improved inhibitory effect on the proliferation of EGFR sensitive (exon 19 del) and resistant (T790 M) cells than osimertinib (Figure 4A). Notably, the inhibitory effect of SH-1028 against WT EGFR cells was slightly weaker than that of osimertinib, indicating that SH-1028 is a selective inhibitor of EGFR mutant cells.

The researchers then measured phosphorylation levels of EGFR *in vitro* using a sandwich ELISA assay with phospho-specific EGFR (pY1068) antibodies (Figure 2B). Analysis revealed that SH-1028 inhibited the phosphorylation of EGFR to a greater degree than osimertinib in PC-9 (EGFR d746–75) and NCI-H1975 (EGFR L858R/T790M) cell lines, demonstrating that SH-1028 has strong inhibitory activity against EGFR mutant kinases (Figure 4B). Consistent with the team's prior selectivity results, in A431 cells (EGFR WT) SH-1028 displayed a weak inhibitory effect on EGFR phosphorylation (Figure 4B). There was no reversion of TKI resistance observed within 24 hours after washing out, confirming the irreversible binding of SH-1028, and inhibition of EGFR phosphorylation was maintained in both EGFR sensitive and L858R/T790M resistant mutations (Figure 4C).

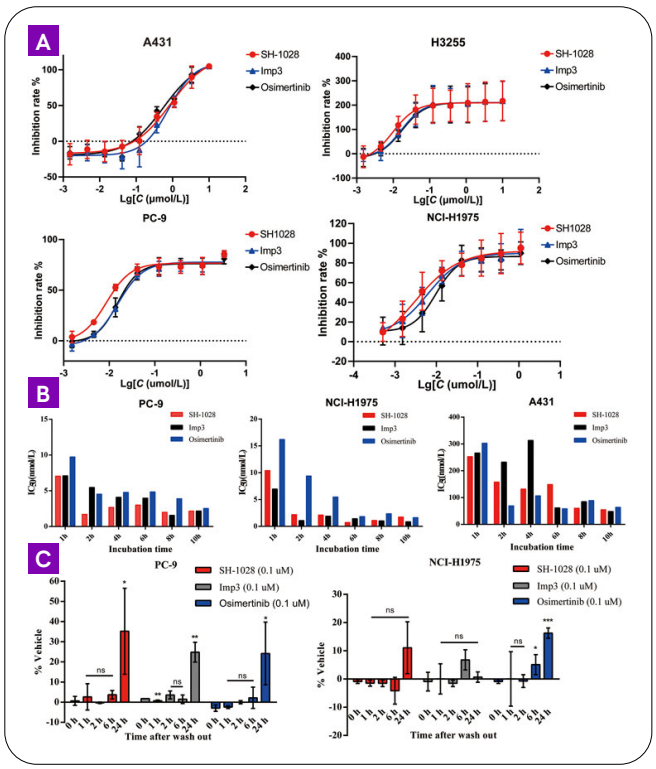


Figure 4: The efficacy of SH-1028 in cell lines. (A) The antiproliferative capabilities of SH-1028 to A431 (EGFR WT), H3255 (EGFR L858R), PC-9 (EGFR d746–750) and NCI-H1975 (EGFR L858R/T790M). (B) Time-dependent inhibition of EGFR phosphorylation in PC-9, NCI-H1975 and A431. (C) Inhibition of EGFR phosphorylation after the drug wash out in PC-9 and NCI-H1975. Data were expressed as mean (n=2; B) and mean ± SD (n=3; A and C); *p < 0.05 vs. 0 h; **p < 0.01 vs. 0 h; ***p < 0.001 vs. 0 h; ns: no significant difference vs. 0 h. (Image credit: Han L, Zhang X, Wang Z, Zhang X, Zhao L, Fu W et al.[8])

Finally, using mouse xenograph models the researchers demonstrated that oral administration of SH-1028 at a daily dose of 5 mg/kg significantly inhibited proliferation of tumor cells with the EGFR sensitive and resistant mutation for 14 consecutive days, with no TKI-induced weight loss. Moreover, the team reports that SH-1028 exhibited good bioavailability and was distributed extensively from the plasma to the tissues. Together, these results show that SH-1028 is able to overcome T790M-mediated resistance in NSCLC due to its distinct structure and metabolite pathway. The researchers suggest that SH-1028 may exert unique therapeutic properties in future clinical trials and be an alternative option for patients who have developed resistance to first-generation EGFR TKIs.

Soft tissue sarcomas

STS constitute a group of more than 50 different subtypes of rare heterogeneous malignancies comprising less than 1% of all adult cancers.[2] STS arise from mesenchymal tissues and can grow into structures such as adipose tissue, muscles, nervous tissue, and blood vessels. The intrinsically complex and heterogeneous nature of STS, and the rarity of the condition, has historically hampered efforts to develop effective targeted therapies and long-term survival still remains poor, especially in metastatic disease.

Although the tumor microenvironment in STS is highly variable, the strong immune presence in certain subtypes suggests that immunotherapy may be a promising treatment approach in many malignancies. Indeed, modified T and NK cell therapies have recently shown encouraging results in STS and other solid tumors.[9] However, treatments are lagging behind other neoplasms and new therapeutic concepts are urgently needed.

Studies have suggested that several ligands for the activating receptor natural killer group 2 member D (NKG2DL) could be potential targets for immunotherapeutic approaches in STS.10 These ligands, including the UL16-binding protein (ULBP) family and MHC class I-related chain (MIC) proteins, are commonly expressed in STS, but generally absent in healthy tissues.[10] NKG2D plays an important role in the immune response, including immune surveillance, antimicrobial immune response, and antitumor effects.[9] The NKG2D pathway has also been shown to modulate tumorigenesis and tumor progression, which is particularly significant for inhibiting tumor cell metastasis.[10]

Immune therapy utilizing bispecific fusion proteins

The use of bispecific antibodies, which directly attract immune effector cells like NK and T cells to the tumor cell, resulting in cell lysis, is an attractive approach for anti-cancer immune therapy. With this method, it is essential to identify reliable target antigens, broadly expressed on tumor tissue while absent in healthy tissues, to ensure treatment efficiency and therapeutic safety of these molecules.

In a recent study, Melanie Märklin, from the University Hospital Tuebingen in Germany, and colleagues developed NKG2D-CD16 and NKG2D-CD3 bispecific fusion proteins (BFPs) to target STS cells.11 These BFPs consist of the extracellular domain of the NKG2D receptor fused to anti-CD16 or anti-CD3 Fab fragments, allowing for recruitment of NK cells and T cells, respectively (Figure 5).

Once the researchers demonstrated that both NKG2D-CD16 and NKG2D-CD3 induced T and NK cell activity, they set out to assess whether this effector cell activation induced lysis of tumor targets. Using cytotoxicity assays at various incubation times, the team evaluated lysis of sarcoma cells using Peripheral Blood Mononuclear Cells (PBMC) of healthy donors in the presence or absence of the fusion proteins (2.5 µg/mL). Sarcoma cells were labeled with DELFIA® BATDA for 1 hour and then incubated with PBMC at different effector to target cell ratios (E:T), with a summary of the overall assay workflow shown in Figure 6.

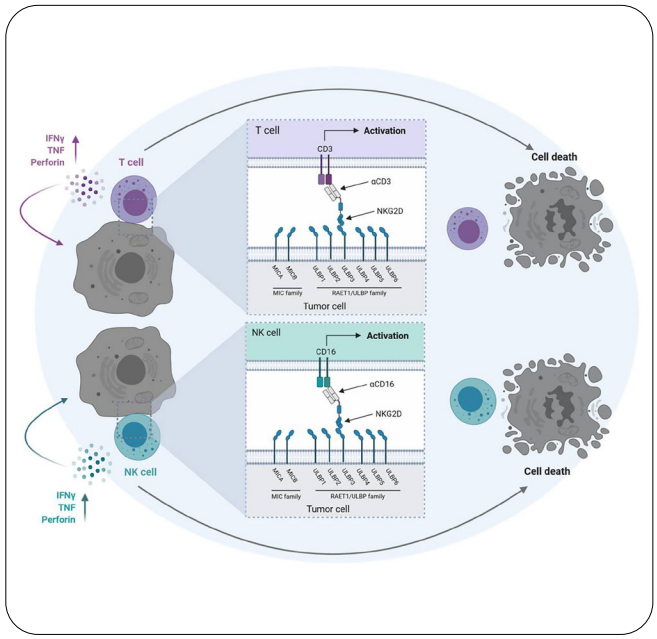


Figure 5: Mechanism of action of NKG2D-CD16/CD3 fusion proteins. Schematic illustration of BFP consisting of the extracellular domain of the NKG2D receptor fused to Fab-fragments directed against CD3 (NKG2D-CD3) or CD16 (NKG2D-CD16). Binding of NKG2D to NKG2DL (MICA/B, ULBP1-6) leads to activation of T cells and NK cells via anti-CD3 and anti-CD16 Fab-fragments and subsequent lysis of tumor cells. The graphic was created with BioRender software (BioRender.com, Toronto, Canada). (Image credit: Hagelstein I, Lutz M, Schmidt M, Heitmann J, Malenke E, Zhou Y et al.11)

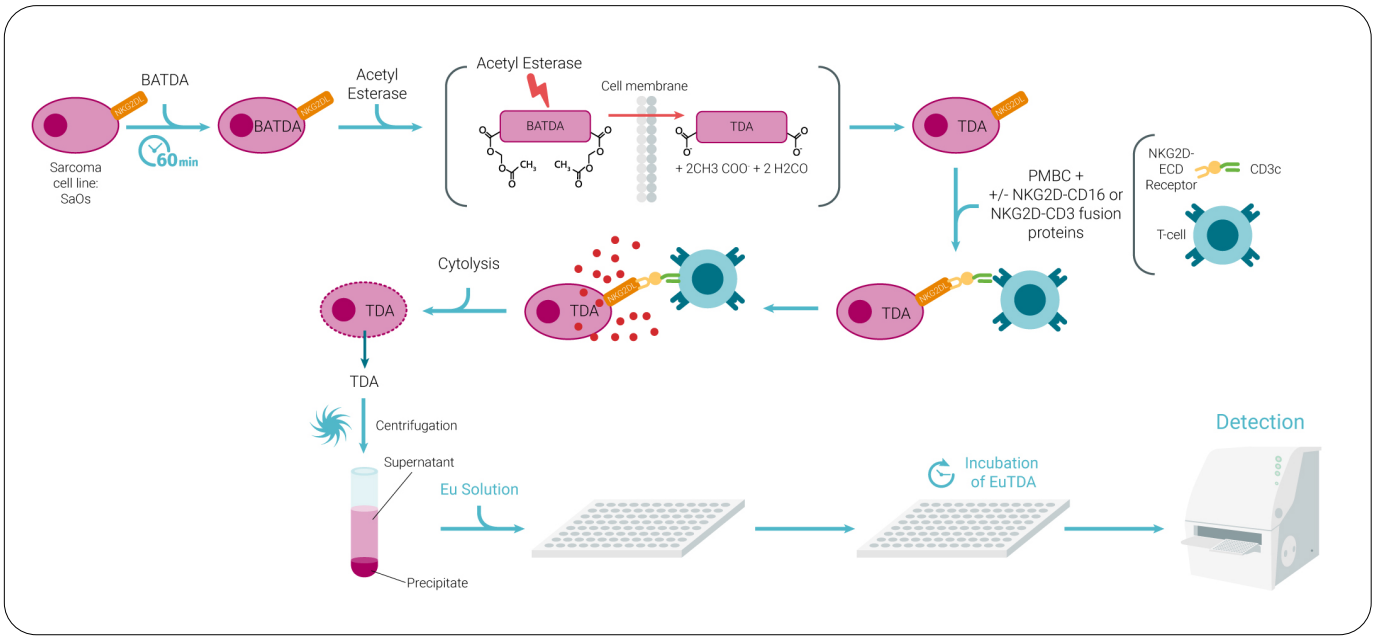


Figure 6: Experimental workflow of the cytotoxicity assay. Using the DELFIA EuTDA Cytotoxicity Assay, target cells are loaded with BATDA reagent. The cells rapidly process the molecule into TDA and are then co-cultured with PBMC cells in the presence or absence of bispecific fusion proteins. When cytolysis occurs in the target cell line, TDA is then collected in the supernatant and mixed with a Europium solution to form EuTDA which is highly fluorescent. The final level of EuTDA is proportional to the amount of cell death from the target cell population.

Analysis revealed that treatment with NKG2D-CD16 resulted in highly effective tumor cell lysis after 2 hours, whereas no significant effect was observed with NKG2D-CD3 at this time point (Figure 7A). However, after 72 hours, flow cytometry-based lysis assays revealed profoundly stronger effects with NKG2D-CD3 (Figure 7B). The finding that NKG2D-CD3 required longer times to reveal its maximum anti-tumor activity was also confirmed by live cell imaging over an incubation period of 136 hours (Figure 7C).

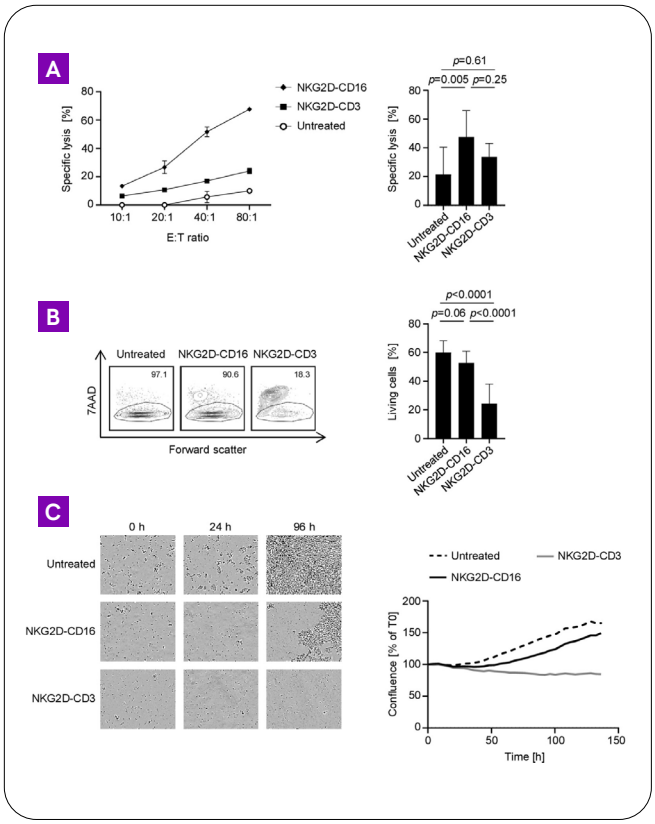


Figure 7: Induction of target cell lysis by NKG2D-CD16/NKG2D-CD3. PBMC of healthy donors (n=4) were incubated with different sarcoma cell lines and treated with the indicated constructs (2.5 µg/mL). (A) Lysis of sarcoma cell lines SaOs, RD-ES and SW1353 (n=3) was analyzed by 2 h Europium cytotoxicity assays. On the left, exemplary data obtained with SaOS with different E:T ratios and on the right pooled data obtained with PBMC of healthy donors at an E:T ratio of 40:1 are shown. (B) Lysis of sarcoma cell lines SaOs, RD-ES and SW1353 (n=3) was determined by flow cytometry based lysis assay (E:T 2.5:1) using PBMC of healthy donors. In the left panel, exemplary dot plots with SW1353 and one PBMC donor are shown; the right panel depicts pooled data. (C) Cell death of sarcoma cells was determined using a live cell imaging system. Cells were incubated with PBMC of healthy donors at an E:T ratio of 5:1 for 136 h. In the left panel, representative pictures at 0, 24, and 96 h are displayed. In the right panel, pooled data with two different cell lines are shown. (Image credit: Hagelstein I, Lutz M, Schmidt M, Heitmann J, Malenke E, Zhou Y et al.11))

According to the authors, these results suggest that combinatorial therapy might be a promising approach with NKG2D-CD16 BFP, after observing that PBMCs from STS patients receiving polychemotherapy showed profound NK and T cell activation after stimulation with NKG2D-CD16. They add that NKG2D-CD3 BFP might be effective as an adjuvant mono- or combination therapy due to its high anti-tumor efficiency. Together, these findings emphasize the potential of NKG2D-CD3 and NKG2D-CD16 BFP to target STS, even in advanced disease.

Conclusion

Although therapeutic options in solid tumors have increased in recent years, NSCLC and STS still present a unique set of challenges to researchers. Here we have highlighted two studies that utilized biochemical and cell-based assays to help characterize and develop new treatments to target NSCLC and STS. Further work is needed to fully characterize the potential of these approaches in the clinical setting and help advance effective treatments for solid tumors.

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