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EpCAM-targeted induction of apoptosis

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1. ABSTRACT

EpCAM is a well-established pancarcinoma-associated target antigen that has been used in a variety of therapeutic approaches. Of particular appeal are those strategies that aim to retarget and locally activate immune effector mechanisms involving apoptosis. Cancer cells typically employ various strategies to evade recognition and elimination by immune effector cells, including low or absent expression of MHCI molecules and active elimination of tumor infiltrating immune cells. In addition, cancer cells show an increased resistance towards endogenous pro-apoptotic stimuli due to aberrancies in their apoptotic machinery. However, compelling evidence indicates that cancer cells are often reliant on these molecular aberrations for continued cell survival. This pivotal role of immune evasion and apoptosis resistance has fueled the quest for therapeutic strategies that can selectively retarget and reactivate immune effector cells or molecules, whereby the balance of cellular fate of cancer cells is selectively tipped towards apoptosis. Here we review and discuss the perspectives for EpCAM-targeted apoptosis induction in cancer by EpCAM-selective bispecific antibodies and TRAIL fusion proteins.

2. INTRODUCTION

Cancer cells often display a qualitatively and/or quantitatively different repertoire of cell surface molecules that can be selectively targeted in cancer therapy using e.g. monoclonal antibodies (mAbs). The Epithelial Cell Adhesion Molecule (EpCAM) has since long been recognized as a suitable target antigen for imaging and immunotherapy of human cancer, since it is overexpressed on a variety of human carcinomas and is not shed into the circulation (1-2). Importantly, in normal cells, EpCAM expression is limited to the basolateral membrane of epithelia (3-4).

The principal feasibility of EpCAM-targeted carcinoma therapy has been demonstrated in various clinical trials using EpCAM-specific mAbs (5-7). EpCAM-specific mAbs are generally well-tolerated and have an acceptable toxicity profile, despite the expression of EpCAM on normal epithelia (8). Furthermore, a variety of EpCAM-targeted immunotoxins (9-12) and gene therapeutic approaches (13-15) have been (pre)clinically evaluated with promising activity. In addition, EpCAM-specific retargeting and reactivation of immune effector

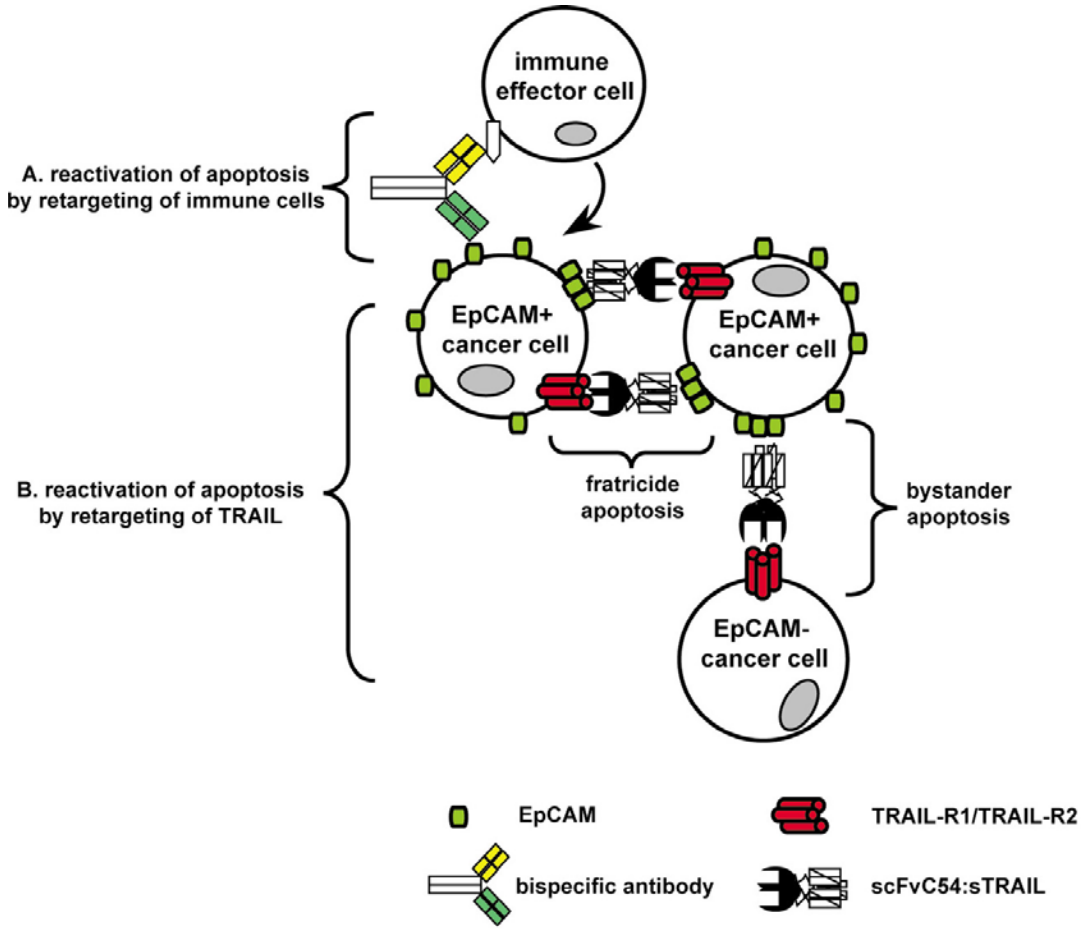

Figure 1. EpCAM-targeted apoptosis induction. A. Reactivation of apoptosis can be achieved using a bispecific antibody that retargets a large populations of predefined effector cells to the predefined target antigen EpCAM. Hereby, an entire cytotoxic effector cell population is retargeted to kill cells they normally would not eliminate by apoptosis induction. **B.** Reactivation of apoptosis can be achieved by the targeted delivery of the immune effector molecule sTRAIL to EpCAM, using the scFvC54:sTRAIL fusion protein. Binding of scFvC54:sTRAIL to EpCAM results in immobilization of scFvC54:sTRAIL at the cell surface of EpCAM-positive cells only. Subsequently, membrane bound scFvC54:sTRAIL induces fratricide apoptosis by reciprocal cross-linking of TRAIL-R1/-R2 on neighboring EpCAM-positive target cells. In addition, membrane bound scFvC54:sTRAIL on target cells can induce cross-linking of agonistic TRAIL receptors on the cell surface of a neighboring EpCAM-negative tumor cell, resulting in apoptosis induction of one or more bystander cells. Diagram is not to scale.

cells and/or effector molecules is of considerable therapeutic potential. This approach is particularly appealing since it aims to utilize and selectively redirect the intrinsic tumoricidal pro-apoptotic potential of the immune system to cancer cells only.

In this review, we will highlight a selection of approaches aimed at targeted apoptosis induction in EpCAM positive tumor cells (see Figure 1). We start by a brief overview of the mechanisms employed by cancer cells to evade immune prosecution and approaches pursued to achieve EpCAM-selective retargeting and reactivation of immune effector cells. Subsequently, we will review the mechanisms employed by cancer cells to evade elimination by programmed cell death, also known as apoptosis, and some of the approaches to achieve EpCAM-selective (re)activation of this process, in particular fusion proteins consisting of a high affinity anti-EpCAM antibody fragment genetically fused to Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL).

2.1. Tumor cell evasion of immune recognition

In actual fact, the immune system is perfectly equipped to selectively eliminate cells with potentially dangerous phenotypes by targeted apoptosis. To this end, the immune system exploits an enormous repertoire of highly selective receptors on immune effector cells (e.g. on T- and B-cells) to identify these cells, whereupon various potent effector mechanisms (e.g. granzymes and fibroblast-associated cell surface ligand (FasL)) are used to eliminate dangerous cells. However, during malignant progression cancer cells acquire a variety of mechanisms to evade recognition by immune effector cells. This so-called immune editing can for instance be due to down regulation

of major histocompatibility class (MHC) I. This can be caused by loss of chromosome loci on which the polymorphic genes are located or by the loss of expression of Beta-2-microglobulin, a protein required for MHC class 1 transport to the cell surface (16). Alternatively, peptide loading into MHC can be abrogated as a result of the loss of the intracellular peptide transporters TAP-1 and-2 (17-18). Another strategy of tumor cells to evade apoptosis induction by the immune system is the active elimination or induction of anergy of tumor infiltrating lymphocytes, a process dubbed as tumor counterattack. In this process tumor cell surface-expressed FasL can selectively activate apoptosis in infiltrating T lymphocytes (19). In addition, it has recently been shown that tumor cell-expressed galectin-1, a beta-galactoside binding lectin, can also selectively induce apoptosis in tumor infiltrating T cells (20). Alternatively, immunomodulatory cytokines such as TGF-beta may reduce T-cell mediated immunity (21).

3. RETARGETING CYTOTOXIC IMMUNE EFFECTOR CELLS TO EpCAM-POSITIVE CARCINOMA

3.1. Retargeting cytotoxic T-cells to EpCAM-positive carcinoma using bispecific antibodies

Bispecific antibodies that bind to both triggering molecules on cytotoxic effector cells and cell surface expressed target antigens on tumor cells can in principal induce the entire cytotoxic effector cell population to kill cells they normally would not lyse. In other words; using bispecific antibodies it is possible to retarget large populations of predefined effector cells to a predefined target antigen like EpCAM.

It is of particular interest to retarget T-cells, since these cells are very motile and possess highly cytotoxic effector molecules. Moreover, T-cells are the most abundant type of immune cell in the body, found not only in blood and lymph, but also in all organs as well as solid tumors.

Several clinical trials have highlighted the considerable promise of T-cell retargeting bsAbs. In early studies, bsAbs were generated by hybrid-hybridomas also known as quadroma technology. In certain studies the bispecific antibody was converted into a $F(ab')_2$ fragment by limited pepsin digestion. Hereby the Fc domain was removed and Fc-mediated interaction with abundantly present Fc-receptors was prevented, thus preventing premature and nonspecific activation and sequestration of effector cells.

Using this technology our lab generated BIS1, $F(ab')_2$ fragments of a bispecific antibody comprised of the high affinity anti-EpCAM mAb MOC31 and an anti-CD3 mAb, that specifically targets the signal transducing CD3-epsilon chain of the T-cell receptor/CD3-complex. Hereby, $CD8^+$ cytotoxic T lymphocytes and to a lesser extent $CD4^+$ T-cells can be specifically retargeted towards EpCAM-positive carcinoma cells in a non-MHC restricted manner. BIS1 was tested in several clinical trials. Although local administration of *ex vivo* activated and retargeted autologous T-cells resulted in promising local inflammation and antitumor activity, direct intravenous injection of BIS1 was not clinically successful. BIS1 in combination with subcutaneously given recombinant interleukin-2 yielded no clinical responses (22). In renal cell carcinoma patients, rapid lymphopenia was observed after BIS1 treatment (23-25), but no accumulation of T-lymphocytes was found in tumor tissue (26). More recently, the feasibility of EpCAM-targeted bsAb therapy was shown in ovarian cancer by Marme *et al* (27). Intraperitoneal injection with a bispecific antiEpCAM x antiCD3 antibody (HEA125xOKT3) in 10 patients with ovarian cancer and ascites resulted in tumor cell lysis *in vivo*.

3.2. Retargeting cytotoxic T-cells to EpCAM-positive carcinoma using recombinant bispecific single-chain antibodies

The production of bispecific antibodies using quadroma technology is inherently subject to the formation of a large fraction of the parental monospecific mAbs and non-cognate combinations of the various Ig light and heavy chains. Recombinant DNA technology has also been used to manipulate the size and shape of bsAb. The major aim is to explore strategies to produce predefined stable bispecific dimers of minimal size which can be easily produced and purified. To date various forms of genetically engineered bsAb closely meeting these criteria have been constructed. However, the need for additional costimulatory immune signals appears to limit the use of bsAbs. Interestingly, an EpCAM-targeted bispecific single-chain Ab (bscAb), generated by Mack *et al* appears to overcome this limitation (28-29). This particular recombinant bispecific antibody format effectively lysed ovarian cancer cells while the cytotoxic activity of the T-cells did not appear to depend on costimulation (30-31). This type of bispecific single-chain antibody was therefore renamed by the authors to bispecific T-cell engager molecules (BiTE) (32) since these bscAbs, in contrast to other bispecific antibodies, do not appear to require co-stimulatory strategies in order to fully activate T-cells. In addition, BiTEs are active at low concentrations and at lower effector to target (E:T) ratios compared with other bscAbs formats. Their efficient tumor cell lysis potential has been attributed to the formation of an immunological synapse on target cells, similar to the normal immunological T-cells synapse, whereupon granzymes and perforins rapidly eliminate the targeted cell by osmotic lysis and apoptosis (32).

3.3. Retargeting of myeloid immune effector cells to EpCAM-positive carcinoma

In addition to T-cells as prime cell type for bispecific antibody therapy, bsAbs with combined specificity for one of the human Fc-receptors (FcR) (e.g. CD16, CD32, and CD64) and EpCAM are also of considerable therapeutic interest. FcR are expressed on many different myeloid effector cells, including monocytes, macrophages, neutrophils and dendritic cells. Triggering of Fc-receptors typically results in Antibody Dependent Cellular Cytotoxicity (ADCC), phagocytosis, and cytokine release. For efficient targeting of FcRs, the FcR-epitope of bsAbs is outside of the Ig ligand binding site, enabling the activation of myeloid effector cells even in the presence of

high concentrations of nonspecific immunoglobulins. With regard to EpCAM-positive carcinoma, some promising results have been reported in ovarian carcinoma cells with a bsAb recognizing EpCAM and the high affinity Fc receptor CD64 (33). Retargeting of activated neutrophils, stimulated to express CD64, to EpCAM using an EpCAM x CD64 bsAb resulted in strong cytotoxic activity towards ovarian carcinoma cells, with comparable efficacy to a T-cell retargeting bsAb, thus clearly establishing the preclinical potential of this approach.

3.4. Trifunctional bispecific antibodies for EpCAM-positive carcinoma

Although the presence of an Fc domain is normally deleterious for the efficacy of bispecific antibodies, reports have emerged on intact bispecific antibodies, also named trifunctional antibodies (trAb), that can simultaneously bind to EpCAM and activate T cells as well as Fc-gamma receptor type I/III positive cells, such as macrophages, NK-cells and DC. The Fc-domain of these trAbs comprises a potent isotype combination of mouse IgG2a and rat IgG2b (34). The presence of this Fc-domain allows for the simultaneous recruitment of T-cells and e.g macrophages and may thus provide optimal costimulatory signals. Indeed, intraperitoneal treatment with an EpCAM-targeted trAb in patients with malignant ascites resulted in the complete elimination of tumor cells in ascites as well as disappearance of ascites accumulation (35). Moreover, a recent pilot study has demonstrated the feasibility using trifunctional antibodies with anti-EpCAM X anti-CD3 and anti-Her2 X anti-CD3 in combination with high-dose chemotherapy and autologous stem cell transplantation in metastatic breast carcinoma (36). Patients treated with the trAbs showed a trend towards improved overall survival. The trAb efficiently activated specific killing of targeted tumor cells without pre- or co-stimulation. However, major concerns regarding systemic application would be the Fc-mediated binding to the ubiquitously present Fc-receptors whereby tumor accretion is limited and side-effects, such as cytokine release syndrome, can be anticipated. In addition, the occurrence of human anti mouse antibodies (HAMA) or human anti rat antibodies (HARA) may further limit the therapeutic applicability.

4. EpCAM-TARGETED (RE-)ACTIVATION OF APOPTOSIS

4.1. Tumor cell evasion of apoptotic elimination

Apoptosis is the key process for the timely and safe removal of aged, superfluous, or dangerously altered cells. Apoptosis is a highly coordinated homeostasis mechanism that centers on the coordinated activation of effector caspases that silently blebs the dying cell to oblivion. Imbalances in the apoptotic machinery have been implicated in a variety of pathological conditions, including autoimmune diseases and cancer.

Typically, cancer cells develop a higher threshold for the normal endogenous pro-apoptotic signals. The most often found aberrancy in cancer is inactivation of the tumor suppressor p53, which is mutated in over 50% of tumors. Moreover, in tumors that express wildtype p53 its function is often inhibited by overexpression of the negative regular HDM2. Additional well established cancer-specific anti-apoptotic mechanisms are the upregulated expression of anti-apoptotic proteins, such as Bcl-2 and XIAP. Bcl-2 expression reduces the sensitivity to apoptosis by limiting one of the main pathways of apoptosis induction, the mitochondrial pathway, whereas overexpression of XIAP blocks the execution phase of apoptosis. The net result of all of these cancer-specific aberrancies is the development of tumor cells with increasingly malignant behavior. In turn these aberrancies are prime targets for the design of novel cancer-selective therapies (37).

A strategy being pursued by our laboratory is to enhance the clinical potential of the immune effector molecules TNF-related apoptosis-inducing ligand (TRAIL) and Fas Ligand (FasL). Both TRAIL and FasL are expressed on the cell surface of activated immune effector cells.

4.2. Target cell-restricted activation of apoptosis by scFv:sTRAIL fusion proteins

TRAIL is normally found as a transmembrane protein on e.g. T and NK-cells, but can also be proteolytically cleaved into a soluble form (sTRAIL). Using recombinant DNA-technology, recombinant forms of sTRAIL have been constructed. Recombinant sTRAIL selectively induces apoptosis in a variety of cancer cell types, with no or minimal activity towards most normal cells (38). TRAIL signals apoptosis by binding to and cross-linking of the agonistic receptors TRAIL-R1 and TRAIL-R2. This interaction leads to the recruitment of FADD and initiator caspase-8 to the intracellular Death Domain (DD) of TRAIL-receptors in the so-called Death Inducing Signaling Complex (DISC) (39-42).

Unfortunately, the clinical efficacy of sTRAIL might be hampered by several factors. For instance, the widespread expression of TRAIL receptors may limit tumor accretion. In addition, conventional sTRAIL preparations are poorly capable of activating TRAIL-R2 signaling, since this receptor only responds well to membrane-bound TRAIL, such as present e.g. T-cells. Moreover, TRAIL-R2 has been shown to be the high affinity receptor of TRAIL (43).

Recently, we have demonstrated that the tumor-selective binding and activity of sTRAIL can be strongly enhanced by genetic fusion to a tumor-selective human antibody fragment that targets EpCAM (44). The resultant fusion protein, designated scFvC54:sTRAIL, selectively bound to EpCAM at the cell surface of targeted cells only, whereby soluble scFvC54:sTRAIL was converted into a membrane-bound form of TRAIL. Consequently, the available membrane-bound sTRAIL domains of scFvC54:sTRAIL efficiently triggered cross-linking of both TRAIL-R2 and TRAIL-R1 on neighboring tumor cells, resulting in EpCAM-restricted reciprocal apoptosis induction.

It has been suggested that in solid tumors apoptosis induction is predominantly engaged via TRAIL-

R2 signaling with minimal involvement of TRAIL-R1 signaling (45). Conversely, apoptotic signaling in Chronic Myeloid Leukemia was reported to be mainly mediated by TRAIL-R1 signaling (46-47).

We reasoned that selective binding of scFvC54:sTRAIL to EGP2-positive tumor cells would also enable the cross-linking of TRAIL receptors on neighboring tumor cells devoid of EGP2 expression (see also Figure 1 for schematic). This principle, also known as the bystander effect, might help to overcome an important limitation to conventional antibody-based approaches, namely the escape from therapy of tumor cells that have lost or down-regulated target antigen expression upon therapy. Such a phenomenon has been reported for treatment of breast carcinoma with anti-EpCAM mAb 17-1A, which resulted in the selection of EpCAM-negative tumor clones (6).

The bystander effect of scFvC54:sTRAIL is based on the principle that targeted tumor cells are not only eliminated, but are also exploited to convey a proapoptotic effect towards neighboring tumor cells that lack EpCAM expression. Using mixed culture experiments we demonstrated that the selective binding of scFvC54:sTRAIL to EGP2-positive target cells conveyed an exceptionally potent anti–tumor bystander effect in EGP2-negative tumor bystander cells (48). This bystander effect of scFvC54:sTRAIL was detectable at target-to-bystander cell ratios as low as 1:100 and was not found in the absence of target cells. Importantly, no innocent bystander activity was detected towards freshly isolated blood cells. Of note, the bystander effect solely depends on accretion of scFvC54:sTRAIL to the cell surface of targeted cells and does not require further cellular processing other than intact death receptor signaling pathways.

Proof of principle for target cell-restricted apoptosis induction by anti-tumor scFv:sTRAIL fusion proteins has been obtained in both solid tumors (49-50) and leukemia (51), with no or minimal activity towards normal cells.

A different approach to activation of TRAIL-receptors is the use of agonistic mAbs, most notably HGS-ETR-1 and HGS-ETR-2 that are currently evaluated in clinical trials (52-53). A recent report highlighted that the use of an agonistic TRAIL-R2 mAb can also help induce potent tumor-specific T-cell immunity (54). In this respect, the targeted delivery of an agonistic mAb to EpCAM, in a bispecific antibody format, might optimize the activation of T-cell mediated immunity as well as the cross-linking and induction of apoptosis of TRAIL-receptors.

5. SUMMARY AND PERSPECTIVES

EpCAM-directed anti-cancer therapeutics have come a long way, starting from naked monoclonal antibodies and immunotoxins to redirecting immune effector cells and targeted delivery of pro-apoptotic ligands like TRAIL. Although the research efforts of the last decades have clearly established the great potential of retargeting immune effector cells and mechanisms to cancerous cells it has also revealed several important limitations.

For instance, bispecific antibodies are monovalent for either target antigen and so will bind substantially less strong compared to the respective parental Abs, due to a reduced avidity effect. Hereby, tumor cell accretion is potentially limited. In this respect it is important to note that a lower affinity for T-cells may actually be beneficial, since high-affinity binding to CD3 may actually target the bsAb to T-cells instead of tumor cells *in vivo* and also may reduce the efficiency of T cell stimulation. To optimize tumor accretion to EpCAM mutant variants of the bscEpCAMxCD3, with lower affinity for CD3, were generated. These variants dissociated more rapidly from CD3 but were efficient in T-cell triggering, in particular on tumor cells with low EpCAM expression (55).

Based on these data, the use of bscAbs with low affinity for CD3 could be exploited therapeutically. In addition, further increasing the affinity for EpCAM could help improve tumor localization. In this respect, the advent of recombinant antibody engineering is yielding ever more promising bispecific molecules with improved properties and enhanced therapeutic potential (56). Worth considering here is the fact that functional activation of T-cells usually requires additional costimulatory signals. BscAbs of the so-called BiTE format appear to be capable of activating T-cells as single agents.

Simple targeted therapies designed to selectively induce apoptosis in cancer cells are currently probably the most promising anti-cancer strategies. These strategies aim to specifically target and kill tumor cells with no or minimal collateral damage. We have provided proof of principle for EpCAM-restricted (as well as EGFR- and CD7-) restricted apoptosis induction using recombinant fusion proteins of a tumor-selective antibody fragments genetically fused to sTRAIL (44,48,49,51). Moreover, we and others have recently reported on a similar strategy in which sTRAIL is swapped for homotrimer soluble FasL (sFasL) (57-58). It has been established that homotrimeric sFasL, in contrast to membrane FasL, is nontoxic to normal cells, but also lacks tumoricidal activity (59). In contrast, sFasL hexamers and secondary aggregated sFasL trimers are highly active towards tumor cells, but are also toxic to liver cells (60-62). Like sFasL trimers, trimeric scFv:sFasL fusion proteins are inactive, but acquire strong tumoricidal activity after specific binding to a pre-selected cell surface-expressed target antigen (57-58). Thus, only upon selective binding to the tumor cell surface the otherwise inactive scFv:sTRAIL and scFv:sFasL fusion protein are activated after which tumor cell apoptosis is induced in an autocrine or paracrine manner.

Further refinement of this strategy might be obtained by using a prodrug strategy, such as for instance described by Gerspach *et al.* for TNF (63-64). The TNF prodrug is a tripartite fusion between a tumor-selective

antibody fragment, soluble TNF, and a TNF receptor-derived inhibitor module. Between the TNF-R inhibitory module and TNF, protease recognition motifs were engineered. Consequently, after tumor-selective binding of the TNF prodrug the inhibitor module is removed by tumor cell-expressed proteases, ensuring strictly antigen-dependent activation of apoptosis.

However, concepts such as targeted delivery of sTRAIL will fail when the targeted tumor cells are resistant to apoptosis due to one or more defects in death receptor or caspase apoptosis pathways. Therefore, single agent therapy is likely to prove not selective enough in most cases. The best way forward appears the combined treatment of cancer cells with therapeutics designed to exploit several cancer-related aberrations, whereby the therapeutic window is increased. However, since both normal and cancer cells critically rely on apoptosis, it is important to consider whether there is a large enough therapeutic window between sensitivity to apoptosis in normal and cancer cells.

In any case, the EpCAM target antigen remains a very promising pan-carcinoma target antigen that allows for studies in the most prevalent forms of cancer in humans. Success or breakthrough in one particular carcinoma type with EpCAM-targeting agents may be easily adaptable to other carcinoma which potentially accelerates clinical application.

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