SLAK-1, a proposed antibody-conjugated illudin analogue that selectively targets breast cancer

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In 1908, Paul Ehrlich promoted a concept termed the "magic bullet": if a compound could be made selective for a pathogen, then a toxin against the pathogen can be combined with this compound to selectively deliver it to its target. Specificity is a crucial consideration in drug design, as adverse side effects are caused by promiscuous off-target interactions. Anticancer drugs are particularly notorious for side effects since they are typically extremely cytotoxic and have poor inherent specificity. Here, the authors describe a strategy to specifically target breast cancer cells by using a Her-2 antibody to deliver a potent cytotoxic illudin analogue optimized via computational modeling. The antibody's specificity to breast cancer cells arises from the differential expression of a membrane receptor, Her-2, which is overexpressed in cancer cells. When the antibody binds to Her-2, a chemically conjugated illudin analogue can be internalized within the cell. This proposal is very current within the field of cancer immunotherapy; cytotoxins conjugated to antibodies, or "magic bullets", are just coming to market in the last few years.

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Our improved understanding behind the molecular mechanisms of tumorigenesis has facilitated the development of novel treatments for cancer. These are often small molecule natural products which have potent cytotoxic activity that inhibit the growth of cancerous cells. However, these chemotherapeutic drugs often have multiple biochemical targets that make it difficult to selectively target cancer cells. Antibody-Drug Conjugates (ADC) are cytotoxic agents that are chemically attached to monoclonal antibodies that target cancer-specific antigens. ADCs using the humanized antibody trastuzumab can facilitate the internalization of the drug by first binding to the human epidermal growth factor receptor 2 (HER2) which are over expressed on the surface of breast cancer cells in aggressive tumour types. With a lower risk of off-target side-effects, we present the use of a more potent cytotoxic agent with dual anticancer modes of action known as the illudin--a novel sesquiterpene that is derived from *Omphalotus* mushrooms with high therapeutic value. We first propose chemical modifications of illudin analogues to maximize both affinity to glutathione reductase and DNA damaging ability. By analyzing the chemical mechanisms of cytotoxicity and carrying out protein-docking simulations, an optimized illudin analogue can be generated. We then propose the construction of SLAK-1, an ADC that consists of a HER2 targeting antibody (Trastuzumab) and an illudin analogue that are attached to each other via a non-reducible thioether linker (SMCC).

L'amélioration de notre compréhension vis-à-vis les mécanismes moléculaires tumorigenèse à faciliter le développement de nouveaux traitements pour le cancer. Ce sont habituellement de petits produits naturels qui détiennent une activité cytotoxique puissante inhibant la croissance de cellules cancéreuses. Toutefois, ces médicaments chimiothérapeutiques ont souvent de multiples cibles biochimiques ce qui rend difficile de cibler sélectivement les cellules cancéreuses. Les conjugués anticorps-médicament (ADC = antibody-drug conjugate) sont des agents cytotoxiques qui sont liés chimiquement

à des anticorps monoclonaux qui ciblent à leur tour des antigènes spécifiques au cancer. L'ADC utilisant l'anticorps humain Trastuzumab peut faciliter l'internalisation du médicament en se liant tout d'abord au récepteur du facteur de croissance épidermique humain 2 (HER2) qui sont surexprimés à la surface des cellules du cancer du sein dans des tumeurs de types agressifs. Avec un risque plus faible d'effets secondaires hors cible, on présente l'utilisation d'un agent cytotoxique plus puissant avec une double action anticancéreuse connu sous le nom d'illudine – un nouveau sesquiterpène dérivé du champignon *Omphalotus* qui détient une haute valeur thérapeutique. Nous proposons d'abord des modifications chimiques à aux analogues d'illudine afin de maximiser à la fois affinité à la réductase glutathion et à sa capacité à endommager l'ADN. En analysant les mécanismes chimiques de la cytotoxicité et en menant des simulations d'amarrage de protéines (« docking »), un analogue d'illudine optimisé peut être généré. Nous proposons ensuite la construction de SLAK-1, un ADC qui consiste d'un anticorps ciblant HER2 (Trastuzumab) et un analogue d'illudine qui sont liés entre-elles par un lien thioéther non réductible (SMCC).

Introduction

Breast cancer is the most prominent form of cancer in women worldwide and contributes to approximately 16% of cancer affecting females. An increase in the prevalence of the disease is evident in developing countries due to an increase in urbanization, life expectancy, and Western lifestyle [1]. The United Kingdom, Canada and United States of America have the highest incidence of breast cancer worldwide followed by the rest of North America, Australia and New Zealand [2]. In the current year, it is estimated that 232,340 females and 2,240 males will be diagnosed with breast cancer in America. Of those diagnosed, 39,620 females and 410 males will not survive [3]. There are several risk factors for developing breast cancer, including familial history, prolonged exposure to endogenous estrogens, alcohol use, obesity, and lack of physical activity.

Currently, there are various strategies to treat breast cancer. When selecting the optimal treatment for a patient, physicians must take into account the stage of the cancer, sensitivity to certain hormones, and whether there is an increased expression of certain surface antigens on breast cancer cells [4]. The standard treatments for patients diagnosed with metastatic breast cancer include chemotherapy, radiation, surgery, and targeted therapy using monoclonal antibodies [5-7]. Approximately 25% of breast cancer cells exhibit between two- and twenty-fold overexpression of HER2, a growth factor receptor whose activation leads to downstream signaling regulating cellular growth. Normal breast cells have approximately 20,000 HER2 receptors, whereas breast cancer cells in aggressive tumors can have over 2 million. The increase in the levels of HER2 receptors on cancer cells results in increased activation leading to excessive cellular division and tumor formation [8]. Common chemotherapeutic drugs such as vinblastine, paclitaxel, anthracyclines, and doxorubicin are not always effective at treating metastatic breast cancer due to multidrug resistance [9,10]. Conventional treatments administered to breast cancer patients have detrimental side-effects that occur due to poor target specificity [11]. One way to overcome this is by conjugating the drug to an antibody that is selective for a surface antigen that can differentiate cancer cells from normal healthy cells. These antibody-drug conjugates (ADCs) will undergo a process of internalization (i.e. receptor-mediated endocytosis) upon binding to the surface antigen (Figure 1a). A wide-range of anticancer compounds can be attached to the antibody of which natural products, metabolites derived from plants, fungi and microbes are among the most promising [12].

Natural products are routinely used in drug development because they have highly potent bioactive properties as antibiotics (vancomycin), antifungals (cyclohexamide), and anticancer drugs (paclitaxel) [13]. For centuries, natural products have been used to treat ailments such as cancers and microbial infections [14]. In the last 50 years, 60% of cancer drugs have been derived from natural products or their analogues [15]. Illudins are a relatively new class of sesquiterpenes – orange-yellow mushrooms found in the woodlands of North America [16]. Illudins possess the ability to inhibit the growth of various types of cancer cells at nanomolar and even picomolar concentrations and are effective in treating multi-drug resistant tumours [17, 18, 19]. Illudins primarily act as an alkylating agent; nucleophilic Illudins also inhibit the function of enzymes such as glutathione reductase (GR) and thioredoxin reductase (TrxR), which protect the cell against oxidative stress [21]. On their own, illudins lack specificity and do not

target cancer cells. However, when conjugated to a specific antibody, illudins may prove to be an excellent anticancer drug with the capability to target and eliminate many types of cancers including breast cancer.

We propose to develop and test a modified illudin molecule through docking simulations and cytotoxicity assays. Subsequently, we can link this illudin analogue to a HER2 targeting monoclonal antibody (Trastuzumab) via a non-reducible thioether linker to produce SLAK-1, an antibody-drug conjugate that has the potential to selectively target and eliminate breast cancer cells.

Main Proposal

R-group modification of illudin S (core) to produce illudin analogue

Illudin S, a natural illudin analogue that possess high cytotoxicity and low target specificity between malignant cells and normal cells, has a narrow therapeutic index (a ratio between the therapeutic effect of the drug versus the cytotoxicity of the drug itself) [22]. Acylfulvenes (AFs) and Hydroxymethylacylfulvene (HMAF), established derivatives of illudin, have improved therapeutic indices. Both illudin S and AFs can react with and damage DNA, leading to cell death. AFs have the additional ability to inhibit GR, an enzyme that is up-regulated in cancer cells to improve the regeneration of glutathione [22-23].

Using computer software, we modified side groups surrounding the core structure of illudin to design an analogue with increased affinity for GR and undiminished DNA cleavage activity. We added a COOCH2CH2SH group on carbon 7 (as labelled in figure 1b) of the main ring so that the maleimide group of SMCC, a crosslinking reagent, can be attached to the illudin via a sulfhydryl group. A strong nucleophilic side group of hydroxyurea acetate was added to carbon 6 (as labelled in figure 1b) of the main ring to increase the toxicity of the molecule. Both of these modifications should increase binding affinity for glutathione reductase (GR). The hydroxyl group was replaced with methyl hydroxyl urea to offer more sites for nucleophilic attack by thiol-containing proteins such as GR while preserving the pharmacophore of the illudin [24]. The mechanism of GR is shown in Figure 2. The addition of a methyl group was conducted to prevent any steric hindrance by the hydroxyurea. The linker region and the addition of an ethyl chain between the SH group and the ester group were designed

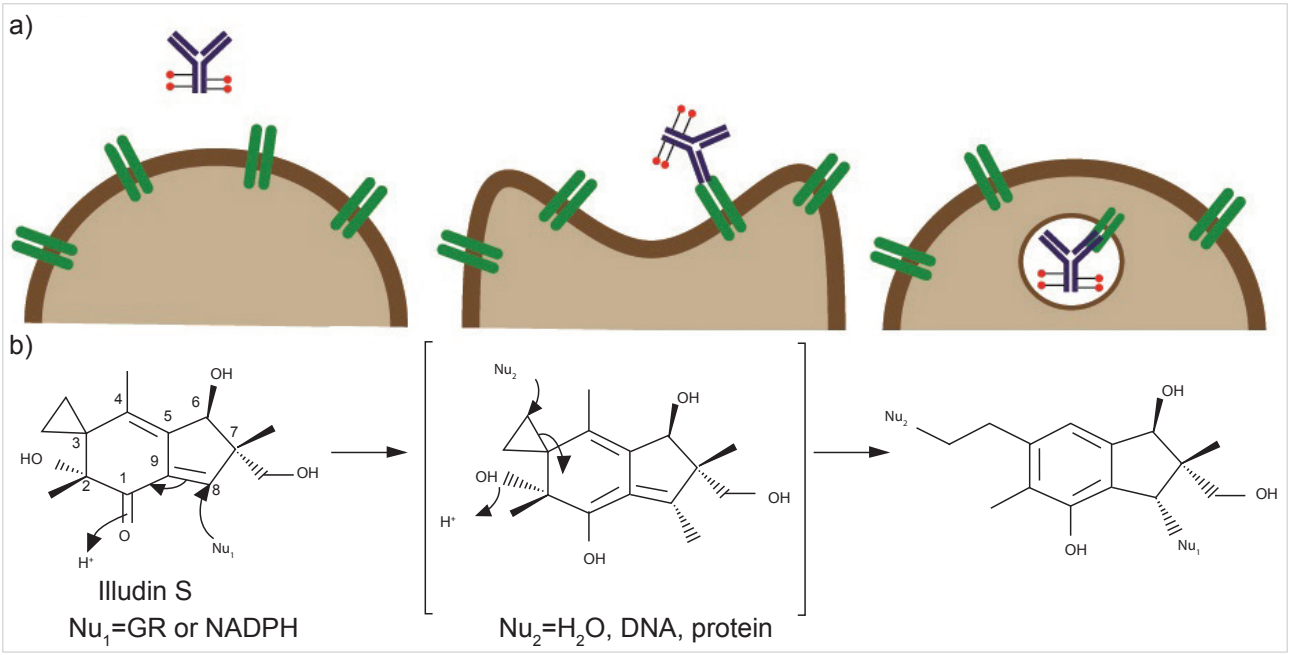

Figure 1. ***Mechanisms of ADC targeting and cytotoxicity by Illudin S.*** (a) ADCs are selective towards cells that express the particular antigen that the monoclonal antibody has a strong affinity towards. Once the target-specific antibody binds to the antigen, it undergoes a process of internalization. (b) Proposed mechanism of action of illudin S where there is first and enzymatic reduction by Nu1 (GR or NADPH) which subsequently reacts with Nu2 (H2O, DNA, protein). The highly unstable cyclopropyl ring increases the reactivity and cytotoxicity of illudin S, making the unstable intermediate a potent alkylating agent.

to prevent steric hindrance interfering with the molecule's pharmacophore, while adding an additional nucleophilic attack site [25]. Additional ester groups were attached to increase the affinity of SLAK-1 for attack by the thiol group of GR to form a stable hydroxy-acyl group. To assess whether the modification increased binding affinity, we used in silico dockings to compare the affinities of our illudin analogue, illudin S, and irofulven for GR. As expected, illudin S had the weakest binding affinity (highest ΔG), whereas our analogue had the strongest (lowest ΔG) (Figure 3).

Conjugation of illudin analogue to trastuzumab to construct SLAK-1

To ensure that our illudin analogue selectively targets breast cancer cells, we propose its conjugation to trastuzumab to create an ADC named SLAK-1. Trastuzumab, also known as Herceptin, is a monoclonal antibody that specifically binds to the HER2 receptors embedded in the membranes of breast cancer cells [28]. The illudin should be attached to trastuzumab using a linker that cannot be cleaved until the ADC is fully internalized. We propose using the non-cleavable and membrane permeable crosslinker succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). SMCC contains a reactive N-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group. To link the antibody trastuzumab to our illudin analogue, SMCC should first be added to the antibody via the NHS ester group. Once the excess crosslinker reagent is removed, the illudin analogue should be added [29]. This two-step reaction will result in the formation of an ADC called SLAK-1 (Figure 4).

Testing the cytotoxicity and specificity of SLAK-1

In order to test SLAK-1's anti-cancer activity, we propose two assays: one for measuring the inhibition of GR, and the other for measuring DNA damage. GR is involved in the redox cycle that protects the cell against oxidative stress. The enzyme reduces glutathione disulfide (GSSG) to glutathione (GSH) by oxidizing NADPH to NADP+ (See reaction below).

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

We can indirectly measure GR inhibition by measuring the rate of NADPH oxidation by a decrease in absorbance at 340 nm that corresponds to NADPH concentration [30]. Thus, one can measure the ability of SLAK-1 and other possible analogues to inhibit GR *in vitro* by measuring NADPH concentration in the presence of various drug concentrations. The comet assay, also known as single cell gel electrophoresis, detects DNA damage in single cells [31]. We propose testing our SLAK-1 analogue against two breast cancer cell lines: KB and SK-Br-3 cells, which will subsequently be lysed and run on an electrophoresis gel. Once visualized, a smear mark resembling a comet will be reflective of DNA damage, when comparing the intensity ratios of the comet's "head" to its "tail".

If SLAK-1 inhibits the activity of GR and damages DNA in vitro, its efficacy should be tested against whole breast cancer cells in vitro. By comparing the growth inhibition of our breast cancer cell lines with that of normal fibroblast cells, we will assess the specificity of SLAK-1. We can also compare the analogue with and without the Trastuzumab antibody attached to the compound within a heterogeneous mixture of diseased and non-diseased cell types to test the efficacy of modified illudin analogue in vitro. Studying the effect of a human breast cancer xenograft in the murine (mouse) model can provide valuable insight into the growth and metastasis of the tumour in vivo. It can also identify host responses and potential side-effects that may occur from the illudin analogue that can be administered as a Trastuzumab-attached compound. Severe combined immunodeficient (scid) [32] mice would be injected with breast cancer cell lines (MCF7, ZR-75-1, and T47d). SLAK-1 can then be administered in a dose-dependent manner within the murine model via injection.

Conclusion

Given the proven clinical efficiency of ADCs against metastatic breast cancer in chemotherapy, the illudin-based drug proposed here might also have the desired effect against breast cancer tumours [33, 34]. We have taken several important parameters into account in developing SLAK-1. First, to ensure potent cytotoxicity against breast cancer cells, we have designed an illudin analogue that is able to efficiently inhibit GR and damage DNA. Assays for GR inhibition by illudin S and acylfulvene analogues have been published in the past [22]; however in silico modeling has not been published by any group. We used docking simulations to compare the binding affinities of several illudin analogues. As expected, illudin S and irofulven had weaker binding affinities (higher ΔG values) than our proposed illudin analogue. Altogether, this suggests that our drug will inhibit GR more efficiently and may prove to be more potent at inhibitingthe growth of breast cancer cells once internalized.

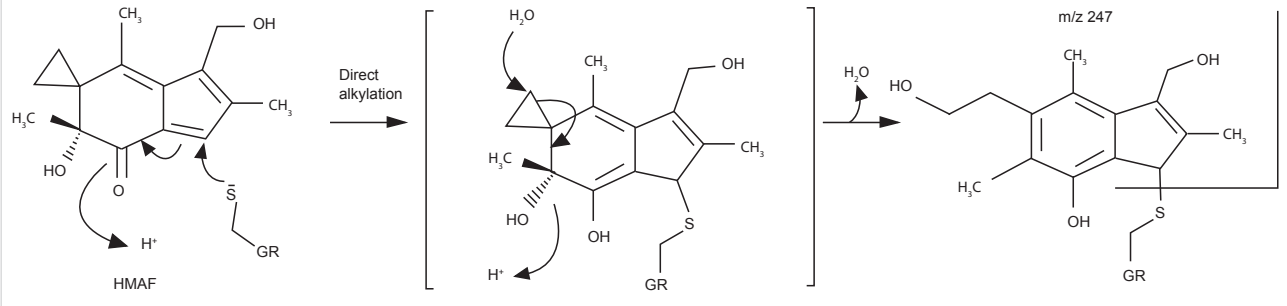

Figure 2. ***Glutathione Reductase Inhibition by HMAF (modified acylfulvene derived from Illudin S).*** Above is the proposed mechanism by Liu and Sturla (2009) showing the inhibition of Glutathione Reductase by HMAF [22]. The sulfur from thiol containing proteins such as GR acts as a nucleophile and is added to the α,β unsaturated ketone; H2O is added to the cyclopropyl group thus inhibiting GR.

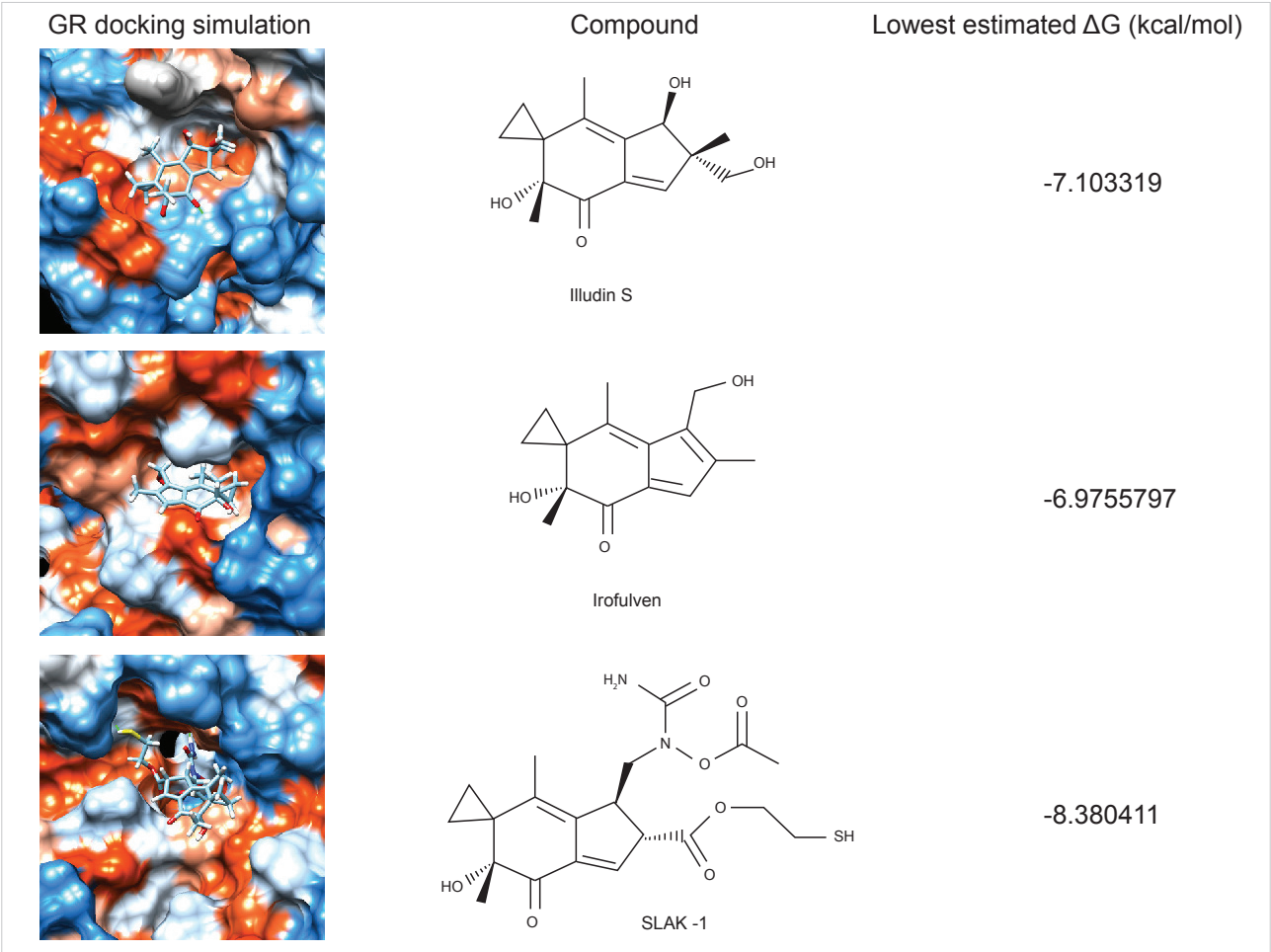

Figure 3. ***In silico docking of Illudin S, Irofulven and SLAK-1 within GR.*** Docking simulations were carried out using SwissDock [26] to assess the ligand binding affinity of the various analogues to glutathione reductase (PDB: 3GRS). Hydrophobicity surfaces (Blue: hydrophilic, white: neutral, red: hydrophobic) were mapped according to the Kyte-Doolittle scale in complex with their respective compounds using UCSF Chimera [27]. Three-dimensional models were generated and respective affinities were estimated based on the lowest Gibbs Free Energy values within the corresponding binding pocket of GR.

Second, we have proposed the conjugation of our illudin analogue to the monoclonal antibody trastuzumab to ensure specificity towards breast cancer cells. Previous studies by Lewis Phillips et al. (2008) have validated the use of maytansenoid, a cytotoxic macrolide that inhibits the formation of microtubules, when conjugated to trastuzumab [35]. The specificity of this ADC for HER2-positive breast carcinomas increases our confidence in the specificity of SLAK-1. If SLAK-1 is synthesized and successfully passes our proposed cytotoxicity and specificity tests, we will have overcome many difficulties associated with current treatments for metastatic breast cancer.

Keywords
Antibody-Drug Conjugate; Illudins; SLAK-1; Breast Cancer; HER 2 receptors; Trastuzumab; conjugué anticorps-médicament; illudines; cancer du sein; récepteurs HER-2.

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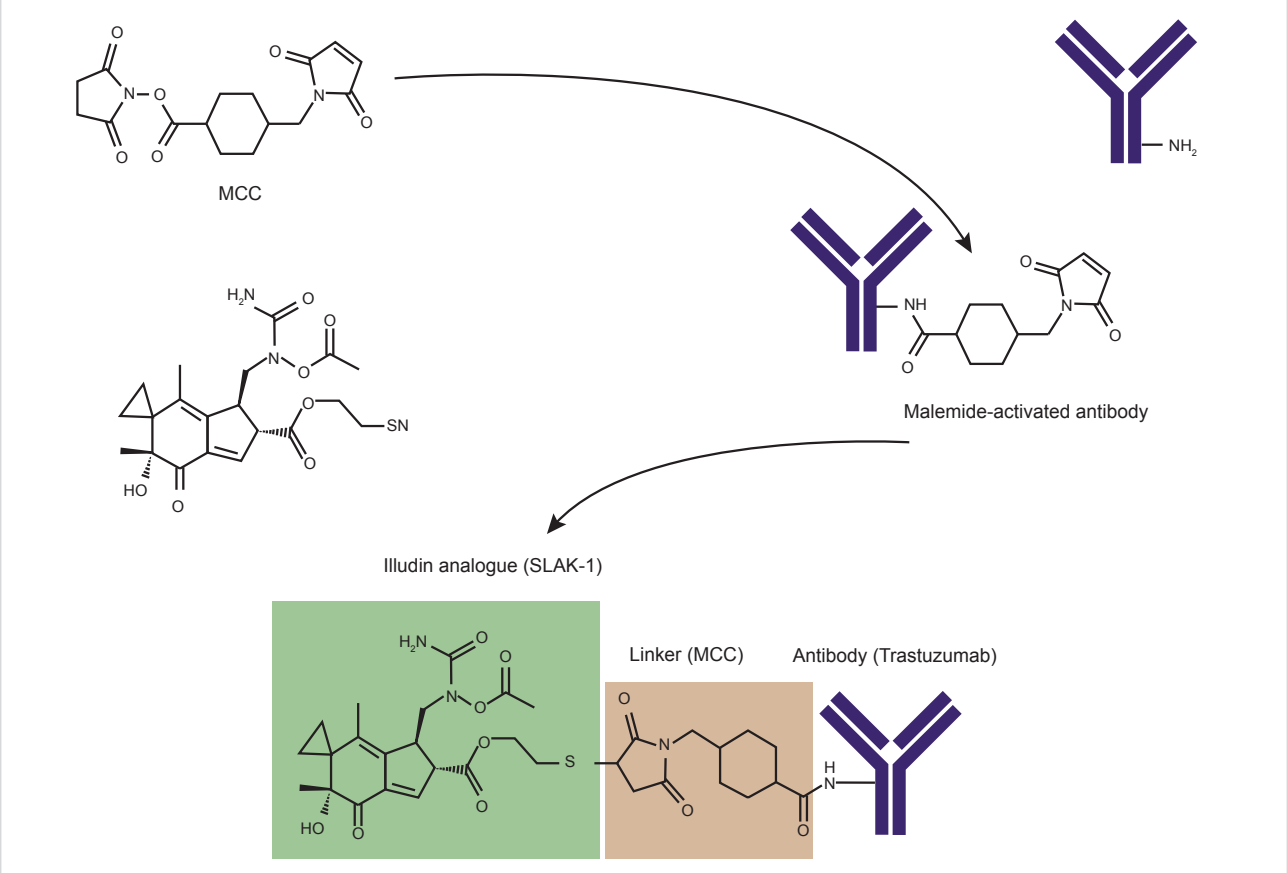

Figure 4. ***Construction of Antibody-Drug Conjugate SLAK-1 to target Her2 positive breast cancer.*** A SMCC non-cleavable linker is first added onto an antibody via NHS ester group. The excess SMCC reagent is then removed. The drug containing a sulfhydryl group is then added. This two-step reaction will result in the formation of an ADC called SLAK-1.

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Review of *Characterizing RNF20 and RNF40 in the Class Switching of B Cells*

This study describes a research plan to conjugate a synthetic proposed analog of the natural product illudin, called SLAK-1, to the monoclonal antibody trastuzumab. The proposal is based largely on the recent success of antibody-drug conjugates (ADCs) that have shown promising clinical results that have led to recent approvals by the FDA. Because illudin functions as a cytotoxic agent through a unique mechanism of reduction by glutathione reductase, which is present in greater quantities in tumour cells versus normal cells, it is hoped that this approach would lead to a more selective ADC with a higher therapeutic index than those based on tubulin targeting agents such as Maytansine.

While there are several interesting concepts described, the manuscript appears to be more of a proposal than a report of actual results. The basis of the actual work done only appears to be a simple molecular modeling study with some proposed analogs showing how they dock into the binding site of glutathione reductase. In order to obtain impactful laboratory experimental results, a major effort would then be required to synthesize these analogs, test them and conjugate them.

The author of this paper should be commended with proposing an extremely ambitious project and the basic idea behind the proposal has merit. In addition, the background and nature of the project are actually quite interesting and could form the basis of a viable proposal. However, some key flaws in the research plan itself make the research proposal, as it is layed out in this manuscript, unlikely to succeed. There are many huge challenges that are glossed over in this proposal-the synthesis of the illudin analog, its characterization, and the challenges of conjugating it to trastuzumab that multiply the difficulty of succeeding in an actual research setting. By removing as many variables as possible (for example sticking with a known drug to use in the conjugation) a complex research plan is more likely to succeed.

The abstract for this paper is interesting and attracts the attention of the reader towards the proposed subject matter. However, it is somewhat misleading in terms of what has been actually accomplished versus what is hypothetical. In particular, by stating “a more potent analog of illudin” will be used as the conjugate it is implied that this analog has already been synthesized and characterized, when in fact it is only proposed based on crude modeling studies. Not only is it not known that this is more potent, the proposed synthetic route to this analog is not disclosed. What is known versus what is hypothetical should be more clearly stated.

The introduction section is generally well written. A great deal of background information is provided to educate the reader about breast cancer, natural products and anti-body drug conjugates. While each of these topics is enormous, the writers do a good job of providing an interesting and concise overview of each. One deficiency of the introduction is an inadequate coverage of the recent advances that have been made in ADC development, both from the standpoint of comparative approaches in design, clinical results, and success in terms of FDA approvals. Only a single reference from 2010 provided. Suffice it to say, a huge number of advances have been made in the past two years that are not described here.

Even if the proposed analog SLAK-1 actually binds as well to GR as it is suggested in the main proposal, it is not clear what iterative process was used to derive this analog. Rather than having just one analog to form the basis of the proposal (SLAK-1) would be of value to see how other designed analogs with various substituents compare so as to assess their potential. In particular, while the rationale for the point of attachment of the thiol linker is good, the functionally dense nitrogen substituent appears to be unstable and synthetically challenging to install. Moreover, the thought process for which this specific analog was designed is poorly conveyed. Figure 3 is not clear in terms of the binding mode of the compounds and why SLAK-1 docks better than the other two compounds . A clearer picture highlighting specific contacts or pockets would be helpful in interpreting the proposed advantages if SLAK-1.

Aside from the above considerations, there are two key flaws to the proposed research. The first is that SLAK-1 is likely to be an unstable, highly enolizable molecule with an acidic proton in the 5-membered ring

(position 8). Because no specific synthetic plans are given for how this compound will be produced, these concerns become greater. The second and perhaps more important issue is that there are no plans described for testing or evaluating this compound on its own, prior to conjugation, to evaluate its potential merits as a conjugation partner. Instead, the proposal appears to entail making this compound (through undisclosed methods), conjugating it and then directly testing the ADC in vivo in tumour bearing mice. This is a highly risky and extremely expensive approach. Prior to carrying out any in vivo experiments, it is essential to evaluate the cytotoxity of the small molecule and compare it to a variety of other analogs to get a clearer understanding of how SLAK-1 is likely to perform. SLAK-1 should be tested preferably first in an in vitro setting such as in cytotoxity assays in tissue culture first, at a minimum. In the section called "Validating the cytotoxicity and specificity of SLAK-1", it is confusing if the authors are describing the SLAK-1 small molecule itself or the ADC in which it is incorporated. If the latter, a new term such as SLAK-1 ADC should be used.

The most favourable part of the chemical aspects of the proposal is the actual plan for conjugating the natural product to trastuzumab. A concise synthetic scheme is provided that appears reasonably viable. However, the precedent for this scheme should be made more transparent in terms of what has been done with other ADCs.

In the conclusion section, some further aspects to ADC design are provided for consideration. A good discussion of the rational for targeting HER-2 in breast cancer is given, and using trastuzumab as the antibody makes reasonable sense based on historical precedent. An opportunity to clarify some key considerations should be taken here, however. For example, it is stated that optimally 2-4 drug molecules /antibody should be used. However, it isn't clear how this will be achieved or if this number holds for all drug molecules. Perhaps more important, criteria for how toxic the small molecule drug needs to be to merit consideration as a viable conjugation partner should be clarified. Not having these criteria defined at the start of the research makes achieving a desired outcome much more difficult.

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