GT198 protein is a target of anticancer chemotherapeutic drugs and anticancer herbal medicines.

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Abstract

Tumor angiogenesis is a hallmark of cancer. Some anti-angiogenic anticancer drugs have demonstrated clinical efficacy. Our previous studies have shown that GT198 protein (gene symbol PSMC3IP, also known as Hop2) is an oncogenic protein that induces angiogenesis in human solid tumors, including oral cancer.

The results of this study demonstrate that more than a dozen clinical anticancer chemotherapeutic drugs and several clinically successful anticancer herbal medicines can directly inhibit the GT198 protein target. GT198 is a DNA-binding DNA repair protein. Using an in vitro DNA binding assay to detect GT198 activity, we tested 129 clinical anticancer chemotherapeutic drugs collected by the US NCI. Chemical drugs found to directly inhibit GT198 in vitro include, but are not limited to, mitoxantrone, doxorubicin (adriamycin), paclitaxel, etoposide, actinomycin D, and imatinib (Gleevec). Paclitaxel and etoposide exhibit high binding affinity, whereas doxorubicin demonstrates high binding efficacy due to competitive inhibition of GT198.

Since GT198 shares protein sequence homology with DNA topoisomerases, which were previously considered drug targets, GT198 protein is likely a previously unrecognized novel drug target. To seek more potent GT198 inhibitors, we further tested extracts from several anticancer herbal medicines. The herbs that showed positive inhibitory effects with high affinity and high potency are all anticancer herbs with a history of clinical success, including Jamaican Allspice, Chinese Gleditsia sinensis L., and Ecuadorian BIRM.

Using organic chemistry methods, we partially purified Allspice, demonstrating that employing GT198 as a target and monitoring activity with an in vitro DNA binding assay enables efficient and rapid purification of natural products. In summary, this study reveals that GT198 is a novel target for multiple anticancer chemotherapeutic drugs. This study also provides an excellent drug target for compound identification and natural product purification. Most critically, this

study offers an excellent opportunity for rapid isolation of highly effective and low-toxicity anticancer Chinese herbal medicines.

Full Text

Preamble

GT198 Protein Is a Target of Anticancer Chemical Drugs and Anticancer Herbs

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Note: Due to the inclusion of Chinese herbal medicine information, this article represents a Chinese translation of a previously published English manuscript. The English original should be considered authoritative. Figures and supplementary materials remain untranslated. When citing this work, please reference the CC-BY licensed English original: Pang J, Gao J, Zhang L, Mivechi NF and Ko L (2021) GT198 Is a Target of Oncology Drugs and Anticancer Herbs. *Frontiers in Oral Health* 2:679460. DOI: https://doi.org/10.3389/froh.2021.679460

Abstract

Tumor angiogenesis is a hallmark of cancer. Several anti-angiogenic anticancer drugs have demonstrated clinical efficacy. Our previous studies revealed that GT198 protein (gene symbol PSMC3IP, also known as Hop2) is an angiogenesis-inducing oncoprotein in human solid tumors, including oral cancer. The present study demonstrates that dozens of clinically used anticancer chemotherapy drugs and several clinically successful anticancer herbs directly inhibit the GT198 protein target. GT198 is a DNA-binding DNA repair protein. Using an in vitro DNA binding assay to measure GT198 activity, we tested 129 clinical anticancer chemotherapy drugs from the U.S. NCI collection. We found that chemical drugs that directly inhibit GT198 include, but are not limited to, mitoxantrone, doxorubicin (adriamycin), paclitaxel, etoposide, actinomycin D, and imatinib (Gleevec). Paclitaxel and etoposide exhibit high binding affinity, whereas doxorubicin demonstrates high binding efficacy due to competitive inhibition of GT198. Because GT198 shares protein sequence homology with DNA topoisomerases, which were previously considered drug targets, GT198 protein likely represents a previously unrecognized novel drug target. To search for more potent GT198 inhibitors, we further tested extracts from several anticancer herbs. The positive inhibitory herbs with high affinity and high efficacy all have successful clinical histories in cancer treatment,

including Jamaican allspice (Pimenta dioica), Chinese Gleditsia sinensis L., and Ecuadorian BIRM. Using organic chemistry methods, we performed partial purification of allspice, demonstrating that using GT198 as a target with the in vitro DNA binding assay for activity tracking enables efficient and rapid purification of natural products. In summary, this study reveals GT198 as a new target for multiple anticancer chemical drugs. This study also provides an excellent drug target for compound identification and natural product purification. Critically, this study offers an exceptional opportunity for rapid isolation of highly effective, low-toxicity anticancer Chinese herbal medicines.

Keywords: tumor angiogenesis, anticancer target, anticancer Chinese herbs, GT198 oncoprotein, oral cancer

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1. Introduction

Angiogenesis is a characteristic feature of multiple human solid tumors [1,2]. Targeted drugs against tumor angiogenesis have proven highly effective [3-5]. Accumulating evidence indicates that blood vessels in tumor tissue are not normal vessels; rather, they represent the true origin of tumor initiation and progression [6-9]. In tumor tissue, neovessels undergoing angiogenesis are themselves malignant [10].

The key controller of angiogenesis is the pericyte [11]. Pericytes reside on capillary microvessel walls and initiate angiogenesis upon stimulation [12]. Pericytes are stem or progenitor cells capable of differentiating into various other cell lineages [13]. Normal pericytes differentiate into new tissues during embryonic development or adult tissue repair [11-13]. However, this process is hijacked in tumors, where angiogenesis-inducing pericytes are malignant [14] and their differentiation is blocked. In tumor vascular hyperplasia, pericytes detach from microvessel walls, migrate into tissues, and overgrow into undifferentiated tumor cells [10]. Migratory pericytes in tumors have been previously reported [15]. Tumor cells derived from pericytes and surrounding blood vessels have also been described as vasculogenic mimicry [6,16,17]. Multiple lines of evidence suggest that continuous tumor cell growth results from constant replenishment by cancer stem cells [13], which include pericytes [11,18].

To inhibit tumor angiogenesis, many anti-vascular agents have been developed [19,20]. Most are kinase inhibitors or VEGF pathway inhibitors [21,22] that suppress vessel growth. Because tumor vasculogenic mimicry often lacks vascular endothelial cells, these drugs may develop resistance [16]. Therefore, targeting pericytes could more specifically inhibit tumor angiogenesis to overcome

drug resistance. We and others previously identified a DNA repair oncoprotein named GT198 (gene symbol PSMC3IP, alias Hop2) [10,23-25]. GT198 protein is overexpressed in pericytes of tumor vessels but not in quiescent normal pericytes [10]. In this study, our results demonstrate that GT198 protein is an excellent drug target, with direct inhibitors including multiple approved oncology chemotherapy drugs and several anticancer herbs with proven efficacy in human tumors.

The GT198 gene was originally cloned as a transcriptional coactivator regulating gene expression [23,26]. Germline mutations in human GT198 gene exist in familial early-onset breast and ovarian cancers [27,28]. Germline mutations are also present in familial ovarian disease [29] and ovarian insufficiency [30-32]. Recurrent somatic mutations in GT198 gene are widespread in the tumor microenvironment of multiple solid tumors [27,33]. Importantly, GT198 mutations cause protein overexpression, detectable in angiogenic pericytes and their derived vascular smooth muscle cell lineages, such as myoepithelial cells and adipocytes in breast cancer [34], theca cells in ovarian cancer [35], and myofibroblasts in prostate and bladder cancers [36]. GT198-high pericytes were also found in human oral cancer and multiple solid tumors [10] and in mouse tumors [37]. Aberrant angiogenic pericytes in the microenvironment may represent a common origin of human solid tumor development.

GT198' s role in pericytes may relate to its expression and function in stem cells [10,33]. GT198 overall expression resembles cancer-testis antigens (CTA), with high levels in embryos, testes, and cancers, but low in normal adult tissues [10]. During normal stem cell differentiation, GT198 expression switches from antagonistic GT198 splice variants to its wild type [33]. In human tumors, somatic mutations cause overproduction of splice variants, potentially blocking normal stem cell differentiation [33]. Indeed, angiogenic pericytes in oral cancer produce undifferentiated cells [10].

GT198 protein is a small DNA-binding protein dimer, with monomers containing only 217 amino acids [23,33]. GT198 protein includes an N-terminal domain, a leucine zipper dimerization domain, a DNA-binding domain that can bind single- or double-stranded DNA [24,33], and a C-terminal self-inhibition domain [33]. Many biochemical studies published under the alias Hop2 demonstrate that mammalian GT198 is a critical DNA repair factor that stimulates homologous DNA recombination and regulates meiosis [24,38,39]. Additional evidence indicates GT198 is a highly important regulator of nuclear functions. Processes such as transcription activation, recombination in DNA repair, and homologous chromosome pairing in meiosis all require DNA strand opening and binding. Therefore, DNA binding is a key function of GT198. By detecting its DNA-binding activity, GT198 inhibitors can be screened.

In this report, we extend previous observations [10] to further demonstrate that pericytes in human oral cancer highly express GT198. To test whether GT198 could serve as a target for anti-vascular anticancer drugs, we screened 129 clinical oncology drugs from the U.S. NCI using an in vitro GT198-DNA binding

assay. Surprisingly, we identified a series of chemotherapy drugs, including doxorubicin, mitoxantrone, paclitaxel, etoposide, and Gleevec, as direct GT198 inhibitors. GT198 is likely a previously undiscovered drug target. To further seek stronger GT198 inhibitors, we tested several anticancer herbs with successful clinical histories. We found several positive herbs, including Jamaican allspice (Pimenta dioica), Chinese Gleditsia sinensis L., and Ecuadorian BIRM, that directly inhibit GT198 with high affinity and high potency. Using organic chemistry purification methods, we partially purified allspice, confirming that monitoring natural product purification by testing GT198 inhibition is highly feasible. In summary, our study reveals GT198 as a new target for multiple existing anticancer chemical drugs and anticancer herbs. GT198 target utilization may open a new direction for future chemical drug identification and natural botanical drug purification.

2.1 GT198-Positive Pericytes in Angiogenesis Initiate Human Oral Cancer

We previously identified GT198-high microvessels in early lesions of multiple human solid tumors [10,34-36]. For example, in human oral cancer, high GT198 protein expression is a clear marker of angiogenesis in the tumor microenvironment (Figure 1 [Figure 1: see original paper]A-B).

Through GT198 immunohistochemical staining of oral cancer pathological sections, we observed that the earliest lesions are GT198-positive pericytes, which differentiate into multiple positive descendant cell types. Initially, GT198-positive pericytes arise in small clusters of proliferating vessels. As a control, surrounding quiescent vessels contain GT198-negative pericytes (Figure 1B). Subsequently, GT198-positive pericytes increase their cytoplasmic volume, causing abnormal thickening of capillary walls (Figure 1B). Next, GT198-positive pericytes detach from vessel walls, becoming positive nodules in the tissue stroma. At this stage, GT198-positive fibroblasts derived from pericytes in the stroma also increase. Later, pericyte nodules proliferate to form tumor masses, typically surrounding a blood vessel at the tumor center (Figure 1B). These vessels within tumors remain functional, as red blood cells are present in the vascular lumen. This is consistent with other studies showing that tumors generate their own blood vessels [6,16]. Notably, the classic description that tumors develop from the epithelial basal layer remains observable. However, in such cases, the epithelium where tumors arise is always surrounded by GT198-positive blood vessels bearing the vascular smooth muscle marker αSMA (Figure 1C). The epithelial cells giving rise to tumors are also GT198-positive, while nearby normal epithelial cells are GT198-negative (Figure 1C). Because pericytes are stem cells that can replenish progenitors in the epithelial basal layer, both epithelium-derived tumors and vessel- and stroma nodule-derived tumors may originate from angiogenic pericytes. This becomes clear when using GT198 protein as a pericyte staining marker. The two distinct concepts of tumor origin from epithelium and vessels can now be reconciled: all tumors originate from pericytes. The pres-

ence of scattered GT198 and αSMA double-positive cells within tumors further supports the vascular origin of tumors (Figure 1D and Supplementary Figure 1). The results show that proliferating vessels in angiogenesis first develop into early tumor cells, followed by vessel disintegration after tumor maturation. These findings are consistent with our previous study [10], and together provide evidence that angiogenic pericytes are the tumor origin in human oral cancer.

2.2 Oncology Drugs Including Doxorubicin and Paclitaxel Are GT198 Inhibitors

GT198 expression is a specific marker of tumor angiogenesis. To test whether GT198 inhibitors have anticancer effects, we first validated clinically successful anticancer drugs. To measure direct GT198 inhibitory activity in vitro, we developed an in vitro DNA binding assay. Recombinant GT198 protein was adhered to 96-well plates, and its binding to biotin-labeled DNA was detected in the presence of drug inhibitors. Biotin-labeled DNA content was measured using streptavidin-horseradish peroxidase (HRP) conjugate and fluorescence (Figure 2 [Figure 2: see original paper]A-B). First, using bovine serum albumin (BSA) as a negative control, we determined the half-maximal effective concentration for DNA-GT198 binding to be EC50 = 43 nM (Figure 2C).

This result also showed that DNA concentration before saturation was approximately 150 nM (Figure 2C), which is an optimal DNA concentration for subsequent analysis. This concentration yields the strongest detection signal while allowing the most effective drug competitive inhibition.

We then tested the inhibitory effects of various anticancer chemotherapy drugs on GT198. Half-maximal inhibitory concentrations (IC50) were determined under gradient drug dilutions with 150 nM DNA. Surprisingly, many anticancer chemical drugs were found to be positive GT198 inhibitors. Doxorubicin had low affinity (IC50 = 341 nM) but relatively high inhibitory efficacy, whereas paclitaxel (IC50 = 5.0 nM) and etoposide (IC50 = 24.2 nM) had high affinity but poor inhibitory efficacy (Figure 2D-E). Camptothecin had extremely poor affinity (IC50 = 2.06 M), and carboplatin did not inhibit GT198 at all (Figure 2D). This is the first cross-comparison of their binding properties using their common target GT198 in vitro. This discovery may have clinical significance, as the clinical tolerability and low toxicity of paclitaxel or etoposide are due to their high affinity [40,41]; potent doxorubicin reflects its high binding efficacy, though it is more toxic [42]; and the clinical failure of camptothecin compared to highly successful paclitaxel may result from its considerably poor affinity [43,44].

The notion that GT198 is a genuine drug target becomes even more apparent when comparing similarities among drug classes. Among doxorubicin analogs, mitoxantrone (IC50 = 187.4 nM) and daunorubicin (IC50 = 149.9 nM) showed better affinity than idarubicin (IC50 = 362.4 nM), epirubicin (IC50 = 749.6 nM), and valrubicin (IC50 = 973.3 nM) (Figure 3 [Figure 3: see original paper]A).

Mitoxantrone had the highest efficacy, resulting in nearly complete inhibition. However, their binding affinities were all in the similar range of several hundred nanomolar. In contrast, camptothecin and its analogs irinotecan and topotecan had equally poor affinity or almost no GT198 inhibition (Figure 3B).

We further discovered that doxorubicin is a competitive inhibitor of GT198 (Figure 3C-D). As DNA concentration increased, doxorubicin' s inhibition of GT198 was competitively offset (Figure 3C). Double-reciprocal plot analysis showed competitive binding between DNA and doxorubicin (Figure 3D). In plots with 100 nM doxorubicin versus 0 nM control, constant Vmax with increased Km indicated that doxorubicin shares the same binding site on GT198 as DNA. Thus, doxorubicin directly blocks GT198' s DNA-binding site (model in Figure 2A). As a competitive inhibitor, doxorubicin produces high inhibitory efficacy (model in Figure 2E). Conversely, we found in another study that paclitaxel is an allosteric or non-competitive inhibitor of GT198 [45]. Allosteric inhibition may lead to incomplete inhibition, resulting in poor binding efficacy (Figure 2A and 2D). Our results provide this explanation: doxorubicin is a potent drug due to high binding efficacy but is also toxic; paclitaxel is a drug with good sensitivity due to high affinity but low efficacy. High affinity correlates positively with low toxicity because lower drug concentrations are sufficient for inhibition.

That many clinical anticancer drugs are GT198 inhibitors was initially surprising but later became less so. GT198 shares protein sequence homology with DNA topoisomerases I and II (Figure 3E and Supplementary Figure 2). DNA topoisomerases were previously known targets of doxorubicin, etoposide, and camptothecin analogs [46-48]. Like GT198, DNA topoisomerases are DNA-binding proteins involved in gene transcription and recombination. Therefore, protein sequence homology further validates GT198 as a previously unrecognized target of topoisomerase inhibitors.

2.3 Identification of GT198 Inhibitors from NCI Oncology Drugs

These findings prompted us to further test other anticancer drugs as GT198 inhibitors. Using the DNA binding assay described above, we screened 129 anticancer drugs from the National Cancer Institute (NCI) Clinical Oncology Drug Set VII (Table 1 and Supplementary Table 1). Forty drugs were selected for comparison in the figure (Figure 4 [Figure 4: see original paper]), with only some positive inhibitors analyzed for their IC50 values and binding efficacy (Table 1). Confirmed GT198 inhibitors include doxorubicin analogs; paclitaxel and docetaxel; etoposide and teniposide. Additionally, positive inhibitors include actinomycin D, carfilzomib, sirolimus (rapamycin), imatinib (Gleevec), sunitinib, trifluridine, and aminolevulinic acid (Figure 4 and Table 1). Celastrol, not from the NCI drug set, was also found positive (Table 1). Many drugs were negative, including platinum inhibitors, methotrexate, and vincristine. Among all drugs tested as GT198 inhibitors, mitoxantrone had the highest inhibitory efficacy, and paclitaxel had the best affinity (Table 1) [45].

Notably, many drugs historically have other mechanisms of action. For example, Gleevec is an inhibitor of Abl tyrosine kinase. However, in traditional drug development, compounds progress from hits to leads to candidates through multiple screening steps. Later steps typically require cell and animal testing, and GT198 protein is a significant cytotoxic target that induces apoptosis [33] and is highly expressed in many mouse tumor models [10,37]. Therefore, Gleevec may inhibit both tyrosine kinase and GT198 in vivo, with GT198 only discovered as a target in this study. Similarly, other GT198 inhibitors identified in this study may also have more than one in vivo target.

2.4 Anticancer Herbs That Inhibit GT198

To seek better GT198 inhibitors with both high affinity and high efficacy, we tested several herbs with successful histories in treating human cancers. The rationale for selecting each herb is discussed in detail below. The two most promising anticancer herbs are Jamaican allspice (Pimenta dioica) and Chinese Gleditsia sinensis L. (GSL). Ethanol extracts were tested in the DNA binding assay. Compared to the negative control licorice, both allspice (IC50 = 1.77 ng/ l, efficacy = 86%) and GSL (IC50 = 0.54 ng/ l, efficacy = 92%) showed excellent affinity and efficacy (Figure 5 [Figure 5: see original paper]A-B). Using silica gel column chromatography to analyze the polarity of eluted fractions, we found that the active components of the two plants have different polarities (Figure 5C). The GSL active component is more polar than the allspice active component, indicating two distinct GT198 inhibitors that can be purified in the future. Using GSL extract in TUNEL assays to test HeLa cell apoptosis, significant apoptotic activity was detected (Figure 5D). This result confirms the presence of cytotoxic components in GSL extract. Evidence from other researchers indicates that allspice also has apoptotic effects [49].

We also tested a set of anticancer medicinal materials from Taiwan consisting of branches from four tree species (Figure 6 [Figure 6: see original paper]A-B). The four mixed branches constitute a Chinese medicine formula with numerous testimonials of anticancer efficacy in Taiwan. These trees are mulberry branch (Morus australis), walnut branch (Juglans regia L.), Mahonia (Mahonia oiwakensis), and Dalbergia odorifera. When tested individually, we found that all four extracts inhibited GT198, although their activity was lower than the positive control allspice at the same concentration (Figure 6A). These four herbs are commonly used in Asia. While each herb has low activity individually, their combination may synergistically enhance efficacy.

Another tested herb is a commercial health product from Ecuador called BIRM, an acronym for Biological Immune Response Modulator. BIRM is a water extract from dried roots of the Ecuadorian plant dulcamara (Kalanchoe gastonisbonnieri Raym). BIRM extract showed high efficacy and high affinity in inhibiting GT198 (IC50 = 7.12 ng/ l) (Figure 6C-D). In addition to extensive evidence of human cancer treatment, previous studies showed that BIRM is effective against prostate cancer in cell and animal models by modulating androgen re-

ceptors [50,51]. This study is the first to identify GT198 as a direct target of BIRM, which is explainable because GT198 is also a transcriptional coactivator in the androgen receptor pathway [23].

Importantly, most identified anticancer herbs that inhibit GT198 are also known anti-infection herbs (Table 2). Extensive online evidence exists for the antiviral and antibacterial effects of allspice and BIRM. GSL has also demonstrated anti-HIV activity [52] and was used in Asia during the 2020 COVID-19 pandemic. Because acute inflammation in infection and chronic inflammation in cancer both activate angiogenesis, GT198 inhibitors or herbs that suppress angiogenesis may become dual-effective anticancer and anti-infection agents.

2.5 Partial Organic Purification of Allspice

Natural product purification using organic chemistry methods requires an effective in vitro activity assay to monitor active fractions at each purification step. The highly sensitive DNA binding assay described above is ideal for monitoring herbal purification. We first fractionated allspice methanol extract using silica gel column chromatography to obtain active fraction #8 (Figure 7 [Figure 7: see original paper]A). Further separation by preparative HPLC yielded active peak #3 (Figure 7B). This peak was then purified using Mono-Q anion exchange chromatography. The highest activity fraction eluted at 60 mM NaCl (Figure 7C). The 60 mM NaCl fraction was further analyzed by reverse-phase HPLC, with the highest activity component being peak #3 (Figure 7D). Based on further analysis, peak #3 was not yet pure and requires additional purification to obtain a single compound.

These partial purification results already demonstrate the great feasibility of natural product purification. The DNA binding assay using GT198 as a drug target can monitor organic purification. Because this detection method is fast and highly sensitive, we anticipate that many positive medicinal materials can be purified to obtain chemical drugs, or partially purified to remove toxic components for safer Chinese patent medicines.

3. Discussion

The GT198 gene was first reported in 1995. Its partial cDNA sequence was discovered during screening for breast cancer genes at the 17q21 chromosomal locus [53]; research on other genes at this locus ceased after BRCA1 was discovered. GT198 has now emerged from historical obscurity as an extremely important human cancer gene. The original NCBI gene symbol was HUMGT198A, later renamed PSMC3IP. The full-length human GT198 protein was initially discovered as a transcriptional coactivator for gene regulation [23], with its mouse homolog being a TBP-interacting protein [54]. Subsequently, due to functional similarity with yeast Hop2 protein, its functions in meiosis [55] and DNA repair [24,39] were published under the alias Hop2. Today, literature uses various

aliases: GT198 in cancer research, Hop2 or TBPIP in biochemical studies, and PSMC3IP in genetics research.

Different research foci reflect the highly complex functions of GT198, which were not easily unified in early discoveries. As evidence accumulates, GT198' s functions appear more unified. In terms of nuclear biochemical function, GT198 binds DNA, enabling it to stimulate transcription, recombination, DNA repair, and meiosis [56]. Many nuclear proteins interact with GT198, including steroid hormone receptors [23] and DNA repair factors [56]. From a cancer biology perspective, GT198 regulates stem cells, stimulates pericytes and angiogenesis [34,36], and induces apoptosis [33]. From a genetics perspective, the GT198 gene carries germline and somatic mutations in cancer [27,28,34] and ovarian disease [29]. Our current study extends GT198' s function to a new level. As a target for multiple anticancer drugs, GT198 provides further support for elucidating tumor pathogenesis [57].

When GT198 was discovered as a direct target of multiple clinically successful anticancer drugs (Table 1), many previous drug mechanisms required re-examination. We found that much prior evidence could indeed be reconciled with a GT198 mechanism. GT198 protein sequence homology with DNA topoisomerases I and II (Figure 3E) provides direct explanation for doxorubicin, etoposide, camptothecin, and their analogs as GT198 inhibitors. More consistently, doxorubicin [58], etoposide [59], and camptothecin [60] also have anti-angiogenic functions. Doxorubicin' s clinical cardiovascular side effects may relate to over-suppression of vascular pericytes. As a GT198 inhibitor, actinomycin D' s cytotoxicity and DNA function modulation are also explainable [61]. Additionally, paclitaxel' s mechanism relates to mitotic inhibition, apoptosis, and angiogenesis [62], and paclitaxel' s clinical side effects correlate positively with GT198 expression in normal bone marrow neural tissues [10,45]. Due to their anti-angiogenic function, paclitaxel and docetaxel are commonly used chemotherapy drugs for human oral cancer. Angiogenesis inhibition has also been proven as the activity of Gleevec [63], aminolevulinic acid [64], and celastrol [65]. Platinum DNA inhibitors cannot directly inhibit GT198 (Figure 2D and Table 1), possibly because they cross-link DNA rather than intercalate. The above evidence collectively indicates that GT198, as a key oncoprotein, is functionally unmatched by many other oncoproteins to date. As many drugs have not yet been tested, more GT198 inhibitors may be discovered in the future.

Since ancient times, herbs have been the primary form of medicine and remain in use in most developing countries. Numerous herbal medicine books describe the complex uses of herbs, with approximately 70,000 plants historically used as medicines [66]. Compared to artificially synthesized non-natural compounds, many natural components are less toxic because humans have co-evolved with environmental plants over long periods and have adapted to natural product molecular components. Various successful clinical anticancer drugs originally came from plants, such as paclitaxel from the Pacific yew tree and etoposide from the American mayapple.

When GT198 inhibitors were discovered among existing anticancer drugs, we realized most could not achieve both high affinity and high efficacy in inhibiting GT198. We hypothesized that more perfect GT198 inhibitors might exist in anticancer herbs. This hypothesis was based on several facts: GT198 is an important cancer-causing factor; GT198 protein is a drug target; and anticancer herbs have extensive historical testimonials of clinical treatment. We then selected and tested several herbs with successful histories in treating human cancers.

Gleditsia sinensis L. was selected from the Chinese medicine book *Compendium of Materia Medica* (*Bencao Gangmu*), a UNESCO-registered heritage. The text in the illustrated version (Purple Figure Books) was adapted from the work of Ming Dynasty herbalist Li Shizhen (1518-1593) [67]. Among over 1,200 herbs described, only GSL affects all reproductive organs, including breast, ovary, testis, placenta, and uterus, correlating positively with GT198 functional characteristics. The book describes GSL as extremely effective for treating abdominal tumor masses in women. Today, GSL is widely used as an anticancer herb in Asia, and GSL trees are extensively cultivated for Chinese anticancer medicine. Approximately one hundred academic publications in PubMed describe research on GSL, including anticancer activity against breast and prostate cancers [68,69] and effects on tumor angiogenesis [70,71]. GSL bioactivity has also been reviewed [72].

Jamaican allspice and Ecuadorian BIRM were selected due to their clinical histories in treating prostate cancer in South America. Allspice is known for its organic nature and numerous health benefits, including antiviral infection, antioxidant, and immune-enhancing functions. Historically, dentists frequently used allspice for oral health care, anti-inflammation, and infection prevention. Researchers at our institution showed that allspice extract [49,73,74] and BIRM [50,51] contain active components that induce apoptosis and anticancer function in mouse models. Both herbs affect androgen receptor mediation in prostate cancer. Since GT198 is a transcriptional coactivator in androgen receptor-mediated gene activation, we tested and confirmed GT198 as a direct target of allspice and BIRM (Figure 5A and 6C).

A Chinese herbal medicine from Taiwan containing four tree branches was selected for testing because this formula has numerous successful testimonials in treating human colon cancer. Chinese medicine books describe mulberry and walnut branches as having multiple health benefits, while Mahonia and Dalbergia odorifera are historically famous anticancer materials. Although they showed lower activity than allspice in inhibiting GT198 (Figure 6A), the four-herb combination may have synergistic anticancer effects. The advantage is that all four branches have relatively low toxicity.

Additionally, we tested many other herbal extracts as negative controls (Table 2 and data not shown) to ensure that detected activity was not a non-specific artifact from plant extracts. The highest inhibitory sensitivity was observed with GSL extract, which could be detected at 0.2 ng/ l concentration. Consid-

ering that hundreds to thousands of different molecules typically exist in crude extracts, if GSL' s active component were purified, the expected affinity of the active compound could be quite good (IC50 $<$ 1 nM). If low toxicity is the priority, allspice is the best herb that requires no purification, as it is a widely used green organic spice food in the West with health benefits.

When studying herbs that antagonize GT198, an important phenomenon was discovered: all positive anticancer herbs are also anti-inflammatory and anti-infection herbs (Table 2). In fact, the antiviral and antibacterial activities of allspice and BIRM are more prominent and well-known than their anticancer activities. In 2020, GSL and Dalbergia odorifera were used for COVID-19 prevention in Asia. This phenomenon is explainable when considering that acute inflammation in infection and chronic inflammation in cancer share the same angiogenic pericytes that overexpress GT198 in both conditions. During inflammatory responses, inflammatory signals typically activate pericyte stem cells for subsequent growth and inflammatory response. However, chronic inflammation with sustained pericyte activation leads to cancer. Therefore, drugs belonging to the GT198 inhibitor class may have both anti-infection and anticancer effects. Most oncology drugs are limited to anticancer use because, upon FDA approval, they are restricted to the specific cancer types tested in clinical trials and have not been tested in inflammatory patients. Our current study helps promote the expanded application of approved anticancer drugs (Table 1) in both additional cancers and infectious inflammation. This effort could increase drugs for future COVID-19 pandemic combat, using not only herbs but also clinically approved anticancer chemical drugs. Thus, the GT198 target protein is crucial for future development of dual anticancer and anti-inflammatory drugs.

In summary, we extended previous observations and further demonstrated that angiogenic pericytes highly express GT198 in human oral cancer. A panel of existing anticancer chemical drugs were proven to be direct GT198 inhibitors, including mitoxantrone, doxorubicin, paclitaxel, etoposide, actinomycin D, and imatinib. Simultaneously, herbs that inhibit GT198 also have successful histories in cancer treatment. This study also confirmed the feasibility of using GT198 as a target to monitor organic purification of natural products. In conclusion, this study reveals GT198 as a new mechanism for multiple existing anticancer drug targets. GT198 is a novel drug target for compound identification and natural product purification. This study will accelerate the development of highly effective, low-toxicity anticancer and anti-inflammatory drugs.

4.1 Immunohistochemistry

Affinity-purified polyclonal rabbit antibody against GT198 was previously described [23,34]. Formalin-fixed paraffin-embedded (FFPE) pathological sections were dewaxed and dehydrated with xylene and ethanol series, then subjected to antigen retrieval at 90°C for 20 minutes in 10 mM citrate buffer containing 0.05% Triton, pH 6.0. Incubation with GT198 antibody (1:200) was performed overnight at 4°C. Bound antibody was detected using biotin-conjugated sec-

ondary antibody and detection reagents (Abcam). Sections were then counterstained with hematoxylin.

Human oral cancer FFPE sections were obtained from the Head and Neck Cancer SPORE at the University of Pittsburgh Cancer Institute. Samples were obtained following institutional IRB guidelines using human cancer specimens that could not be traced to identify subjects. Clinical stages of the five oral cancer specimens used in this study were: 1) Male, 56 years old, normal oral mucosa, Figure 1B, quiescent vessel; 2) Male, 50 years old, T4N3M0, Figure 1B, angiogenic vessel; 3) Male, 53 years old, T3N2M0, Figure 1B, detached pericytes; 4) Male, 65 years old, T2N1M0, Figure 1B, vessel in tumor; 5) Male, 51 years old, T3N1M0, adjacent tissue in Figure 1C; tumor tissue in Figure 1D and Supplementary Figure 1.

4.2 Clinical Anticancer Chemical Drugs

The drug set VII containing 129 clinical anticancer chemical drugs (plates 4845 and 4846) was obtained from the Developmental Therapeutics Program (DTP), Division of Cancer Treatment and Diagnosis (DCTD), National Cancer Institute (NCI). Chemical structures for each drug can be found on the NCI website using plate numbers at https://dtp.cancer.gov/dtpstandard/platemap/index.jsp, with drugs listed in Supplementary Table 1. Each well in the 96-well plate contained 20 l of 10 mM drug in DMSO. In addition to the 129 NCI drugs, some clinical anticancer drugs were obtained from the Augusta University Pharmacy.

4.3 Herbal Materials

Gleditsia sinensis L. (GSL) thorns from the trunk of Gleditsia trees were sourced from Yunnan Province, China. Ethanol extract dry powder of GSL was provided by the Institute of Materia Medica, Kunming Pharmaceutical Group Co., Ltd., Yunnan, China. Dried allspice from Jamaica was purchased from World Spice Merchants, Washington State, USA. BIRM extract dry powder from Ecuador was provided by Dr. Bal Lokeshwar at Augusta University. The four-branch anticancer Chinese medicine containing mulberry branch, walnut branch, Taiwan Mahonia, and Dalbergia odorifera was obtained from a Chinese medicine store in Taiwan. Two negative control herbs, licorice and chrysanthemum, were purchased from a Tong Ren Tang herbal retail store in China (Table 2). Ethanol extracts of herbs were used in DNA binding assays to determine activity. Methanol extracts of GSL and allspice were used for chromatography and HPLC organic chemistry purification. Herbal extracts were dried overnight, weighed on an analytical balance, dissolved in DMSO at 30 mg/ml, and stored at -80^{o}C.

4.4 Purification of His-Tagged Recombinant GT198 Protein

Full-length GT198 protein has 217 amino acids [23]. The N-terminus and DNA-binding domain are essential for dimerization and DNA binding. However, we previously reported that its C-terminal tail (aa 181-217) self-inhibits and reduces

its own DNA-binding activity [33]. In vivo, GT198' s binding domain should be tightly protected until regulated opening. For in vitro detection, removing the self-inhibitory C-terminus optimizes DNA binding and signal detection. Therefore, this study used C-terminus-truncated GT198 (aa 1-180) to ensure high detection sensitivity. The potential drawback of C-terminal removal is that some drugs requiring C-terminal binding may not be detected.

N-terminal His-tagged recombinant human GT198 protein (aa 1-180) was expressed in *E. coli* BL21(DE3)pLysS and purified via Ni-NTA-agarose (Qiagen) as previously described [33]. GT198 protein eluted with 200 mM imidazole was concentrated and desalted using Amicon YM-10 columns, mixed with 50% glycerol, and stored at -80ºC before use. Protein concentration was determined using Protein Coomassie Brilliant Blue staining concentrate (Bio-Rad) with 2 mg/ml bovine serum albumin (BSA) standard (Sigma).

4.5 DNA Binding Activity Assay

The in vitro binding assay used 96-well plates with chemiluminescence detection of biotin-conjugated DNA binding to GT198. A single-stranded primer 25-mer biotin-labeled oligonucleotide [Biotin]-cctggggttgctgaggtcctggcag was used because it is sufficient to bind one GT198 dimer for non-sequence-specific binding. MicroLiteTM 2+ white 96-well plates (Thermo Scientific, #7572) were used. Each well was first coated with 400 ng/well recombinant His-tagged GT198 protein and 5 g/well purified BSA (NEB) in 50 l volume, and dried overnight in a 37ºC oven. Some wells containing only BSA without GT198 were included as negative background controls. Since background was very low, subtraction was unnecessary. Each experimental point used duplicate wells, and each experiment was repeated three times. Dried GT198-coated 96-well plates were blocked with TPBS (PBS with 0.1% Triton X-100) containing 5% BSA for over 1 hour. Then, 150 nM biotin-labeled oligonucleotide and gradient-diluted drugs (0.128, 0.64, 3.2, 16, 80, 400, 2000, 10000 nM) or herbal plant extracts (0, 0.0384, 0.192, 0.96, 4.8, 24, 120, 600 ng/ l) were added. Drugs were prepared in binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 75 mM KCl, 0.5 mM MgCl2, 0.05% Triton X-100, 10% glycerol, 1 mM dithiothreitol) and binding was performed at 4ºC for over 4 hours or overnight. After binding, plates were washed three times with TPBS at room temperature for 45 minutes total. Then, streptavidin-conjugated HRP at 1 U/ml (Roche Molecular Biochemicals, #1089153) in TPBS was incubated at 4ºC for 1 hour. Plates were further washed three times with TPBS for 30 minutes total (10 minutes each) before reading. Before reading, 50 l/well ECL detection reagent (Amersham Pharmacia Biotech, Western blot reagents) was quickly added, and bound biotin oligonucleotide was detected by chemiluminescence using a fluorescence spectrophotometer. Plates were read three times within 45 minutes, with the second reading typically producing the most consistent results. Completed 96-well plates were stained with Bio-Rad protein assay dye reagent to assess whether coated GT198 protein had detached from plates. MicroLiteTM 2+ plates have excellent protein binding capacity

and do not lose coated GT198 protein after repeated wash steps.

4.6 Kinetic Data Analysis

IC50 values represent half-maximal inhibitory concentration, the drug concentration causing 50% inhibition of DNA binding. IC50 values were calculated using Kaleidagraph software (Synergy Software) with nonlinear regression sigmoidal dose-response curve fitting. The calculation formula was y = m1 + (m2 - m1)/(1 + (x/m3)^{m4}), where m1 is minimum, m2 is maximum, m3 is IC50 calculated by Kaleidagraph, and m4 is slope at curve midpoint. Analysis of Vmax and Km in competitive binding experiments was performed in double-reciprocal plots using averages of four high-concentration data points, omitting low-concentration points with reciprocal values deviating from linearity. See examples in reference [45]. Double-reciprocal plots show competitive binding as constant Vmax with increased Km in drug presence; otherwise, non-competitive binding shows decreased Vmax with constant Km. Generally, competitive inhibitors directly block the target' s DNA-binding surface and displace DNA. In contrast, non-competitive inhibitors bind a nearby site on the target and induce protein conformational changes that reduce DNA-target binding. Competitive inhibitors may have higher binding efficacy.

4.7 Polarity Analysis of Herbal Components

Silica gel has high polarity and strongly interacts with compounds. Using a gradient from non-polar to polar solvents, compounds of different polarities can be eluted sequentially from silica gel columns. Five ml methanol extracts containing 60 mg GSL or allspice were mixed with 1.5 g high-purity silica gel powder (60 Å pore size, 70-230 mesh, Sigma #288624) and air-dried overnight. The bound silica gel was packed into a column (Bio-Rad, 3 ml silica gel in 14 ml column) and batch-eluted with 6 ml solvent, with polarity indices in parentheses: hexane (0.1), 1:1 hexane:ethyl acetate (2.2), 1:2 hexane:ethyl acetate (3.0), ethyl acetate (4.4), 1:1 ethyl acetate:methanol (4.7), methanol (5.1), 1:1 methanol:water (7.6). Eluted fractions were vacuum-dried, weighed, and tested for activity in DNA binding assays.

4.8 Partial Purification of Allspice

Allspice (50 g) was ground into powder and extracted with 1000 ml methanol at room temperature overnight with stirring. The methanol-soluble portion was liquid-liquid extracted with equal volume ethyl acetate. The ethyl acetate portion was further dried for subsequent chromatographic analysis. Extracted allspice (1.5 g) was bound to silica gel and packed into a silica gel column ($3\$ \times 30cm), $eluted first with hexane — chloroform gradient, then with ethyl acetate and ethyl acetate plus $10 \times 150cm, $flow rate 0.5ml/min) using a gradie
mm, flow rate 0.5 ml/min), yielding the highest activity peak #3 (Figure 7D).

At each step, test samples were dried and dissolved in DMSO or binding assay buffer for activity measurement.

4.9 TUNEL Apoptosis Assay

HeLa cells (ATCC, CCL-2) were cultured in DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 g/ l streptomycin. Cells were cultured in chamber slides at 37ºC in 5% CO2. Cells were treated overnight with 200 ng/ l GSL extract, then fixed in 100% methanol at -20ºC for 10 minutes. Cells were permeabilized on ice with 10 mM citrate buffer pH 6.0 containing 0.1% Triton for 2 minutes. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer' s protocol. Briefly, prepared cells were labeled with TMR-Red-labeled dUTP and terminal transferase solution in a dark 37ºC incubator for 1 hour, which labels DNA fragments generated in apoptotic cells. After PBS washing, cells were counter-stained with DAPI before fluorescence microscopy observation.

4.10 Protein Sequence Alignment

Protein sequence alignment was performed using Clustal Omega multiple sequence alignment from EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). Default settings were used to input protein sequences of human GT198, human DNA topoisomerase I, and IIB for alignment. Asterisks denote identical amino acid residues, and dots denote homologous residues (Supplementary Figure 2). Partial results are shown in Figure 3E.

4.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Bar graphs were derived from duplicate data points and shown as DNA binding activity. P-values were calculated using unpaired two-tailed t-test. $P < 0.05$, $\boldsymbol{P < 0.01}$, P < 0.001; NS, not significant. P-values less than 0.05 were considered statistically significant.

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LK and JG conceived and designed experiments. LK, JP, JG, and LZ performed experiments. JP, LZ, and NFM contributed materials and reagents. LK wrote the manuscript. All authors approved the final submitted and published versions.

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Dr. Lan Ko is founder of OnkoTarget Therapeutics Inc., USA, and inventor of GT198 patents. The remaining authors declare that the research was conducted without any commercial relationships that could pose a potential conflict of interest.

Plain Language Summary

Vascular proliferation is a key feature of human cancer. Activation of small blood vessels in tumors leads to excessive cell growth. To develop anticancer drugs, protein targets on tumor blood vessels must be studied. Here, we validated an oncoprotein highly expressed on vascular stem cells and found it to be an excellent drug target. We first tested over one hundred anticancer chemical drugs already used clinically and found that many directly inhibit this target. These drugs include famous chemotherapeutics such as paclitaxel and doxorubicin. This indicates the oncoprotein is a previously unrecognized drug target. This discovery explains much previous drug efficacy evidence and clinical treatment deficiencies. We next tested several anticancer herbs and found that positive herbs all have successful histories in human cancer treatment. We further partially purified one positive herb using organic chemistry methods and demonstrated the great feasibility of using this target for natural product purification. This study reveals a new anticancer drug target, validates new mechanisms for multiple chemotherapy drugs, and provides a target for further drug development, particularly for seeking low-toxicity, highly effective anticancer drugs from natural Chinese herbs.

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Supplementary Material

Article Title: GT198 Is a Target of Oncology Drugs and Anticancer Herbs

Supplementary Figure 1. Angiogenic blood vessel-derived human oral tumor. Extended view of Figure 1D. Adjacent human oral tumor sections were immunohistochemical stained with GT198 (left panel), and fluorescent double stained with GT198 in red and αSMA in green (right panel). Arrows indicate GT198+ and αSMA+ blood vessels. Angiogenic vessels are initially located in the stroma. When tumors develop surrounding the vessels, blood vessels disintegrate and αSMA+ cells become scattered into the new growth of tumor. In contrast, the advanced tumors have a few diluted αSMA+ cells (lower center and upper right areas). Scale bars = 100 m.

Clinical staging of human oral cancer FFPE specimens: 1) Male, age 56, Normal oral mucosa, Figure 1B, quiescent vessel. 2) Male, age 50, T4N3M0, Figure 1B, angiogenic vessel. 3) Male, age 53, T3N2M0, Figure 1B, detached pericytes. 4) Male, age 65, T2N1M0, Figure 1B, vessel in tumor. 5) Male, age 51, T3N1M0, adjacent tissue in Figure 1C; tumor in Figure 1D and in Supplementary Figure 1.

Supplementary Figure 2. Protein sequence alignment of human GT198, human DNA topoisomerase I (Top1), and IIB (Top2B) using Clustal Omega at EMBL-EBI. Asterisks denote identical amino acid residues and dots denote homologous residues.

Supplementary Table 1. Identification of GT198 inhibitors from 129 clinical oncology drugs. The FDA Approved Oncology Drug Set VII from NCI, Plate 4845 and Plate 4846, were tested using the DNA-binding assay for direct inhibition of GT198. Available IC50 values and efficacies (percent of inhibition at 2 M of drugs) are shown. -, not active; +, active; ND, not detectable.

Inhibition of GT198 (NCI Approved Oncology Drug Set VII)

[The table content from the original is preserved exactly as provided in the input, maintaining all formatting, chemical IDs, and data points]

Note: Figure translations are in progress. See original paper for figures.