Targeting SUMO signaling to wrestle cancer

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Chapter 1

Targeting SUMO Signaling to Wrestle Cancer

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Trends in Cancer, 2021

doi.org/10.1016/j.trecan.2020.11.009

ABSTRACT

The Small-ubiquitin-like-modifier (SUMO) signaling cascade is critical for gene expression, genome integrity and cell cycle progression. In this review, we discuss the important role SUMO may play in cancer and how to target SUMO signaling. Recently developed small molecule inhibitors enable therapeutic targeting of the SUMOylation pathway. Blocking SUMOylation not only leads to reduced cancer cell proliferation but also to an increased anti-tumor immune response by stimulating interferon signaling, indicating that SUMOylation inhibitors have a dual mode of action that can be employed in the fight against cancer. The search for tumor types that can be treated with SUMOylation inhibitors is ongoing. Employing SUMO conjugation inhibitory drugs in the years to come has potential as new therapeutic strategy.

Keywords: SUMO, ubiquitin, cancer, cell-cycle, mitosis, inhibitor

SUMOYLATION AND ITS TARGETS

Small-ubiquitin-like-modifiers (SUMOs) are post-translational modifications (PTMs) involved in various cellular processes, including cell cycle progression and the DNA damage response [1]. The conjugation of SUMO proteins to substrate proteins, called SUMOylation, occurs via an enzymatic cascade consisting of a dimeric SUMO activating enzyme **E1** (**SAE1/UBA2**), a single **E2 (UBC9)** and a limited set of E3 ligases. Mammals have up to five SUMO family members. Mature SUMO2 and SUMO3 have 97% sequence similarity, whereas SUMO1 and SUMO2/3 have only 53% sequence similarity. SUMO2 and SUMO3 form chains in an efficient manner via an internal SUMO consensus site [2,3].

The reversible nature of SUMOylation is facilitated by SUMO-specific proteases (SENPs), which are able to deconjugate SUMOs from substrate proteins. SENPs are responsible for the deconjugation of mono-SUMO, di-SUMO or poly-SUMO chains from target proteins. Additionally, the SENPs are crucial for maturation of precursor SUMO proteins by cleaving their carboxyl termini to expose their di-Gly motif required for conjugation.

The majority of SUMO targets is localized in the nucleus and is thought to undergo rapid cycles of SUMO conjugation and de-conjugation, with a low overall stoichiometry of SUMOylation [4–7]. SUMO is thought to play important roles in different types of cancer due to the critical function of SUMOylation in cell cycle progression and genome integrity. Therefore, SUMOylation inhibitors have potential for anti-cancer therapy. In recent years, novel SUMO pathway inhibitors have been developed to target cancer and recently promising data on this topic have been published [8–11]. This review will focus on the role of SUMO in cancer progression and the use of SUMOylation inhibitors to halt cancer progression (Figure 1). In addition to the topics and targets discussed in this review, SUMOylation modulates many other proteins [12]. Furthermore, control of the DNA damage response and gene transcription by SUMOylation are important topics that have been discussed in more detail elsewhere [6,13–17].

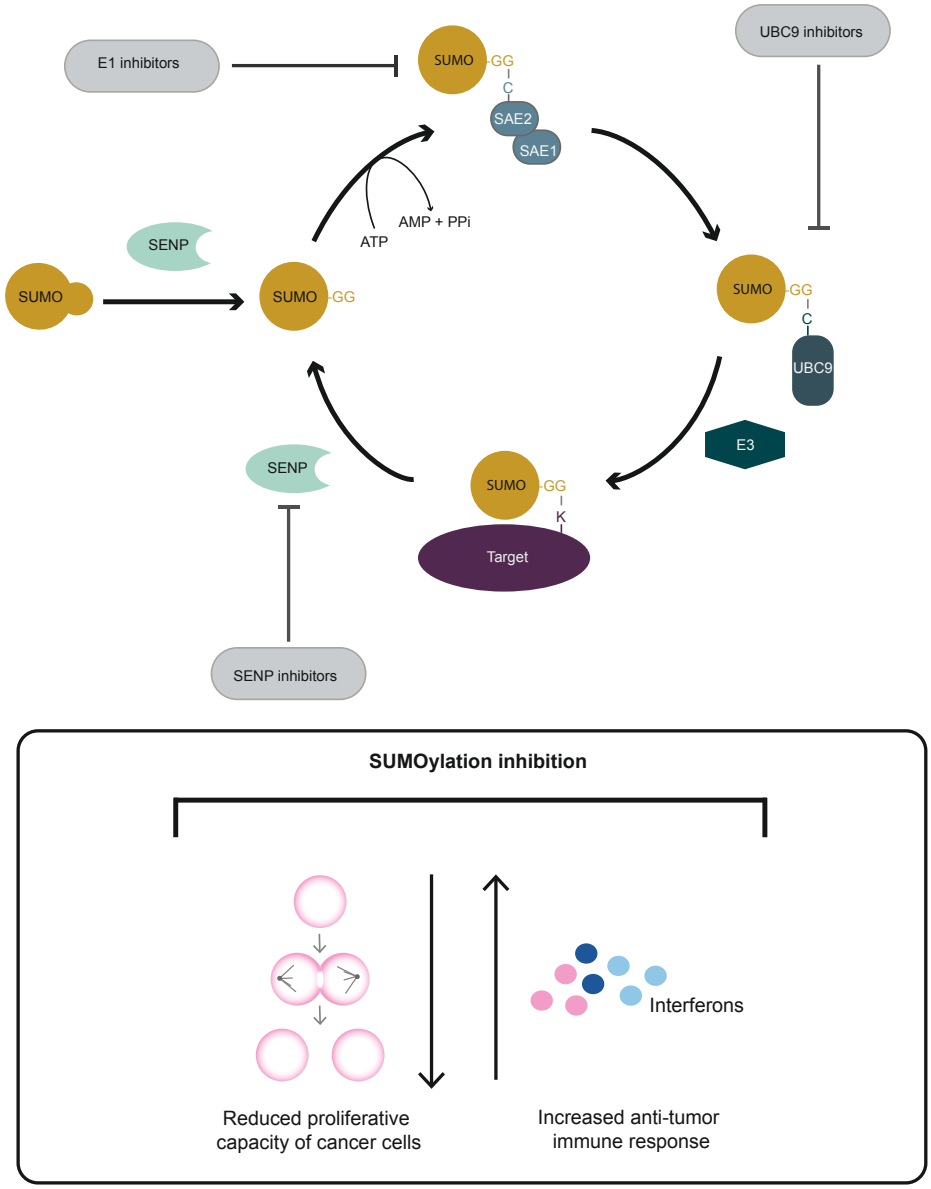


Figure 1 SUMO-cycle inhibitors reduce proliferation and increase anti-tumor immune responses. SUMO proteins mature via cleavage of their carboxyl termini, exposing their di-Gly motif needed for conjugation to target proteins. SUMO is conjugated to target proteins via its enzymatic cascade including E1, E2 and E3-ligases. SENPs deconjugate SUMO proteins from their targets. Inhibitors of the E1 and E2 can block the SUMOylation cascade and inhibitors of SENPs can block deSUMOylation of subsets of targets and prevent maturation of SUMOs. A block in the SUMOylation cascade leads to impaired cell proliferation and induces interferon production to stimulate anti-tumor immune responses, indicating its dual potential to target cancer cells.

SUMO IS IMPORTANT FOR CELL CYCLE PROGRESSION

The critical role of SUMOylation in cell cycle progression was uncovered by silencing several components of the SUMOylation cascade. Upon knockout or conditional knockdown of UBC9 in mammalian cell lines, severe nuclear defects were observed, including multi-nucleated cells, anaphase bridges, reduced chromatin condensation and apoptosis [18,19]. UBC9 depletion blocks cell proliferation via chromatin and nonchromatin associated pathways [20]. Similar effects as seen for loss of UBC9 are also observed when knocking down the SAE1/2 enzyme, including strong reductions in cell proliferation [21–23]. Dynamic regulation of substrates by SUMOylation in all phases of the cell cycle has been identified via a proteomics approach [24]. SUMOylation thus appears crucial throughout the entire cell cycle, particularly in mitosis.

From the perspective of mammalian development, removal of several components of the SUMOylation cascade has extensive effects on their development. Mouse embryos deficient for UBC9 harbor severe mitotic defects, including anaphase bridges, an increased amount of polyploid cells, and hypo-condensation, resulting in embryonic lethality at the early post-implantation stage [18]. In particular, SUMO2-deficient mouse embryos do not develop past early stages of embryonic development (E10.5), in contrast to SUMO1 or SUMO3 deficient mouse embryos, indicating the critical role of SUMO2 in development [25].

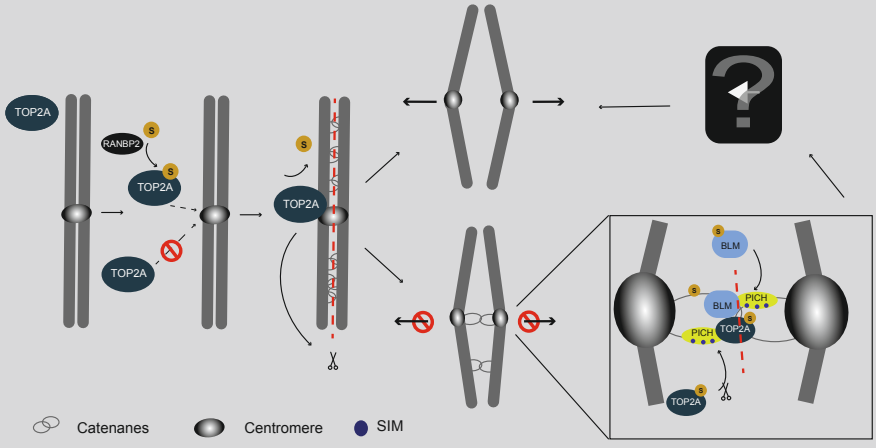
Phenotypical characteristics illustrating loss of SUMOylation in cells are aneuploidy (the presence of abnormal numbers of chromosomes per cell) [18] and **chromatin bridge formation** [23,26–28]. The remaining stretch of DNA between two daughter-cells in the case of chromatin bridge formation prevents cells to properly divide and start their own independent cell cycle. To unravel the role of SUMO in the development of chromatin bridges and aneuploidy, it is important to understand the role of SUMOylation in chromosome segregation. This is detailed in Box 1.

As long as incorrectly attached chromosomes remain, the **spindle assembly checkpoint (SAC)** halts mitosis via inhibiting the anaphase promoting **complex/cyclosome (APC/C)-CDC20**. The SAC is an essential feedback-control system for proper chromosome segregation and is responsive to disruptions of microtubule-kinetochore attachment to prevent premature dissociation of sister chromatids [29,30]. Several proteins involved in the SAC are targets for SUMOylation. For example, a SUMO mutant of BubR1 cannot be removed from the kinetochores during metaphase, resulting in delayed mitosis and chromosomal mis-segregation [31].

Box 1 | SUMO and UFBs

TOPIIα is crucial for decatenating chromosomes prior to chromosome segregation, to prevent impaired cell division. The SUMO E3-ligase RanBP2 is responsible for the SUMOylation of the C-terminal part of TOPIIα in mammalian cells, which is required for proper localization of TOPIIα to inner centromeres [115,116]. Furthermore, SUMOylation reduces the activity of TOPIIα until anaphase, when decatenation of centromeric DNA is required [27]. Loss of TOPIIα SUMOylation will compromise the decatenation of DNA at the centromere and cause impaired cell division, via concatenated sister-chromatids.

In anaphase a cell is presented with a "final chance" to resolve concatenated sister-chromatids. Sister-chromatids in anaphase can still be connected via catenates, including centromeric catenates, which are also known as ultra-fine bridges (UFBs) [117]. When UFBs remain unresolved, they can lead to chromatin bridges, which is the phenotype observed upon loss of SUMOylation as mentioned before. UFBs are coated with amongst others the SUMO targets Polo-like kinase 1-Interacting Checkpoint Helicase (PICH) and the Bloom syndrome helicase (BLM). PICH was identified as an interaction partner of SUMOylated poly (ADP-ribose) polymerase 1 (PARP1) at the centromere [118]. The same study also identified the preferential binding of PICH to SUMOylated TOPIIα. Furthermore, PICH itself can also be SUMOylated, which reduces its affinity for DNA binding [118]. PICH comprises 3 SIM domains with distinct functions; two domains influence the enzymatic activity on the chromosomes, for example via attenuating SUMOylated TOPIIα activity [119], whereas the most C-terminally located SIM domain is crucial for centromeric localization of PICH [118]. PICH is known to recruit BLM and TOPIIα to UFBs; however it remains unclear how these proteins act together in resolving UFBs [117,120,121] (Figure I).



Box 1, Figure I. Role of SUMOylation in sister-chromatid decatenation. TOPIIα is SUMOylated at its C-terminal domain via the SUMO E3 RanBP2. SUMOylated TOPIIα localizes to the centromere. TOPIIα SUMOylation reduces its decatenating activity until anaphase onset upon which TOPIIα is deSUMOylated and decatenation can occur, leading to chromosomes segregation. Impaired SUMOylation potentially reduces decatenation, resulting in ultra-fine bridges (UFBs). TOPIIα, BLM and PICH are recruited to and resolve the UFBs in a SUMO-dependent manner. The exact molecular mechanism to resolve these UFBs in a SUMO-dependent manner is still unclear.

BubR1 together with Bub3 and Mad2 bind CDC20 to form the mitotic checkpoint complex (MCC) in the presence of unattached kinetochores, which acts as an effector of the SAC and blocks mitosis via interfering with the formation of the APC/C-CDC20 [32]. The APC/C is a multi-subunit ubiquitin E3-ligase that facilitates metaphase to anaphase transition. The APC/C amongst others ubiquitylates securin and cyclin B, resulting in their proteasomal degradation. Securin degradation leads to the activation of Separase, which cleaves cohesin, resulting in sister-chromatid separation [29,32,33]. The APC4 subunit of the APC/C can be SUMOylated on two acceptor lysines, K772 and K798. SUMOylation of the APC4 subunit occurs preferentially in mitosis and is critical for timely metaphase to anaphase transition [34]. SUMOylation of the APC/C increases its ubiquitylation activity towards selected substrates [23], demonstrating the importance of SUMOylation for proper chromosome segregation.

Aberrant expression of SUMO proteases halts cell proliferation and results in defective nuclear morphology and binucleated cells [35]. During mitosis, SENP1 and SENP2 are localized at the kinetochore, while deregulating SENP1 and SENP2 leads to chromosomal segregation errors [36]. Phosphorylation of SENP3 inhibits its deSUMOylating activity towards chromosome associated proteins, resulting in a mitotic arrest via upregulation of Mad2, a member of the MCC [28]. In addition, a knockdown of SENP7, which together with SENP6 is able to process SUMO chains, also delayed mitotic progression [37]. SENP7 depletion results in delocalization of HP1α from the pericentromeric heterochromatin where it is important for centromeric cohesion [38,39]. SENP6 is crucial for the assembly of the inner kinetochore [40]. Depletion of SENP6 decreases the stability of the constitutive centromere-associated network (CCAN)[40,41].

SUMO REGULATES P53 AND C-MYC

One of the most well-known tumor suppressors is **p53**, a transcription factor that inhibits cell cycle progression or induces apoptosis upon genotoxic stress [42]. p53 is mainly SUMOylated on lysine 386 by the PIAS-ligases and SUMOylation of p53 contributes to its activation [4,43]. The ubiquitin E3 ligase and oncoprotein MDM2 is the key p53 regulator that ubiquitylates p53, targeting it for proteasomal degradation [44]. More specifically, high levels of MDM2 are responsible for p53 degradation, whereas low levels of MDM2 cause mono-ubiquitylation and consequently nuclear export of p53 [45]. SUMOylation of MDM2 leads to p53 degradation via increasing MDM2 levels by decreasing its auto-ubiquitylating activity and consequently degradation [46]. SUMO

specific proteases can counteract MDM2 SUMOylation. UV radiation induces SUSP4, a mouse SUMO protease that removes SUMO from MDM2, leading to stabilization of p53 [47,48]. Lastly, RPL11 a regulator protein of the MDM2-p53 axis is also SUMOylated and potentially suppresses MDM2, via a currently unknown mechanism [49].

The protein product of the oncogene **c-Myc** is a transcription factor that has been linked to SUMOylation. c-Myc is a SUMO target that is specifically detected after heat shock or proteasome inhibition, implying that SUMOylation of c-Myc leads to its proteasomal degradation [50–52]. Interestingly, loss of SUMO via SAE1/2 knockdown is lethal for cells with high c-Myc expression, for example in the classical c-Myc-driven Burkitt lymphoma [53,54]. Still, the precise mechanisms underlying the SUMOylation dependence of c-Myc-driven tumor development is not completely clear. So far, contradicting results regarding SUMOylation and c-Myc have been published. One study showed that SUMOylation of c-Myc via the SUMO E3 ligase PIAS1 leads to transcriptional repression and subsequent proteasomal degradation [55], whereas another study shows that PIAS1 positively regulates c-Myc transcriptional activity in B cell lymphomas [56]. Recently, it has been found that SENP1 is responsible for deSUMOylating c-Myc, resulting in stabilization of c-Myc and consequently enhanced transcriptional activity [57,58], which supports the idea that SUMOylation suppresses c-Myc transcriptional activity. The inhibitory effect of SUMOylation on c-Myc transcriptional activity results in downstream repression of Pol I and Pol II dependent transcription activity [58,59]. The dependence of c-Myc-driven tumors on SUMOylation provides the opportunity to employ this weakness for therapeutic purposes [54,60].

INHIBITING THE SUMOYLATION CASCADE

Inhibiting proteins in the SUMOylation cascade could be beneficial for the treatment of malignancies. The expression of SUMOylation cascade proteins, SENPs, SAE1/2, UBC9 and E3-ligases, are upregulated in multiple cancers [7,61] and SUMOylation affects some proteins encoded by oncogenes and tumor suppressor genes. Combined, this paves the way for implementation of SUMOylation cascade inhibitors in the treatment of cancers. A considerable set of natural and synthetic compounds have been reported to inhibit the SUMOylation cascade (table 1).

Table 1 | SUMOylation cascade inhibitors

COMPOUND	NATURAL OR SYNTHETIC	TYPE OF MOLECULE	TARGET	IC50 (INHIBITION OF IN-VITRO SUMOYLATION)	PUBLICATION
GINKGOLIC ACID (15:1)	Natural product small-molecule	Alkylphenol	E1	3.0 μM	[64]
ANACARDIC ACID	Natural product small-molecule	Structural analog of Ginkgolic acid (15:1)	E1	2.2 μM	[64]
KERRIAMYCIN B	Natural product small-molecule	Antibiotic	E1	11.7μM	[65]
SUMO-AMSN SUMO-AVSN	Protein-based	c-terminally modified SUMO proteins with 5'-sulfonyladenosine based molecules	E1		[134]
COMPOUND 21	Synthetic small-molecule	Phenyl Urea	E1	14.4 μM	[70]
DAVIDIIN	Natural product	Ellagitannin	E1	0.15 μM	[66]
TANNIC ACID	Natural	Gallotannin	E1	12.8 μM	[67]
SEVERAL COMPOUNDS IDENTIFIED IN A THIAZOLE UREA AND PYRAZOLE UREA BASED SCREEN	Synthetic small-molecules	Thiazole Urea and Pyrazole Urea	E1	30 - 100 μM	[135]
ML-792	Synthetic small-molecule	Pyrazole- carbonylpyrimidine	E1	0.003 μM (SUMO1) 0.011 μM (SUMO2)	[8]
COH-000	Synthetic small-molecule	dimethyl 1-((R)-1-(phenylamino)-2-(p-tolyl) ethyl)-7-oxabicyclo [2.2.1]hepta-2,5-diene-2,3-dicarboxylate	E1	0.2 μM	[11,71]
TAK-981	Synthetic small-molecule	Pyrazole- carbonylpyrimidine	E1		[9]
ML-93	Synthetic		E1	0.037 μM	[10]
GSK145A	Synthetic small-molecule	Diamino-pyrimidine	E2	12.5 μM	[75]
2-D08	Synthetic small-molecule	Flavonoid	E2	6 μM	[136]
SPECTOMYCIN B	Natura product small-molecule	Antibiotic	E2	4.4μM	[78]

Table 1 *(continued)*

COMPOUND	NATURAL OR SYNTHETIC	TYPE OF MOLECULE	TARGET	IC50 (INHIBITION OF IN-VITRO SUMOYLATION)	PUBLICATION
COMPOUND 2	Synthetic small-molecule	Pyridine	E2	75μM	[137]
SUBINS	Protein-based	SUMO2 variants	E2	0.025 μM	[76]
COMPOUND 38	Synthetic small-molecule	Benzodiazepine	SENP1	9.2μM	[138]
TRIPTOLIDE	Natural product small-molecule	Tripterygium wilfordii Hook F	SENP1	0.009754 – 0.0203 μM (*in vivo*)	[83]
COMPOUND J5	Synthetic small-molecule	2-(4-Chlorophenyl)-2-oxoethyl 4-benzamidobenzoate derivative	SENP1	2.385 μM	[139]
GN6958	Synthetic small-molecule	Phenyl Urea	SENP1	29.6 μM	[87]
COMPOUND 69 AND 117	Synthetic small-molecules	Oxadiazoles	SENP2	5.9 μM, 3.7μM	[140]
COMPOUND 13M	Synthetic small-molecule	Phenyl	SENP1	3.5 μM	[85]
MOMORDIN IC (MC)	Natural product small-molecule	Pentacyclic triterpenoid	SENP1	15.37 μM	[84]
COMPOUND 3	Synthetic small-molecule	Phenyl	SENP1/2	3.55 μM, 2.98 μM	[86]
STREPTONIGRIN	Natural product small-molecule	Antibiotic	SENP1	0.518 μM	[141]
EBSELEN	Synthetic small-molecule	Organo-selenium	SENP2	2 μM (*in vivo*)	[89]
COMPOUND 6, 7 AND 10	Synthetic small-molecules		SENP1	3.7 μM, 0.99 μM, 7.5 μM	[88]

SAE1/UBA2

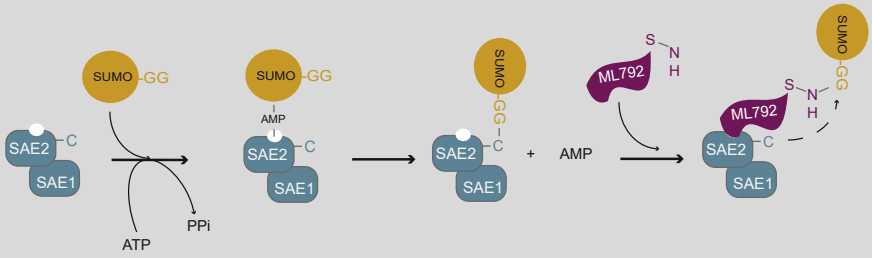
The SUMO E1 is a dimer consisting of the SAE1 and UBA2/SAE2 subunits. Knockdown of these subunits blocks the proliferative capacity of cancer cells, as shown in for example HCT116 colon cancer cells, U2OS osteosarcoma cells, Raji Burkitt lymphoma cells, Eμ-*Myc* lymphoma cells, and U87 and U251 human glioma cells *in vitro. In vivo* subcutaneous tumor models for glioma and Burkitt lymphoma and an orthotopic xenograft model for HCT116 showed a decrease in tumor growth upon shRNA-induced knockdown of SAE2 [22,53,54,6263]. The first reported SAE1/2 inhibitors (table 1) are natural compounds, including ginkgolic acid, its structural analog anacardic acid and kerriamycin B [64,65]. Treatment with ginkgolic acid and anacardic acid decreased cancer cell growth of Eμ-Myc lymphoma cells, and P493-6, BL70, Raji and Daudi Burkitt lymphoma cells *in vitro* [54]. These compounds inhibit formation of the SAE1/2-SUMO intermediate, consequently blocking the conjugation of SUMO to target proteins. Other natural compounds blocking SAE1/2 are Davidiin [66] and tannic acid [67] that employ a similar mechanism of action. Both compounds inhibit cancer cell growth, respectively for NCI-H460 lung cancer cells, MKN-45 gastric cancer cells, DU-145 prostate cancer cells [66] and YD-38 cells, a gingival squamous cell carcinoma [68]. The limitations of these natural products are that they mostly function in the micromolar range and that they do not singly target SUMOylation. Ginkgolic acid is known to target pro-inflammatory molecules like prostaglandins and leukotrienes [69], and tannic acid can also induce cancer cell death via activation of apoptosis rather than via inhibition of cell cycle progression as expected for a SUMOylation inhibitor [68]. The broad range of targets affected by these natural compounds, complicates the mechanistic understanding of their anti-cancer effects.

Synthetic inhibitors of the SUMO E1, including compound-21 [70], ML-792 [8], its derivative TAK-981 (Takeda) and COH-000 [11] have been developed to tackle this problem. Compound-21 and COH-000 act similar to the natural compounds via interaction with the SAE1/2. Compound-21 specifically interacts with the ATP binding site in the SAE2 and COH-000 via binding to Cys30 in an allosteric site of the SAE2. ML-792, TAK-981 and ML-93 inhibit SAE1/2 activity by forming an adduct with SUMO in an ATP dependent manner catalyzed by the enzyme itself [8–10], as detailed in Box 2. These single molecule compounds are highly specific and do not have side effects on for example ubiquitylation and neddylation up to the micromolar range [8,71]. For COH-000, ML-792, TAK-981 and ML-93 it was reported that they inhibit cancer *growth in vitro* in for example, HCT116 and Colo-205 colon carcinoma cell lines, MDA-MB-231 and MDA-MB-249 breast cancer cell lines and pancreatic ductal adenocarcinoma (PDAC).

In vivo the syngeneic immunocompetent BALB/c A20 lymphoma model treated with TAK981 and a HCT116 subcutaneous colon cancer xenograft treated with COH-000 exhibited reduced growth [8–10,71]. In the PDAC model it was observed that Myc hyperactivation sensitizes PDAC cell lines including PSN1 and primary huPDAC cells towards SUMO inhibition via ML-93. Furthermore, a PaTu- 8988T xenograft model showed dose dependent sensitivity towards SUMO E1 inhibition [10].

Box 2 | ML-792/TAK-981 SAE1/2 inhibition

The selective SAE inhibitor ML-792 and its functional analogue TAK-981 are structurally related to adenosine 5'-monophosphate (AMP) and are expected to bind in the nucleotide binding pocket of SAE. For the identification of selective SAE inhibitors, a pyrazole carbonylpyrimidine-based scaffold was used [8]. Inhibitory activity occurs via the formation of an adduct between ML-792 or TAK-981 and SUMO. The sulfamate ester in ML-792 forms a covalent irreversible adduct with the C-terminus of SUMO, catalyzed by the SAE enzyme itself in an ATP-dependent manner. This ML-792-SUMO conjugate subsequently binds tightly to SAE2, also known as UBA2, the catalytic subunit of SAE and inhibits its activity [8,122] (Figure I). Specificity was confirmed via screening ML-792 against a panel of ATP-dependent enzymes, which did not demonstrate any significant off-target effects. Furthermore, E1 activity of NAE, the E1 for Neddylation was only affected by ML-792 at very high doses [8]. UAE, the ubiquitin E1 was not inhibited by ML-792.



Box 2, Figure I. Mechanism of SAE inhibition by ML-792. This graphical overview shows the enzymatic mechanism of SUMO-ML792 adduct formation in an ATP-dependent manner, via the SAE1/UBA2 enzyme. The SAE1/UBA2 enzyme catalyzes the formation of a SUMO-AMP intermediate, binding to the ATP binding pocket in SAE2 and releasing inorganic pyrophosphate. Next, SUMO-AMP reacts with the active site cysteine to form a SAE2-SUMO thioester, releasing AMP. ML-792 binds to the ATP binding-site in the SAE2-SUMO thioester complex and subsequently, the sulfamate ester in ML-792 forms a covalent adduct with the C-terminus of SUMO. The ML792-SUMO adduct occupies the SAE1/UBA2 enzyme and impairs its activity. This mechanism is analogous to the MLN4924-Nedd8 adduct formation inhibiting NAE, as described in Brownell et al. [142].

Interestingly, SUMOylation modulates type I interferon signaling [72,73]. Consistently, the activity of TAK-981 against the murine A20 lymphoma is dependent on IFN alpha/beta receptor 1 (IFNAR) activity, linking the activity of TAK-981 to interferon signaling *in vivo* [9]. Box 3 describes the interaction between SUMOylation and the interferon pathway. Furthermore, it is suggested that TAK-981 promotes anti-tumor immune responses via enhanced cross-presentation of exogenous antigens released by dying tumor cells, leading to cytotoxic T cell priming and activation in mice [74], implying a role for innate and adaptive immunity in TAK-981 anti-tumor activity. Notably, TAK-981 is currently in a phase I clinical trial in patients with metastatic solid tumors or lymphomas (ClinicalTrials.gov Identifier: NCT03648372)[I], focusing on safety, tolerability and pharmacokinetics of the compound. Additionally, an early phase I clinical trial with TAK-981 in combination with Cetuximab or Avelumab for intra-tumoral micro-dosing in patients with head and neck cancer has started (ClinicalTrials.gov Identifier: NCT04065555)[II]. This trial aims to study the biological effects of TAK-981 within the tumor microenvironment. Lastly, a phase Ib/phase II interventional clinical trial with TAK-981 in combination with Rituximab in patients with Non-Hodgkin lymphoma (ClinicalTrials.gov Identifier: NCT04074330)[III] focusing on safety of the drug, followed by an intervention study evaluating the efficacy of TAK-981 in combination with rituximab. All three clinical trials are currently recruiting patients and no results have been listed yet. Taken together, TAK-981 is the SUMOylation cascade inhibitor that is most advanced and up to now shows to be the most promising candidate for clinical purposes.

UBC9

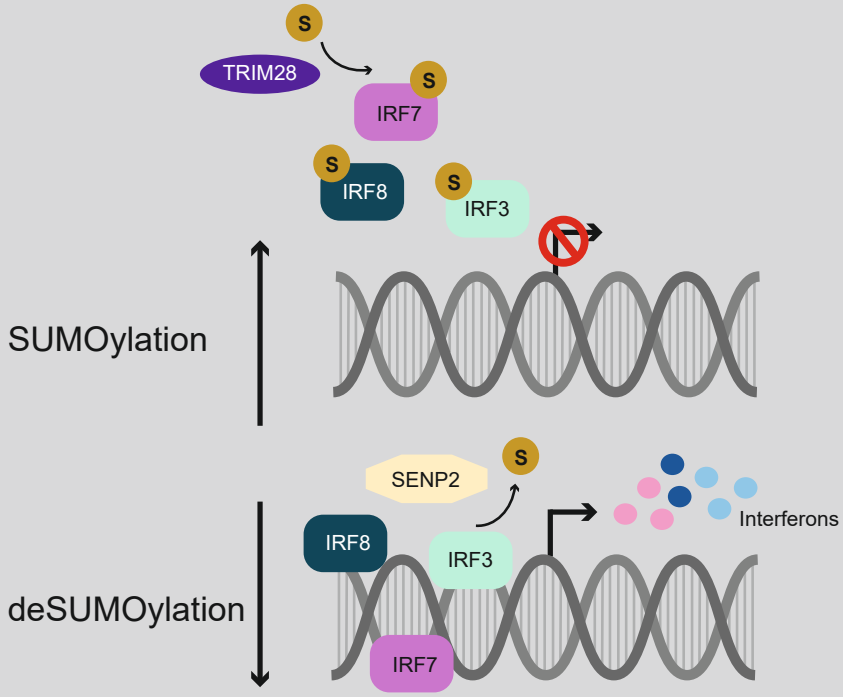
Ubiquitin-conjugating enzyme 9 (UBC9) accepts SUMO proteins from the E1 and catalyzes their covalent attachment to target proteins. UBC9 is the sole E2-enzyme in the SUMOylation cascade and contributes to substrate specificity together with the E3-ligases. Knocking down UBC9 reduced the proliferative capacity of for example HCT116 colon cancer cells *in vitro* and *in vivo* in an orthotopic xenograft model [22]. Several UBC9 inhibitors (table 1) have been identified through multi compound screening approaches, including GSK145A in a specific screen to detect inhibitors of TRPS1 SUMOylation [75], and SUBINs, which are SUMO-based UBC9 inhibitors which specifically inhibit SUMO-chain formation [76]. Spectomycin B1, a known antibiotic for gram-positive bacteria [77], binds to UBC9 and inhibits its interaction with SUMO, which is possibly unrelated to its antibiotic activity. Estrogen-dependent proliferation of human breast-cancer cells is inhibited by spectomycin B1 in a manner similar

to UBC9 knockdown [78]. Lastly, 2-D08 (2',3',4',,-trihydroxyflavone) is an inhibitor that specifically blocks the transfer of SUMO from UBC9 to substrates [79]. 2-D08 reduces growth and induces apoptosis in non-APL AML cells and inhibits cell migration in K-Ras mutated pancreatic cancer cells [80–82]. However an *in vivo* effect of 2-D08 was only shown in a combination approach with all-trans retinoic acid (ATRA) in non-APL AML cells [82]. Thus, a variety of UBC9 inhibitors are able to block the SUMOylation cascade and have potential for being employed to target cancer cells.

Box 3 | SUMO and the immune system

The SUMOylation cascade balances innate immune signaling via regulating type I interferon responses and NF-kappa-B (NF_kB) activity. As reviewed in [123], SUMOylation affects multiple regulators in type I IFN production to act in a stimulatory or in an inhibitory manner. SUMOylation of the interferon regulatory transcription factor IRF3 negatively regulates interferon beta (IFNß) transcription. Consequently, deSUMOylation of IRF3 by SENP2 induces IFNß transcription [124,125]. Interferon alpha (IFNα) transcription is regulated in a similar manner by IRF7 SUMOylation via the E3 ligase TRIM28 [126,127]. In addition, IRF8, a transcription factor activated by IFNs and interleukin-12 is regulated by SUMO in a similar manner as IRF3 and 7 [128] (Figure I). Recent literature has shown that *in vivo* efficacy of SUMOylation inhibition is dependent on active interferon signaling [97–99]. Upon SUMOylation inhibition, active infiltration of the tumor by immune cells has been uncovered. Furthermore, MHC class I is upregulated upon TAK981 treatment, most likely in an interferon dependent manner [129]. Increased antigen presentation via MHC I upregulation is important for anti-tumor immune response. Release of SUMO's repressive effect on interferon transcription by SUMOylation inhibition enhances anti-tumor immune response.

Another effector of our innate immunity is the GMP-AMP synthase (cGAS), which senses viral DNA and consequently activates the stimulator of interferon genes (STING). Subsequently, STING stimulates type 1 IFNs. The E3 ligase TRIM38 SUMOylates cGAS and STING, resulting in their stabilization at early stages after infection. At late infective state, SENP2 deSUMOylates cGAS and STING, which leads to their degradation and thus diminishes the immune response [130]. In contrast, it has also been found that SUMOylation suppresses cGAS DNA sensing potential, which can be relieved by SENP7, showing a dampening effect of SUMOylation on immune activation [131]. The difference in SENP2 and SENP7 function can potentially be explained by the specificity of SENP7 for SUMO chains, whereas SENP2 can remove mono-SUMO conjugates and can also cleave poly SUMOs [132]. Combined, this suggests a differential effect for mono-SUMOylation and poly-SUMOylation on the cGAS-STING pathway. Furthermore, the NF_kB pathway can also be regulated by SUMOylation in an inhibitory and in a stimulatory manner [133]. Overall, it shows that SUMOylation is important for restraining the immune response by its predominant repressive effect on the pathways mentioned above. Loss of SUMOylation left mice susceptible to septic shock and increased protection against viral infection [72]. Thus, SUMOylation is a "master repressor" of gene expression in response to immune activating triggers. SUMO inhibition therefore has the potential to enhance anti-tumor immune responses.



Box 3, Figure I. The role of SUMOylation in interferon production. Increased SUMOylation halts interferon production, via SUMOylation of transcription factors IRF7, IRF8 and IRF3. De-SUMOylation of these proteins results in active transcription of interferons.

To date no small molecule inhibitors for SUMO E3-ligases have been identified. Future development of SUMO E3-ligase inhibitors will potentially add a level of specificity to inhibition of the SUMOylation cascade, since different E3-ligases are responsible for SUMOylation of subsets of target proteins.

SENPs

SUMO specific proteases (SENPs) are responsible for the maturation of SUMO and for the deconjugation of SUMO from substrate proteins. The SENP family comprises six members, SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7. Interestingly, SENP1 is upregulated in various cancers and SENP2 and SENP3 to a lesser extent as mentioned previously. Therefore, SENPs might represent targets for anti-cancer therapies. Several SENP inhibitors have been identified, targeting SENP1 or SENP2 (table 1). SENP inhibitors derived from natural products include triptolide and Momordin Ic, both potential SENP1 inhibitors that were found to reduce LNCaP and PC3 prostate

cancer cell proliferation. PC3 cells express higher levels of SENP1 and correspondingly are more sensitive towards Momordin Ic treatment. PC3 xenograft models show sensitivity towards both Momordin Ic and triptolide [83,84]. More SENP1 inhibitors have been developed, including compound 13m through in silico screening [85], compound 3 in an effort to develop a highly reliable assay for SENP inhibitors [86] and GN6958 [87]. These inhibitors show proper SENP1 inhibitory potential *in vitro.* However, they have not been tested *in vivo* as has been done for the two natural SENP1 inhibitors. The development of SENP inhibitors is ongoing, with as latest result the identification of a novel class of non-competitive SENP1 inhibitors [88]. As shown for the SENP2 inhibitor Ebselen, inhibition of SENPs will lead to an increase in overall SUMO conjugation in B35 cells and *in vivo* after injection in to mouse brains [89]. An increase in overall SUMOylation is proposed as a mechanism to protect the brain from ischemic damage [89]. Ebselen might therefore be suitable to prevent ischemic brain damage and be useful in the clinic outside of the oncology field. These examples highlight the potential for SENP inhibitors as cancer therapy and as treatment for potential other diseases.

POTENTIAL TOXICITY AND RISKS OF SUMO INHIBITION

Potential toxicity and adverse effects need to be taken into account for SUMO cascade inhibitors. SUMOylation inhibition is probably not simply beneficial as a therapeutic approach in cancer therapy. Knockout of UBC9 in adult mice resulted in loss of intestinal epithelium stability and function, leading to diarrhea and death [90]. Embryonic lethality at the early post-implantation stage is the phenotype of a full UBC9 or SUMO2 knockout [18,25]. Interestingly, UBC9 haploinsufficiency promotes malignant phenotypes and cell growth in Lgr5+ CBC cells *in vitro* and as an intestinal cancer mouse model. This intestinal cancer model indicates a tumor suppressive role for fully functional UBC9, which seems in conflict with the established role of SUMO in cancer [91]. Extensive understanding of cell type specific effects of SUMO inhibition is of major importance for therapeutic implementation.

Side effects of the selective SAE inhibitor ML-93 were irritation and ulceration at the injection site observed in a PDAC xenograft model [10]. A counterindication for the use of SUMOylation inhibitors is the downregulation of SUMO proteins or SUMO conjugating enzymes or the upregulation of SUMO-deconjugating enzymes in some tumors [7,61]. Furthermore, combinations of SUMOylation inhibition with other drugs should be carefully analyzed for potential adverse effects. For example, proteasomal degradation of PML-RARα upon As_2O_3 treatment is dependent on its hyper-SUMOylation [92–94].

Naturally, inhibiting SUMOylation in combination with As_2O_3 to treat APL would thus be counterproductive. In addition, IRC117539 promotes SUMOylation and ubiquitylation of the androgen receptor (AR) as potential therapy for prostate cancers, reminiscent to As_2O_3 therapy in APL. IRC117539 reduced cell growth in AR positive prostate cancer cells, whereas combining IRC117539 with ML-792 left the drug completely ineffective [95]. The above-mentioned considerations need to be taken into account for SUMO inhibition as tailored therapeutic option for a subset of malignancies.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

To implement SUMOylation inhibitors in clinical practice, a careful evaluation of cancers responsive to treatment has to be made. TAK-981 is the only SUMOylation inhibitor that is currently evaluated in clinical trials for a broad range of cancers. To identify types of cancer that can be treated with TAK-981, research should focus on identifying biomarkers that sensitize cancer cells for SUMOylation inhibition. As mentioned before in this review and in other reviews, cancer cells overexpressing c-Myc are sensitive to SUMO inhibition and are thus potentially suitable for treatment with SUMO inhibitors [60]. Ultimately, SUMO inhibitors should be used as components of combination therapies. Deep biological insight in SUMO biology should enable the identification of potential combination therapies. Given the key role of SUMOylation in cell cycle progression, combination therapies with cell cycle inhibitors could be explored. For this purpose, inhibitors of CDK4/6, Wee1 and Aurora kinases could be considered [96]. Furthermore, it was reported that SUMO inhibition by TAK-981 activates the immune system to target tumors via the interferon pathway [9] (Box 3). Recently, several research groups have shown the *in vivo* potential of TAK981 for human pancreatic cancer and B cell lymphoma, and mouse B cell lymphoma and colorectal cancer [97–99]. These research projects highlight the potential of TAK981 combination therapies with immune checkpoint inhibitors to stimulate anti-tumor immunity [100]. This strategy is also employed in several phase I and II clinical trials (NCT03648372[I], NCT04065555[II], NCT04074330[III], NCT04776018[VIII], NCT04381650[VIIII]).

However, careful risk analysis is required to avoid combination therapies that induce adverse effects. Accurate predictions to identify responsive tumor types are challenging to make. A recent example is a prediction model for chemotherapy resistance in AML based on PTMs including SUMO [101]. The authors identified a new class of biomarkers based on ubiquitin and SUMO conjugation, by comparing PTMs

in extracts of chemosensitive and chemoresistant AML and identified 122 proteins whose conjugation to SUMO or ubiquitin marks resistance. These biomarkers could potentially predict responses of AML patients to standard chemotherapies based on PTMs [101]. Extending this method could help to propose optimal therapeutic options, for example novel PTM-based drugs, including SUMO inhibitors.

In addition to the emerging development of SUMOylation inhibitory drugs, mainly targeting SUMO E1, inhibitors of similar PTMs like ubiquitin and Nedd8 are also being developed for clinical purposes and being tested in clinical trials. The most well-known cancer therapeutics based on the ubiquitin system are proteasome inhibitors. These inhibitors were initially developed to prevent cancer-induced cachexia. Pre-clinical studies showed that in cultured cancer cells and murine cancer models, proteasome inhibitors induce apoptosis. In clinical trials, proteasome inhibitors emerged to enable the treatment of myelomas and lymphomas, establishing new standards of care [102]. More recent developments are focused on direct inhibition of ubiquitin conjugation and de-conjugation. Inhibition of the ubiquitin activating enzyme (UAE) is one example and Pyr41 was the first UAE inhibitor established [103]. TAK-243 is a potent small-molecule inhibitor of UAE, which shows anti-tumoral activity via amongst others ER stress induced apoptosis [104–106]. Until now two phase I clinical trials have been listed for TAK-243. One study focusing on the dosage and side effects of TAK-243 for patients with acute myeloid leukemia (AML) or chronic myelomonocytic leukemia unresponsive to conventional treatment has not yet started recruiting patients (ClinicalTrials.gov Identifier: NCT03816319) [IV]. The second study listed for TAK-243 focusing on dosage and side effects in patients with advanced solid tumors listed several adverse effects and has been terminated in 2019 (ClinicalTrials.gov Identifier: NCT02045095) [V]. Future research has to demonstrate if TAK-243 or other new drugs targeting the ubiquitin system are suitable for treatment of cancers.

Epigenetic drugs focused on PTM modulation have large potential for the treatment of malignant diseases. For example, MLN4924 is a selective small-molecule inhibitor of the Nedd8 Activating Enzyme (NAE). The Neddylation pathway regulates to a large extent the turnover of a subset of proteins upstream of the proteasome via controlling the activity of the Cullin-RING ubiquitin ligases [107]. These ligases are known to play important roles in cellular processes associated with cancer growth and survival [108]. MLN4924 disrupts Cullin-RING ligase related protein turnover, resulting in apoptosis in human cancer cells, via dysregulating S-phase DNA synthesis *in vitro,* leading to endoreplication. The use of MLN4924 in a xenograft model decreased the growth of the engrafted tumor [109–111]. This research suggests that Neddylation is a promising

therapeutic target. Phase I clinical trial data demonstrate that MLN4924 is generally well tolerated and preliminary evidence suggests modest activity in refractory lymphoma [112–114]. Currently, MLN4924 is in Phase III clinical trials and is thus the most clinically advanced E1 drug. Both studies investigate the combination of MLN4924 with azacytidine. Enrolled patients will be randomly assigned to single treatment with azacytidine or to the combination therapy group. These studies investigate improved event free survival in AML, Myelodysplastic syndromes (MDS) or chronic myelomonocytic leukemia (CMML) upon combination therapy (NCT03268954)[VI] and the efficacy of MLN4924 with azacytidine in patients with AML not eligible for standard chemotherapy (NCT04090736)[VII]. For both studies no results have been published.

For precision therapeutic purposes, it is important to understand the mechanisms driving malignant diseases to identify the most promising therapy for individual patients. Utilizing the potential of PTM drugs by identifying novel combination therapies will strengthen the outcome for patients. Commemorating the developments made in the past years, large advances have been made in the development of SUMO and other PTM inhibitory drugs. Developing and investigating SUMO conjugation inhibitory drugs in the coming years has the potential to lead to new therapeutic strategies.

Acknowledgements

The laboratory of A.C.O.V. is supported by the Netherlands Organization for Scientific Research (NWO) and the Dutch Cancer Society.

Resources

[I] https://clinicaltrials.gov/ct2/show/NCT03648372
[II] https://clinicaltrials.gov/ct2/show/NCT04065555
[III] https://clinicaltrials.gov/ct2/show/NCT04074330
[IV] https://clinicaltrials.gov/ct2/show/NCT03816319
[V] https://clinicaltrials.gov/ct2/show/NCT02045095
[VI] https://clinicaltrials.gov/ct2/show/NCT03268954
[VII] https://clinicaltrials.gov/ct2/show/NCT04090736
[VIII] https://clinicaltrials.gov/ct2/show/NCT04776018
[VIIII] https://clinicaltrials.gov/ct2/show/NCT04381650

Declaration of Interest

The authors have received ML-792 and TAK-981 from Millennium Pharmaceuticals, Inc. -Takeda for preclinical research purposes.

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