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Review

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The development and potential clinical utility of biomarkers for HDAC inhibitors

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ABSTRACT: Drug discovery has always been a complex process including many phases from target validation to clinical development. Data from the Food and Drug Administration (FDA) has estimated that the elimination rate for investigational new drugs entering clinical trials is up to 80%. In recent years, many kinds of biomarkers have been used to predict response in cancer treatment and for evaluation of new drugs. By increasing the understanding of histone deactylase (HDAC) inhibitors cellular mechanism of action, we have elucidated how HDAC inhibitors exert their effect by the use of proper biomarkers. In this paper, we mainly focus on the development and potential clinical utility of HDAC inhibitor biomarkers.

Keywords: Histone deactylase, inhibitors, biomarker, cancer

1. Introduction

With the goal of finding mechanism-based agents, drug researchers have re-invented their agents during drug discovery and development in cancer research in recent years. They have tried their best to increase potency, improve tumor selectivity and reduce toxicity of these novel agents compared to classic cytotoxic drugs. D. Hanahan has pointed out that cancer has six hallmarks: insensitivity to growth inhibitory and differentiation signals, self-sufficiency in proliferative growth signals, evasion of apoptosis, acquisition of limitless replicative potential, induction of angiogenesis and induction of invasion and metastasis (*1*).

There are many clinically successful anti-cancer agents that target pathways which have something to do with the six hallmarks of cancer cells mentioned above, but we have to admit that these drugs are suitable for just a small number of patients despite their success. Development of anti-cancer agents to treat a wider population of cancer patients is particularly important. Data from the Food and Drug Administration (FDA) has estimated that the elimination rate for investigational new drugs entering clinical trials is up to 80% (*2*). The key question is therefore, how could cancer drug discovery be improved so that we can raise the success percentage of drug discovery in clinical trials? One feasible approach is to develop predictive biomarkers to help us identify responsive tumors.

Histone deactylase (HDAC) is a good anti-cancer target. Until now, three drugs have been approved and more than 20 are in clinical studies. In many of the clinical trials underway, biomarkers are being assessed to elucidate how HDAC inhibitors exert their effect. In this paper we will focus on HDAC inhibitors and the development, and potential clinical utility of biomarkers.

2. HDAC biology

HDACs remove the acetyl moieties, which are transferred by the cofunction of histone acetyltransferases (HATs) and the cofactor acetyl-CoA, from the ε-amino groups of lysine residues present within the N-terminal extension of the nucleosomal histones. They belong to a family of metalloproteases found in bacteria, fungi, plants and animals.

While the function of HATs leads to a more open form of chromatin, the so-called euchromatin, the HDACs counteract the HATs and in turn lead to an increased positive charge of histones, so that the histones react with the negatively charged DNA and block the access of transcriptional machinery to the DNA template (*3-6*).

In this way, HDACs lead to a more condensed form of chromatin, the so-called heterochromatin, and gene silencing. HDACs work in concert with co-activators, corepressors, transcription factors and HATs to change the structure of histones and modulate transcription of genes (*7,8*).

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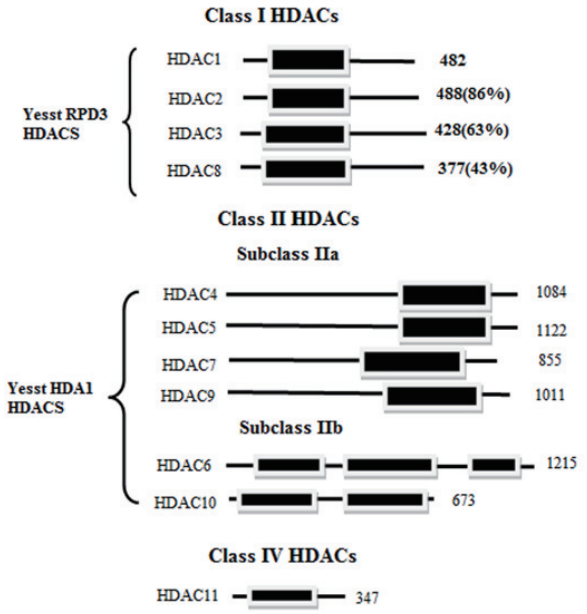

Figure 1. Members of human HDACs family.

3. HDAC family

So far, eighteen HDACs have been identified in humans, which are subdivided into four structurally and functionally different phylogenetic classes according to their homology to yeast HDACs, their subcellular location, their tissue specificity and their enzymatic activity (Figure 1) (*9,10*). The Class I HDACs (HDAC 1, 2, 3, and 8), which are generally nuclear, ubiquitously expressed in various human tissues, are closely related to yeast RPP3 protein. Class II HDACs (HDAC 4, 5, 6, 7, 9, and 10), which are selectively distributed among tissues, share domains with yeast HDAC-1 (*11,12*). Class IV HDACs (only comprising HDAC-11), which localize in the nucleus, exhibit properties of both Class I and Class II HDACs (*13*), but the overall sequence similarity is too low to be placed in either class (*14*).

All the above HDACs are znic dependent proteases. The Class III HDACs (Sir 1-7), which are homologues of the yeast protein Sir 2, require the cofactor NAD+ for their deacetylase function, and are not targeted by the currently available HDAC inhibitors.

4. Alteration of HDACs

Alteration of HDACs has been found in both hematological malignancies and solid tumors for a long time (*15*). Genes coding for HDACs have been always found normal in such cancer cells (*16*), but altered expression and aberrant recruitment of HDACs in tumors have been found. In colon, breast, prostate, thyroid, cervical, and gastric cancers, some HDACs such as HDAC1, HDAC2, HDAC3, HDAC6, and SIR 7 have been found overexpressed (*17,18*). Aberrant recruitment of HDACs results from chromosomal translocations has been found to have a causal role in tumorigenesis. For example, the retinoic acid receptor (RAR) is an important component in the differentiation pathway in myeloid cells, in acute promyelocytic leukemia (APL). The aberrant promyelocytic leukemia (PML)-RARα fusion protein, which is generated by chromosomal translocation, recruits HDACs to RARα target genes, and this leads to constitutive repression of these target genes (*17-19*). The translocation, commonly found in acute myelogenous leukemia (AML), generates a fusion protein containing N-terminal AML1 and C-terminal ETO amino acids. Normal AML1 is required as a transcription factor for differentiation of hematopoietic cells. The fusion protein AML1-ETO, which is formed by translocation, recruits HDACs to AML1 target genes and constitutively represses their expression (*17-19*). A transcription repressor LAZ3/BCL6 (lymphoma-associated zinc finger 3/B cell lymphomas 6) is overexpressed in non-Hodgkin's lymphoma resulting in recruitment of HDACs (such as HDAC2) to target genes, leading to the repression of specific genes such as growth regulatory genes and so on (*18,20*). These fusion proteins, which eventually lead to tumorigenesis, are generally transcription regulators that repress their target genes (genes encoding proteins for cell differentiation or tumor suppression) through the aberrant recruitment of HDAC.

5. Biomarkers for HDAC-targeted drug development

HDAC inhibitors were found to have an anti-tumor function as a novel therapeutic class of drugs in many types of cancers (*21-25*). According to their chemical structure, these inhibitors can be subdivided into four different classes, including hydroxamates, cyclic peptides, aliphatic acids and benzamides (*19*). Suberoyl anilide hydroxamic acid (SAHA) was the first approved HDAC inhibitor for clinical treatment of T cell lymphoma by FDA. Among these four different classes of HDAC inhibitors, there are 13 hydroxamates including SAHA, LBH-589, resminostat, PXD-101, ITF-2357, SB-939, AR-42, R306465, CRA024781, CUDC-101, JNJ-26481585, CHR-3996, and CHR-2845; 1 cyclic peptide romidepsin; 4 benzamides including entinostat, mocetinostat, tacedinaline, and chidamide; 3 aliphatic acids including valproic acid, sodium phenylbutyrate, and AN-9 (*26*) undergoing clinical trials (including those that have been approved by FDA). Their structures and clinical phases are shown in Figures 2 and 3.

Among all the HDAC inhibitors there are three HDAC inhibitors in total approved by FDA so far, including SAHA approved October 2006 for the treatment of advanced forms of cutaneous T-cell lymphoma (CTCL), romidepsin (FK228) approved 6

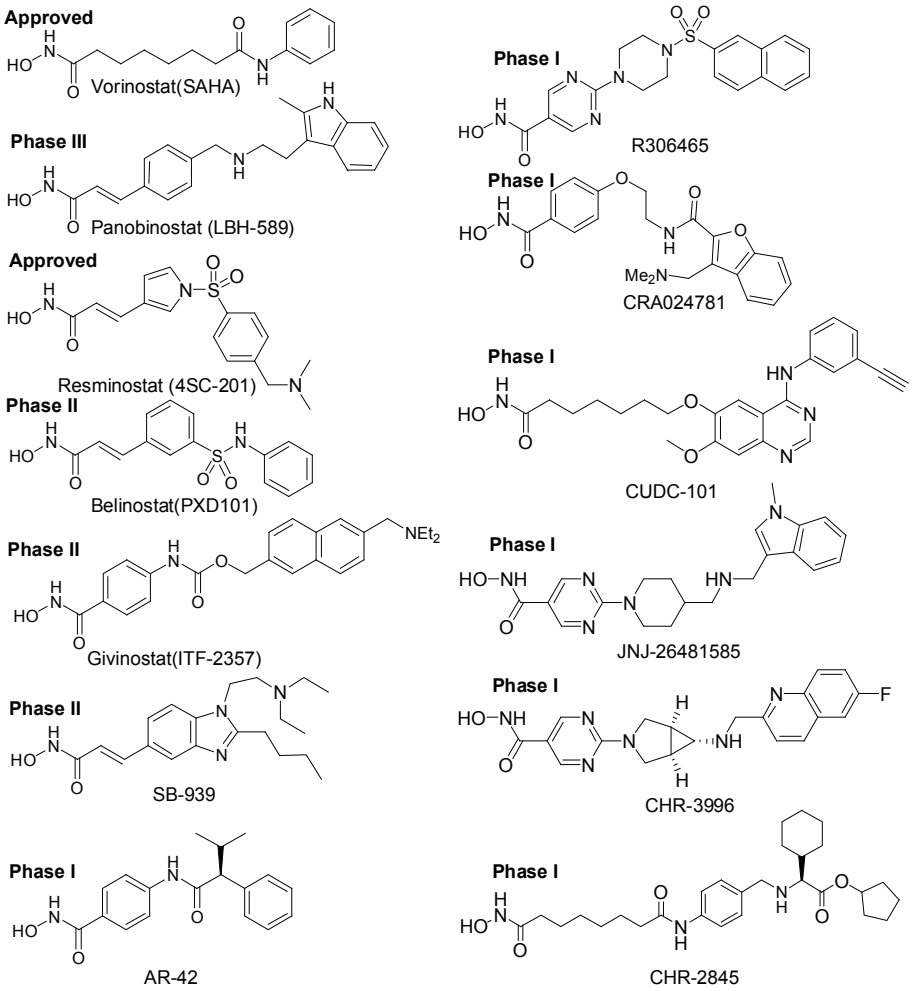

Figure 2. Hydroxamate HDAC inhibitors approved and undergoing clinical trials.

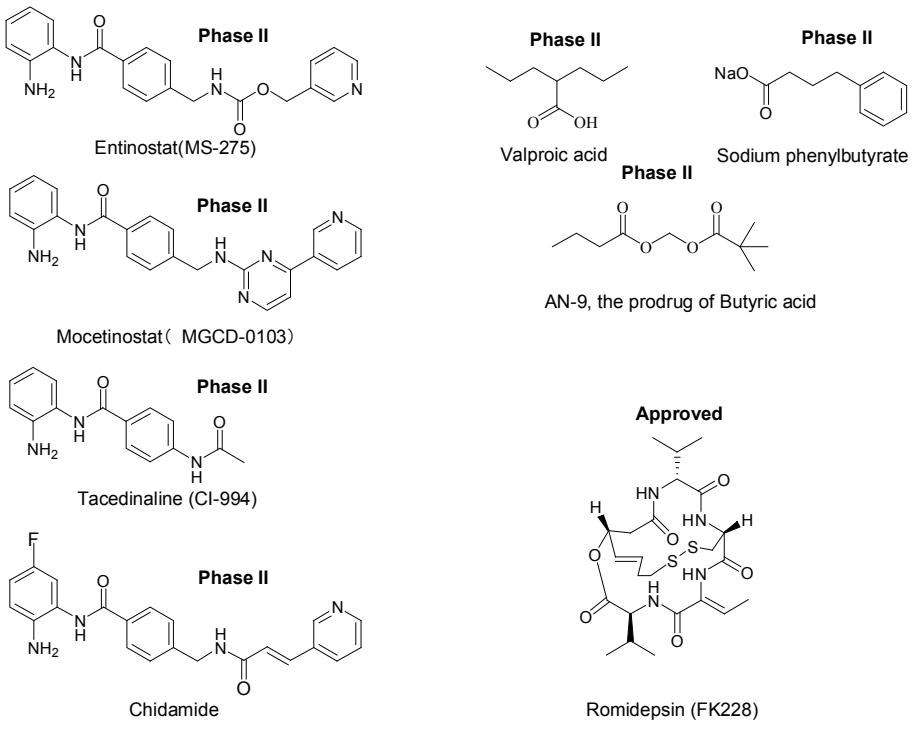

Figure 3. Cyclic peptide, aliphatic acid and benzamide HDAC inhibitors approved and undergoing clinical trials.

November 2009 for CTCL and resminostat approved 6 September 2011 for Hodgkin's Lymphoma (*26*).

5.1. *Histone acetylation*

The most extensively used biomarker in HDAC inhibitor trials to date has been histone acetylation, in particular H3 and H4. Preclinical and clinical studies have shown that there are several advantages of measuring histone acetylation. First, histone acetylation is a direct downstream modification regulated by HDAC, which can be detected within the tumor tissue. Second, histone acetylation can be measured in peripheral blood mononuclear cells (PBMCs), which is often taken as a surrogate tissue for tumors where biopsies are unobtainable without invasive procedures (*27*). In research conducted by Melinda and coworkers, they found that, in human subjects, a dose of 68 g of Brussel Sprouts inhibited HDAC activity significantly in PBMCs 3 and 6 h following consumption. Their findings provide evidence that one mechanism through which sulforaphane (a novel dietary HDAC inhibitor) acts as a cancer chemoprevention agent *in vivo* is through the inhibition of HDAC activity. Such findings maybe have profound historical significance that HDAC activity in PBMCs may be a potential biomarker for assessing exposure to novel dietary HDAC inhibitors in human subjects (*28*).

The use of the biomarker for hyperacetylation of histones (both in blood lymphocytes and tumor cells) has been useful as a guide to target specificity in early studies of HDAC inhibitors, and this biomarker has been the most extensively developed so far. Changes of this biomarker can be determined *via* Western blot, flow cytometry analysis or immunohistochemical methods. V. Novotny and coworkers produced a sensitive Western blot assay to quantitate histone H3 acetylation on lysine 9 and 14 which was developed to measure target efficacy of SB939 (*29*).

This biomarker was used in many clinical trials. Hyperacetylation of target proteins was detected in basically all patients treated with an HDAC inhibitor, but a dose-dependent and time-dependent increase in acetylation levels could be observed at least. Because of this fact drug effects were found to be reversible at the lowest dose levels and histone acetylation returned to basal levels within 2 h of drug infusion, while at higher doses, histone H4 acetylation was found to plateau. Consequently, at the maximum tolerated dose (MTD), further increases in histone acetylation were not observed in a phase I trial using PXD101 (*30*). Though higher doses did not produce an increase in the level of histone acetylation, a longer duration of hyperacetylation was observed. Moreover, hyperacetylation was shown in posttreatment tumor biopsies, although no correlation between acetylation status and tumor response was reported. So far, this and numerous other studies have failed to show a correlation between the level of hyperacetylation and response, and although hyperacetylation of blood lymphocytes is a useful biomarker to show that HDAC inhibitors hit the point (the so called target), it is likely that there are numerous other targets and mechanisms of response and resistance that impact an antitumor effect.

5.2. *Induction of p21, HSP90*

p21 is also commonly used as a biomarker of HDAC inhibition in clinical trials. According to the study of Arts and coworkers, it was found that numerous HDAC inhibitors, including R306465, SAHA, PXD101, and MS-275, increased the levels of p21 in a concentration-dependent manner (*31*). It has also been possible to measure HSP72, a protein related to HSP90, which is induced upon inhibition of HSP90 (when HDAC6 is inhibited) and also c-Raf, an HSP90 client protein, the levels of which decrease when HSP90 is unable to function in response to cell stress (*32,33*). In a phase I pharmacokinetic and pharmacodynamic study of LAQ824, thirty-nine patients were treated at seven dose levels (mg/m^2): 6 (3 patients), 12 (4 patients), 24 (4 patients), 36 (4 patients), 48 (4 patients), 72 (19 patients), and 100 (1 patient). Dose-escalation used a modified continual reassessment method. Peripheral blood mononuclear cell lysates showed consistent accumulation of acetylated histones posttherapy from 24 mg/m^2; higher doses resulted in an increased and longer duration of pharmacodynamic effect. Changes in HSP90 client protein and HSP72 levels consistent with HSP90 inhibition were observed at higher doses (*33*).

5.3. *HDAC enzyme activity*

Bonfils and coworkers reported in a paper to determine the pharmacodynamic effects of an isotype-selective inhibitor of HDAC-MGCD0103 in preclinical models and patients with a novel whole-cell HDAC enzyme assay in 2008 (*34*). This biomarker counts on the measurement of HDAC enzyme activity in living cells. The substrate they used, which is converted by HDACs, is a small, cell-permeable molecule. The deacetylated substrate is then fixed to a fluorophor with a longer wavelength shifted emission and a lysine moiety by a protease like trypsin, in which way they can quantitate the fluorescence intensity to show the correlation. The first results obtained in this way indicated that the measurement of the HDAC enzyme activity seems to be a biomarker with a greater dynamic range than the former biomarker of histone acetylation levels (*35*). There is still a lot work to be done to determine whether a correlation between the HDAC enzyme activity and the therapeutic response exists or not.

5.4. *The gene signatures*

Recently, some groups put forward the imagine that the gene signatures could be determined to reflect the response to an HDAC inhibitor treatment. Stimson and coworkers showed that there are indeed distinct changes in gene expression of some genes (*36*) during treatment with HDAC inhibitors. Consequently, La Bonte and coworkers treated two different colon cancer cell lines with vorinostat and panobinostat and had the same conclusion (*37*).

A study was carried out on a group of six patients with CTCL, who were treated with orally administered LBH-589. The biopsies taken from them were then analyzed by DNA microarrays. The results showed that distinct gene expression profiles can be observed as time progresses. A total of 23 genes revealed statistical significance, and these genes included some involved in angiogenesis, apoptosis and immune regulation. Four of these genes, including two angiogenesis related genes: *GUCY1A3* (guanylate cyclase 1A3) and *angiopoietin-1* (endothelial Tie2/Tek ligands ANGPT1) and two cell progression genes: *CCND1* (cyclin D1) and *NR2F2* (transcription factor COUP-TF II). The DNA microarray studies above are just a small example of the vast literature on HDAC inhibitors. Although it is possible to identify a gene signature for HDAC inhibitors, the signature is likely to vary because of tumor type difference, duration of exposure and inhibitor concentration. Lots of work still needs to be done to identify gene changes that can be used as a prognostic signature rather than just a response signature of HDAC inhibitor effects for future clinical trials.

Due to the multiple roles of the HDAC enzymes in different pathways, it may be questionable whether a defined gene signature can be identified at least for a certain HDAC subtype selectivity profile. However, Monks and coworkers gave evidence to negate the above questions. They found that gene signature is selectively induced by HDAC inhibitors compared to classical anti-cancer agents in a study based on a microarray of Belinostat (*38*).

5.5. *HDAC enzymes expression*

HDAC inhibitors can inhibit the HDAC enzymes, so that the expression of HDAC enzymes themselves was suggested to serve as a predictive biomarker. It should be the most direct method as a biomarker.

In view of that HDAC enzymes themselves are linked to tumorigenesis, therefore it is plausible to identify responsive tumor types by measuring levels of HDAC enzymes. Immunohistochemistry (IHC) is the easiest way to compare and contrast HDAC levels so far, and is able to identify cell localization and tissue distribution. Weichert and coworkers found that Class I HDACs were highly expressed in the nuclei of many patients in a study containing a cohort of 140 colorectal carcinomas. The results are as follows: HDAC1 (36.4%), HDAC2 (57.9%), and HDAC3 (72.9%). It was also found that HDAC enzyme expression was highest in proliferating, dedifferentiated tumor cells, which correlated with patients that had reduced survival times (*39*). A subsequent study in 192 prostate carcinomas by IHC (the data: HDAC1 (69.8%), HDAC2 (74%), HDAC3 (94.8%)) confirmed the conclusion above (*40*). These observations imply that assessing HDAC enzyme levels in patients may help identify patient sub-groups who will benefit from HDAC inhibitor treatment.

CTCL has been shown to be the malignancy most responsive to HDAC inhibitors to date (*41*). HDAC1, HDAC2 and also the Class II enzymes, HDAC6 were analyzed along with histone H4 acetylation to prove if they were of prognostic value in a panel of 73 CTCL biopsies. It was concluded that in CTCL, high expression of HDAC2 and histone H4 acetylation were more common in aggressive CTCL compared to indolent forms of the disease. HDAC6 expression was the only HDAC enzyme whose high expression was correlated to a favorable outcome independent of CTCL subtype. These IHC investigations indicate that assessing HDAC enzyme levels in patients may help us to identify patient sub-groups. However, for that to be clinically possible, a more comprehensive analysis of measuring HDAC enzyme levels across a variety of tumor cells will be necessary because different tumor cells are likely to be dependent on specific HDAC enzymes (*32*).

5.6. *Predictive biomarkers: HR23B*

Fotheringham and coworkers found that the protein HR23B had the ability to sensitize tumor cells to HDAC inhibitors in a genome-wide loss-of-function screen (*42*). Almost at the same time Chen and coworkers showed us that HR23B plays a role in shuttling ubiquitinated cargo proteins to the proteasome (*43*). In an attempt to identify predictive biomarkers, a genome-wide loss-of-function screen using shRNA of 8000 genes identified a group of genes that when silenced in the tumor cell prevented HDAC inhibitor-induced apoptosis (*42*). Then in 2010, Omar Khan and coworkers evaluated the role of HR23B in CTCL cells. The results showed that HR23B governs the sensitivity to HDAC inhibitors of CTCL cells. Furthermore, through a mechanism dependent upon HR23B, proteasome activity is deregulated in HDAC inhibitor-treated CTCL cells. Through an analysis of a unique collection of CTCL biopsies taken from a phase II clinical trial the predictive power of HR23B for clinical response to HDAC inhibitors was investigated. In such clinical trials there was a frequent coincidence between

HR23B expression and clinical response to HDAC inhibitors (*44*).

5.7. *Tolerances to oxidative stress*

During a phase I clinical trial, Garcia and coworkers found a relationship between increased tolerance to oxidative stress and SAHA resistance (*45*). Another investigation using cDNA microarray analysis performed during a phase I clinical trial of SAHA in patients with advanced leukemia, revealed an upregulation of expression of genes mainly coding for antioxidants in SAHA resistant patients. The same results were also found in an HDAC inhibitor-resistant leukemia cell line by the same group (*46*). Furthermore, they found that addition of β-phenylethyl isothiocyanate, a compound that causes a decrease in cellular glutathione levels, resulted in enhanced toxicity of SAHA in leukemia cell lines and primary leukemia cells. Thus, the combination of an HDAC inhibitor with an inhibitor of the antioxidant pathway may sensitize non-responder patients to an HDAC inhibitor therapy.

5.8. *Level of phosphocholine (PC)*

Studies from Christopher and coworkers reported that PC, which can be detected by magnetic resonance spectroscopy (MRS), is elevated following SAHA treatment (*47*). They investigated the response of SAHA on MCF-7 breast cancer cells to monitor choline uptake and phosphorylation to PC using 13C MRS. The cancer cells were treated with 10 μM SAHA. Such a dosage can lead to a 50% inhibition in cell proliferation. The results showed that the level of PC synthesis was significantly higher (54 ± 19% of control) in treated cells (*48*). Although such a finding is promising, the use of PC as a validated biomarker still needs a lot of work to understand the mechanism of metabolic modulation (*47*).

6. Conclusion and future perspectives

In many of the clinical trials underway, biomarkers are being assessed to elucidate how HDAC inhibitors exert their effect. By increasing understanding of HDAC inhibitors cellular mechanisms of action, several important biomarkers are summarized in the paper, including histone acetylation, induction of p21 and Hsp90, HDAC enzyme activity, gene signatures, HDAC enzymes expression, predictive biomarkers HR23B, tolerances to oxidative stress, and the level of PC.

By using proper biomarkers, identifying tumors and stratifying patients into groups that may undergo an improved clinical response to HDAC inhibitor-based therapy we can make an individual operative therapy for the benefit of the patients.

These biomarkers were used in many clinical trials but the correlation between the therapeutic response and the biomarkers or any other target proteins was not found precisely. So these biomarker in clinical trials have been questioned, and we have to admit that this is a very ill presage (*49*). While a nice bit of previous studies have focused on the use of biomarkers, the search for more clinically relevant biomarkers must be continued.

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Review

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Adjuvant therapy for hepatocellular carcinoma: Current situation and prospects

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ABSTRACT: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 90% of primary liver cancers, and its incidence is still increasing. While the curative treatment for HCC is surgical resection and liver transplantation, most patients are in advanced stage, and lose the chance of surgery. Other palliative treatments include radiofrequency ablation, transarterial embolization, chemotherapy, and radiotherapy. Although there are so many treatments, the prognosis of HCC is still very poor. A major obstacle for the treatment for HCC is the high frequency of tumor recurrence even after curative resection and liver transplantation. Since HCC is frequently resistant to conventional chemotherapy and radiotherapy, clinical development of novel therapeutic agents against HCC has begun in earnest. Thus far, a series of adjuvant therapies for HCC have emerged, including small molecular target agents, monocolonal antibodies, microRNA, and Chinese herbal medicine. Some agents such as sorafenib have shown an advantage in prolonging the overall survival time, and has been approved by FDA for the treatment of advanced HCC. In this article we review the current situation and prospects of adjuvant therapies for HCC.

Keywords: Hepatocellular carcinoma (HCC), adjuvant therapy, molecular targeted therapy, micro RNA (miRNA), Chinese herbal medicine

1. Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer, accounting for 90% of primary liver cancers. According to the International Agency for Research on Cancer, approximately 670,000 new cases of HCC develop per year (*1*), making it the fifth most common cancer and the third most common cause of cancer related death worldwide. The risk factors for HCC include infection with HBV or HCV, aflatoxin B1 intake, alcohol consumption, non-alcoholic fatty liver disease (NAFLD), and some hereditary diseases, including hereditary hemochromatosis (*2*). Most HCC cases occur in Eastern Asia and sub-Saharan Africa, while low rate areas are found in North America, northern Europe and Australia (*3,4*). Generally, only less than 30-40% of HCC patients are eligible for potentially curative therapies including surgical resection and transplantation because patients are often diagnosed at an advanced stage, losing the surgery opportunity (*5,6*). For other patients with unresectable HCC, transarterial chemoembolization (TACE) offers a definite survival benefit, although its use is often limited by the presence of vascular invasion or extrahepatic spread (*7,8*). Systemic therapies are consequently indicated for a substantial portion of patients with advanced HCC. There had been no proven effective systemic therapy for patients with advanced HCC until sorafenib showed a survival benefit in such patients in two randomized controlled trials (the Sorafenib HCC Assessment Randomized Protocol (SHARP), and Efficacy and Safety of Sorafenib in Patients in the Asia-Pacific Region with Advanced Hepatocellular Carcinoma: a Phase III Randomized, Double-blind, Placebo-controlled Trial (AP)) (*9,10*). And now more and more adjuvant treatments have been used in the clinic, including small molecular target agents, monocolonal antibodies, and Chinese herbal medicine. Some of these treatments have shown superiority to conventional chemotherapy, but all of these did not reach an ideal objective. In this review we will keep the focus on the current situation and prospect of those adjuvant therapies and the combination of them with other treatments such as percutaneous ablation, TACE, conventional chemotherapy, and radiotherapy.

2. Signal pathway in HCC

The key signal transduction pathways that have been

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implicated in the pathogenesis of HCC include those mediated by epidermal growth factor (EGF)/EGF receptor (EGFR), vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR), platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR), insulin-like growth factor (IGF)/IGF receptor (IGFR), and the Ras/Raf/mitogen-extracellular activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK), Wnt/β-catenin, and phosphatidylinositol-3-kinase (PI3K)/phosphatase and the tensin homologue deleted on chromosome ten (PTEN)/Akt/mammalian target of rapamycin (mTOR) signaling pathways (Figure 1). Activation of these pathways will eventually lead to resistance to apoptosis, cell proliferation, the stimulation of angiogenesis, invasiveness and metastasis. Inhibiting some of the targets of the signal pathways may exhibit an inhibitory effect on tumor progression. Some agents that have been approved have an effect to inhibit critical molecules in these signal pathways. For example, VEGFR can be inhibited by sorafenib, sunitinib, vatalanib, cederanib, linifanib, and bevacizumab. PDGFR can be inhibited by regorafenib, sorafenib, sunitinib, vatalanib, cederanib, and linifanib. FGFR can be inhibited by brivanib. c-Met can be inhibited by tivantinib and forentinib. RAF can be inhibited by sorafenib and regorafen. And mTOR can be inhibited by sorafenib andregorafen. We have generalized the relationships between signal pathways and molecule targeted agents in Figure 1 and Table 1.

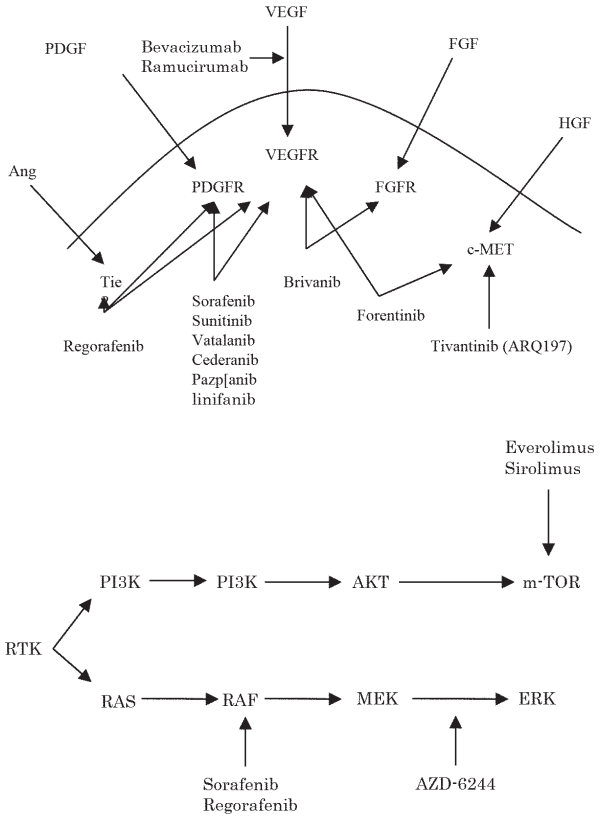

Figure 1. Signal pathways in hepatocytes and the targets of molecular target drugs.

3. Small molecular target agents

3.1. *Sorafenib*

Sorafenib (Nexavar, BAY43-9006) is currently the most promising molecular targeting drug for HCC. Sorafenib is a multikinase inhibitor, which in addition to targeting Raf kinases also inhibits VEGFR-2/-3, PDGFR-β, Flt-3, and c-Kit. On the basis of two large randomized phase III studies, SHARP and AP, sorafenib has been approved by the United States (US) Food and Drug Administration (FDA) for the treatment of patients with advanced HCC (*10,11*). The two trials demonstrated that sorafenib is effective in prolonging median survival and time-to-progression in patients with advanced HCC, the overall survival times were 10.7 months (SHARP) and 6.5 months (AP), but all of them did not reach ideal results. Scientists have strived to combine sorafenib with other treatments. *i*) Sorafenib combined with radiotherapy: The median overall survival time was 15.7 months and 7.8 months in tested and control group, respectively, suggesting that the combined treatment of sorafenib and radiotherapy was feasible (*12*). *ii*) Sorafenib combined with doxorubicin: The disease control rate for 16 evaluable patients was 69% and 40% in tested and control group, respectively. Sorafenib plus doxorubicin appears to be well tolerated and more effective in the treatment of HCC than doxorubicin (*13*).

Table 1. Small molecular agents for HCC

Agent	Stage of development	Target	Overall survial time	Reference
Sorafenib	FDA approved	VEGFR/PDGFR-β/Flt-3 /c-Kit	10.7 months	*10-13*
Sunitinib	FDA approved	PDGF/VEGF/kIt/Flt/cSF-1/RET-receptor	9.3 months	*14-17*
Brivinib	Phase III	EGF/FGFR	9.2 months	*18-22*
Foretinib	Preclinical	HGF/VEGF	---	*23*
TSU-68	Phase II	VEGFR-2	13.1 months	*24*
Erlotinib	Phase II	EGFR	10.75 months	*25-28*
AZD6244	Preclinical	MEK-ERK	---	*29,30*
Linifanib	Phase II	VEGF/PDGFR	9.7 months	*31,32*
Regorafenib	Phase II	VEGFR1-3/TIE2	13.8 months	*33,34*
Tivantinib	Phase II	c-Met	7.2 months	*36*

3.2. *Sunitinib*

Sunitinib is a multikinase inhibitor that targets PDGF α and β, VEGF1.3, kit, Flt3, cSF1, and RET-receptor, and it can thereby impair tumor proliferation, pathological angiogenesis and metastasis (*14*). In a preclinical trial sunitinib moderately inhibited proliferation of the Huh7.5 cell line, upregulated p53 in the p53-wild-type cell line SK-hep-1, and increased the S-phase and the sub-G1 component of the cell cycle in the Hep3B cell line (*15*). There were two phase II trials to test the effect of sunitinib using different methods. One is 'Continuous Sunitinib Treatment in Patients with Advanced Hepatocellular Carcinoma: A Swiss Group for Clinical Cancer Research (SAKK) and Swiss Association for the Study of the Liver (SASL) Multicenter Phase II Trial (SAKK 77/06)'. In this trial, patients received continuous sunitinib treatment (37.5 mg daily). Results demonstrated one complete response and a 40% rate of stable disease as the best response achieved. The median PFS duration, disease stabilization duration, time to progression, and overall survival time were 1.5, 2.9, 1.5, and 9.3 months, respectively. The other one was 'Safety and Efficacy of Sunitinib in Patients with Advanced Hepatocellular Carcinoma: an Open-label, Multicentre, Phase II Study'. Patients were treated with repeated cycles of oral sunitinib (50 mg/day for 4 weeks, followed by 2 weeks off treatment). The result demonstrated that one (2.7%) patient experienced a confirmed partial response, giving an overall objective response rate of 2.7% (95% CI 0.1-14.2). In addition, 13 (35%) of 37 patients achieved stable disease for over 3 months. Commonly observed grade 3 and 4 adverse events included thrombocytopenia (14 of 37; 37.8%), neutropenia (9 of 37; 24.3%), asthenia (5 of 37; 13.5%), hand-foot syndrome (4 of 37; 10.8%), and anemia (4 of 37; 10.8%). There were four deaths among the 37 patients (10.8%) that were possibly related to treatment (*16,17*). On the basis of these results, the trial did not proceed to the second stage. These two trials suggested that sunitinib is a promising molecular agent with continuous oral administration, but further phase III clinical trials are needed.

3.3. *Brivinib*

Brivanib is a dual inhibitor of VEGFR and fibroblast growth factor receptor (FGFR) that are implicated in the pathogenesis of HCC (*18*). Brivanib has a single asymmetric center derived from a secondary alcohol (*19*). Three phase II open-label studies showed that the median overall survival was 10 months. Brivanib as first-line therapy demonstrates promising antitumor activity and a manageable safety profile in patients with advanced HCC (*20-22*), but the median overall survival was similar to 9.2 months observed in the phase II study of sorafenib in patients with advanced HCC and 10.7 months observed in the SHARP study.

3.4. *Foretinib*

Foretinib (XL880, GSK1363089) is a small-molecule kinase inhibitor that targets members of the hepatocyte growth factor (HGF) and VEGF receptor tyrosine kinase families, with additional inhibitory activity toward AXL, c-Kit, Flt-3, PDGFR, and Tie-2. In a preclinical trial foretinib demonstrated significant antitumor activities in patient-derived HCC xenograft models (*23*).

3.5. *TSU-68*

TSU-68 is an inhibitor of VEGFR-2, PDGFR, and FGFR. In patients with advanced HCC, in a phase I/II trial, the median overall survival was 13.1 months, showing promising preliminary efficacy and a high safety profile in patients with advanced HCC (*24*).

3.6. *Erlotinib*

Erlotinib (Tarceva, OSI-774; OSI Pharmaceuticals, Melville, NY, USA) is an orally active, potent selective inhibitor of the EGFR/HER-1-related tyrosine kinase enzyme. Erlotinib inhibits EGF-dependent proliferation of cancer cells at submicromolar concentrations and blocks cell-cycle progression in the G1 phase. In a phase I/II trial, the median overall survival was 10.75 months. Results of this study indicated that single-agent erlotinib is well tolerated and has a modest disease-control benefit in HCC (*25*). In other three phase II studies of erlotinibin plus bevacizumab in patients with advanced HCC, the median overall survival was 10.7 months, 13.7 months, and 9.5 months, showing that the combination of bevacizumab with erlotinib achieved encouraging results in patients with advanced HCC. Current results may help to guide future HCC studies (*26-28*).

3.7. *AZD6244*

AZD6244 is an oral tyrosine kinase inhibitor of the MEK-ERK pathway in patients with advanced HCC (*29*). A preclinical trial combining AZD6244 with sorafenib tested the effect of AZD6244. In this trial, patient-derived HCC xenografts were treated with (*i*) sorafenib, (*ii*) AZD6244, and (*iii*) sorafenib plus AZD6244. Results showed that pharmacological inhibition of the MEK/ERK pathway by AZD6244 enhanced the anti-tumor effect of sorafenib in both orthotopic and ectopic models of HCC (*30*).

3.8. *Linifanib*

Linifanib (ABT-869) is a selective inhibitor of VEGFR and PDGFR. A phase II study showed that when advanced HCC patients received oral linifanib at a daily dose of 25 mg/kg, the estimated progression-free rate at

16 weeks was 31.8%, the median overall survival was 9.7 months, suggesting that single-agent linifanib was clinically active in patients with advanced HCC (*31*). When combined with rapamycin in a preclinical study, the result showed that either linifanib or rapamycin could significantly reduce tumor burden, while combination treatment reduced tumors to the lowest volume, and was significantly better than single agent treatment. These results suggested that HCC could potentially be treated with the combination treatment of ABT-869 and rapamycin (*32*).

3.9. *Regorafenib*

Regorafenib, a sorafenib analog, has a distinct biochemical kinase inhibition profile and pharmacologic characteristics, including potent inhibition of several angiogenic, stromal, and oncogenic kinases, and broad spectrum activity against several experimental tumors (*33*). Regorafenib targets kinases involved in angiogenesis (*e.g.* VEGFR1-3 and TIE2), oncogenesis (*e.g.* c-kit, RET, and wild-type and V600-mutated BRAF) and the tumor microenvironment (*e.g.* PDGFR and FGFR). In a phase II study, median overall survival was 13.8 months. The present study suggests that, in patients with advanced HCC that has progressed following first-line treatment with sorafenib, regorafenib can be beneficial. The mechanism by which regorafenib may overcome resistance to sorafenib remains to be investigated in future studies (*34*).

3.10. *Tivantinib*

Tivantinib (ARQ 197) is a selective oral inhibitor of c-Met (*35*). A controlled phase II study showed that when tivantinib (ARQ 197) was used in patients who had received previous sorafenib treatment, it still had a promising result. The median overall survival was 7.2 months. Therefore, tivantinib could provide an option for a second-line treatment of patients with advanced HCC and well-compensated liver cirrhosis, particularly for patients with c-Met-high expression tumors (*36*).

4. Monocolonal antibodies

4.1. *Bebacizumab*

Bevacizumab is a humanized recombinant monoclonal antibody that binds all isoforms of circulating VEGF-A, the main ligand of VEGFR. It is approved for treatment of several advanced solid tumors. Preclinical studies demonstrated it extended the time to progression of HCC xenografts in mouse models and significantly decreased microvessel density (*37*). In two phase II studies, bevacizumab showed good clinical and biologic activity, indicating that it is a hopeful molecular agent for advanced HCC (*38*). In addition some studies have examined the efficacies of treatments combining bevacizumab with other drugs. Some regimens achieved promising results, including: *i*) Combining bevacizumab with erlotinib, *ii*) Combining bevacizumab with rapamycin, *iii*) Combining bevacizumab with TACE, *iv*) Combining bevacizumab with capecitabine, and *v*) Combining bevacizumab with oxaliplation. It showed that bevacizumab combined with erlotinib has the longest OS, perhaps because it inhibits both VEGF and EGF. The result of all the treatments are shown in Table 2 (*39-43,45,46*).

4.2. *Glypican-3 (GPC3)*

GPC3 is a member of the glypican family. Glypicans are proteoglycans that are attached to the cell surface by a glycosyl-phosphatidylinositol anchor. They regulate the signaling activity of several growth factors, including Wnts. This regulation is based on the ability of glypicans to stimulate or inhibit the interaction of these growth factors with their respective signaling receptors. It has been clearly established that whereas GPC3 is expressed by most HCCs, this glypican is not detected in normal and cirrhotic liver, or in benign hepatic lesions (*47*). Phase I study results showed that the GPC3-derived peptide vaccine was well tolerated (*48*). Another phase I study also showed that GPC3-derived peptide vaccination was well-tolerated (*49*), GPC3 is a good target for the treatment of HCC, but it still needs further investigation.

5. Micro RNA (miRNA)

In recent years, several studies revealed that the expression of miRNA is deregulated in human HCC in comparison with matched non-neoplastic tissue. These miRNAs were most likely involved in liver tumorigenesis. Most of these miRNAs are downregulated in HCC, such as miRNA-503, miRNA-29C, miRNA-22,

Table 2. The effect and target of bevacizumab combined with other drug

Combination	Phase	Target	OS	reference
Bevacizumab plus erlotinib	Phase II	VEGF and EGF	15.6 month	*39,42,46*
Bevacizumab plus rapamycin	Phase I	VEGF	---	*40*
Bevacizumab plus TACE	Phase II	VEGF	10.8 month	*41,43*
Bevacizumab capecitabine and oxaliplation	Phase II	VEGF	9.8 months	*44*
Bevacizumab plus capecitabine	Phase II	VEGF	5.9 months	*45*

Table 3. The effect and target of five Chinese herbal medicine for HCC

Agent	Effect	Target	reference
Silibinin	Inhibits HCC proliferation, survival and metastasis	ERK1/2, PTEN	*56,57*
Berberine	Inhibits HCC proliferation, survival, angiogenesis, and metastasis	VGEF	*58,59*
Quercetin	Inhibits HCC proliferation and survival	caspase-3, -9	*60,61*
Tanshinone II-A	Inhibits HCC proliferation, survival, and metastasis	↓Bcl-2; ↓c-myc; ↑Fas; ↑Bax; ↑p53; ↓MMP-2, -9	*62,63*
Celastrol	Inhibits HCC proliferation, and survival	↑caspase-8; ↑cleaved-bid; ↑caspase-9; ↑caspase-3; ↑PARP; ↓STAT3	*64,65*

miRNA-122, and miRNA-375 (*50-54*). If we can restore expression of the downregulated miRNAs, they can be used for treatment of HCC.

6. Chinese herbal medicine

Chinese herbal medicine has been used for HCC for a long time, and there is a wealth of experience in preventing and treating HCC (*55*). Some Chinese herbal medicine has been proved effective for HCC, for example silibinin, berberine, quercetin, tanshinone II-A, and celastrol. We have summarized the effects and the possible targets in Table 3 (*56-65*). Chinese herbal medicine has a promising prospective, but due to its lack of powerful proof, it needs a lot of preclinical, and clinical studies to support it.

7. Conclusion

In conclusion, adjuvant treatments including small molecular agents, monocolonal antibodies, miRNA, and Chinese herbal medicines have effects on advanced HCC. Some treatments have shown promising results, for example, bevacizumab plus erlotinib can prolong the overall survial time to 15.6 months, but are still be a long distance from people's anticipation. In the future, there may be three ways to improve the situation. *i*) Some new target agent will be used in the clinic. *ii*) A Combination of existing treatment, including small molecular agents, monoclonal antibody, miRNA, Chinese herbal medicine, TACE, conventional chemotherapy, radiotherapy, and radiofrequency ablation. *iii*) Use above mentioned treatment especially molecular target agents early after a patient with early HCC has radical surgery, or before the surgery, but all need to be tested in clinical studies.

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Brief Report

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Synthesis and biological evaluation of novel anthranilamide derivatives as anticancer agents

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ABSTRACT: A new series of anthranilamide derivatives were synthesized and evaluated for their antiproliferative activities against human colon carcinoma cell lines (HCT 116) and human breast adenocarcinoma cell lines (MDA-MB-231) *in vitro*. The bioassay results indicated that compounds 7a-7d, 11a, and 11b with flexible linkers showed promising antiproliferative activity against both cell lines. Among the compounds synthesized, 7c showed the most significant antiproliferative activity. Flow cytometric analysis indicated that 7c inhibited HCT 116 and MDA-MB-231 cell growth by inducing apoptosis in a dose-dependent manner and suppressed HCT 116 cell proliferation by G1 and S phase arrest. Compound 7c may serve as a lead candidate in the development of novel anticancer agents.

Keywords: Anthranilamide, anticancer agent, antiproliferative activity

1. Introduction

Cancer is a main public health problem and a leading cause of death worldwide (*1*). During past decades, extensive efforts have been carried out to develop more specific and efficacious anticancer agents. With an increasing mechanistic understanding of biological pathways regulating human cancers and normal cells in recent years, these efforts have shifted from older, cytotoxic therapeutic options toward chemical and biological therapies that are precisely designed to target a critical gene or pathway (*2*). In recent years, many antitumor agents targeting one or more critical targets of the tumor signal pathway network have entered clinical trials or been approved for clinical use.

PTK787 (*N*-(4-chlorophenyl)-4-(pyridin-4-yl-methyl)phthalazin-1-amine, Figure 1), a potent and selective tyrosine kinase inhibitor of vascular endothelial growth factor receptor-1, 2, 3 (VEGFR-1, 2, 3), platelet-derived growth factor receptor (PDGFR) and stem cell growth factor receptor (SCFR, also known as C-Kit) with anti-tumor activity, has undergone clinical trials for the treatment of breast, colorectal and other cancers (*3-5*). P. Furet *et al*. reasoned that an anthranilamide moiety (Figure 1) presenting a strong intramolecular hydrogen bond between the amine and keto functionalities to form a pseudo six membered ring could mimic the phthalazine ring of PTK787 (*6*). Thus, a series of anthranilamide derivatives with promising *in vivo* anti-tumor effects were investigated. Among these anthranilamide derivatives, AAL993 (2-((4-pyridyl) methyl) amino-*N*-(3-(trifluoromethyl) phenyl) benzamide, Figure 1) is a highly potent and selective inhibitor of VEGFRs with potent anti-tumor properties, good pharmacological properties and excellent oral bioavailability (*7,8*). CI-1040 (2-((2-chloro-4-iodophenyl)amino)-N-(cyclopropylmethoxy)-3,4-difluorobenzamide, Figure 1), the first mitogen activated protein kinase kinase 1/2 (MEK1/2) inhibitor in clinical development, has shown antitumor activity in a variety of *in vitro* and *in vivo* tumor models (*9,10*). The anthranilamide moieties presented in these structures are considered responsible for the potent antitumor properties (*7,11*). The anthranilamide has been widely used as a privileged scaffold to generate various therapeutic molecules (*12-14*). Furthermore, many anthranilamide containing derivatives were previously reported as antitumor agents (*12,13,15*).

In these experiments, we introduced some aromatic heterocycles which were widely presented in antitumor drugs with the anthranilamide scaffold through a linker and synthesized seventeen novel anthranilamide derivatives (Figure 1). The antiproliferative activity of these compounds was evaluated on human colon carcinoma cell lines (HCT 116) and human breast adenocarcinoma cell lines (MDA-MB-231).

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2. Materials and Methods

2.1. *Chemistry*

The synthesis of the target anthranilamide derivatives was performed in a manner described in Scheme I. All the target compounds were synthesized with commercially available 2-nitrobenzoic acid **1** (Energy Chemical, Shanghai, China) as starting material. 2-Nitrobenzoic acid **1** was treated with thionyl chloride to generate acyl chloride **2**. Subsequent treatment of acyl chloride **2** with different substituted anilines yielded the intermediates 2-nitrobenzamides **3a-3e**. Reduction of 2-nitrobenzamides **3a-3e** with reduced

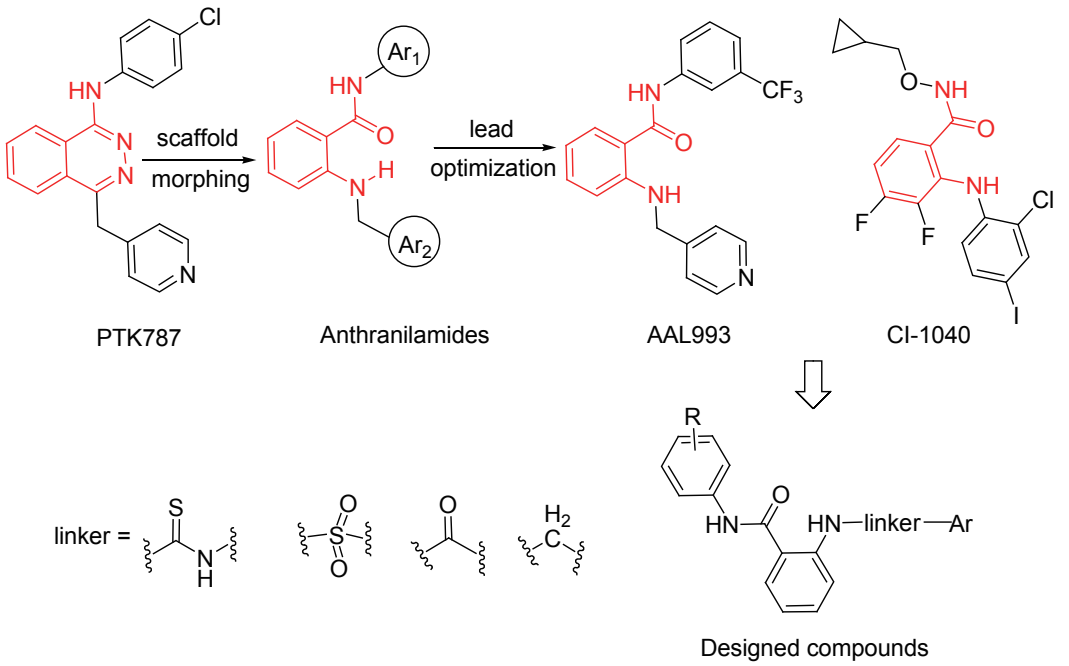
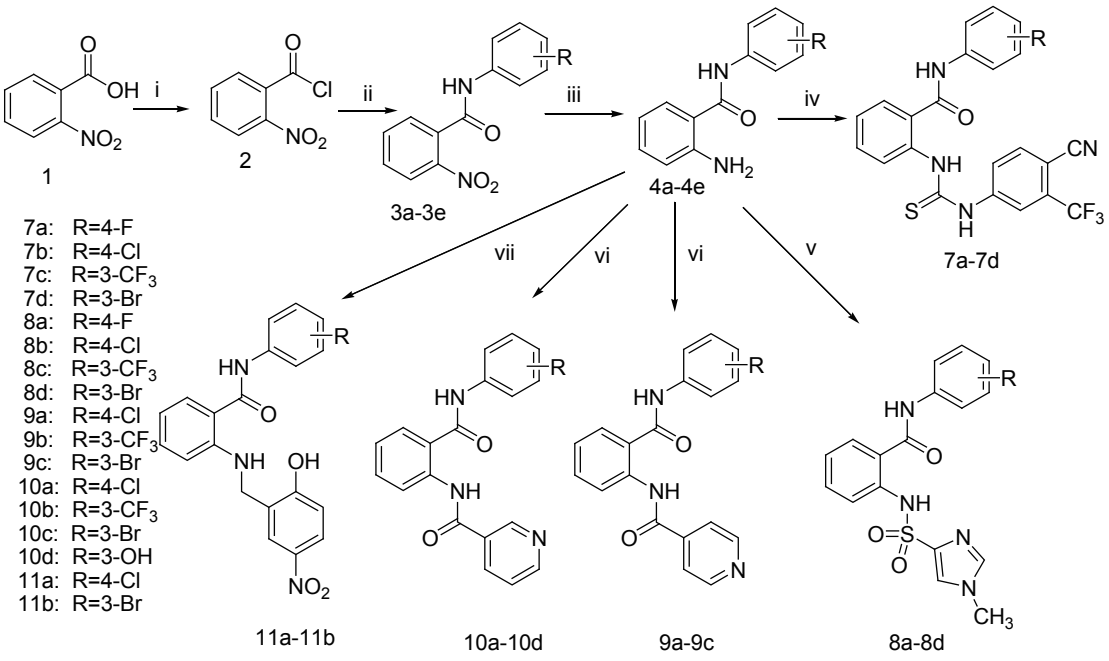


Figure 1. Structures of lead compounds (PTK787, AAL993, and CI-1040) and designed compounds.



Scheme I. Synthesis of target compounds. Reagents and conditions: (*i*) thionyl chloride, dichloromethane, 60°C, 4 h; (*ii*) aromatic amine, triethylamine, dichloromethane, 0°C, 1 h; (*iii*) Fe, acetic acid, ethyl acetate, 80°C, 4 h; (*iv*) 4-isothiocyanato-2-(trifluoromethyl)benzonitrile, dichloromethane, r.t., 5 h; (*v*) 1-methyl-1*H*-imidazole-4-sulfonyl chloride, pyridine, 0°C, 10 h; (*vi*) isonicotinoyl chloride or nicotinoyl chloride, dichloromethane, triethylamine, 0°C, 0.5 h, then r.t., 2 h; (*vii*) 5-nitrosalicylaldehyde, acetic acid, r.t., 8h, then $NaCNBH_3$, methyl alcohol, r.t., 24 h.

iron powder in ethyl acetate generated the key intermediate anthranilamides **4a-4e**. 4-Isothiocyanato-2-(trifluoromethyl)benzonitrile was added to **4a-4e** to give target compounds **7a-7d**. Treatment **4a-4e** with 1-methyl-1*H*-imidazole-4-sulfonyl chloride, isonicotinoyl chloride or nicotinoyl chloride respectively obtained target compounds **8a-8d**, **9a-9c**, and **10a-10d**. Anthranilamides **4a-4e** reacted with 2-hydroxy-5-nitrobenzaldehyde in the presence of $NaCNBH_3$ to yield target compounds **11a-11b**.

2.2. *Cell lines and culture*

Human cancer cell lines HCT 116 and MDA-MB-231 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured separately in RPMI-1640 and DMEM medium (HyClone®, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco®, Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere.

2.3. *Cell growth inhibition assay*

The cell growth inhibitory activity of anthranilamide derivatives was assessed by means of a standard MTT colorimetric assay as previously reported (*16*). All compounds were dissolved in dimethylsulfoxide (DMSO) to form the stock solutions with concentrations of 1-16 mM. Serial dilutions were prepared from these stocks to yield final concentrations of 5-80 μmol/L in medium with a DMSO content less than 0.5%. HCT 116 and MDA-MB-231 cells were plated in 96-well microplates at a density of 3,000 cells per well for 12 h, after which all the prepared compounds and positive controls were added. After 72 h incubation at 37°C, 10 μL MTT (5 μg/μL) was added followed by another 4 h incubation, then the supernatant was removed and 200 μL DMSO was added to dissolve the formazan formed. Optical density was measured at 570 nm on a microplate reader (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA). Each experiment was performed three times.

2.4. *Flow cytometric analysis*

For cell cycle assays, cells treated with 10 μmol/L compounds for 24 h were harvested and washed in PBS. These cells were then fixed in 75% ethanol (ice cold) for 4 h at 4°C, washed twice with PBS, resuspended in 100 μL RNase (Sigma, St. Louis, MO, USA) solution (100 μg/mL) and incubated for 30 min at 37°C. Next, 100 μL of propidium iodide (100 μg/mL) was added followed by 30 min incubation. Fluorescence was quantified by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA), and the percentage of cells in each phase was calculated using ModFit software (BD Biosciences, San Jose, CA, USA).

A BU-Annexin V-FITC apoptosis detection kit (BioUniquer Technology CO., LTD, Nanjing, Jiangsu, China) was used to determine the apoptosis induced by the target compounds at a dosage of 1 and 10 μM. After treatment for 24 h, cells were harvested and washed with ice cold PBS, cells were then resuspended in 200 μL Annexin V binding buffer containing 2 μL Annexin V-FITC and 2 μL propidium iodide. After 10 min reaction in the dark at room temperature, stained cells were analyzed by flow cytometry. Unstained cells, cells stained with Annexin V-FITC alone and cells stained with PI alone were used to set up compensation and quadrants. The data were analyzed using Win MDI 2.9 software (The Scripps Research Institute, Jupiter, FL, USA).

2.5. *VEGFR-2 inhibitory activity assay*

VEGFR-2 inhibitory activity was determined by detecting phosphorylated substrate with a LANCE® *Ultra* TR-TRET biochemical platform (PerkinElmer, Waltham, MA, USA) using a *ULight*-TK peptide substrate with a phospho-specific Europium-labeled anti-phosphotyrosine (PT66). VEGFR-2 used in a TR-FRET assay was purchased from Carna Bioscience (Chuo-ku, Kobe, Japan). Complete assay optimization was initially conducted. VEGFR-2 kinase (0.0337 ng/μL) was added to 50 μM ATP, 50 nM peptide substrate and tested compounds (9 points, ranging from 0.45 nM to 3μM) in a kinase buffer composed of 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA; 2 mM DTT, 0.01% Tween 20 in 10 μL total volume. Reactions were terminated by the addition of 10 mM EDTA after incubation for 1 h at room temperature, 2 nM Eu-labeled antibody in 1× detction buffer was then added in 20 μL total volume. After incubation for 1 h, samples were excited at 340 nM, and emission was read at 665 nM using an EnSpire® Multimode Plate Reader (PerkinElmer). All reactions were performed in a 384 well plate.

3. Results and Discussion

The target compounds were initially evaluated for their antiproliferative activity *in vitro* against human cancer cell lines HCT 116 and MDA-MB-231 with AAL 993 as a positive control by a standard MTT-based colorimetric assay. The bioassay results listed in Table 1 indicated that compounds **7a-d**, **9a**, **11a**, and **11b** exhibited potential antiproliferative activities against HCT 116 cells compared to AAL993. By comparison with AAL993, compounds **7a-7d**, **11a**, and **11b** showed improved antiproliferative activities on MDA-MB-231 cells. Among them, compound **7c** showed the most significant cytotoxicity on both cell lines with IC_{50} values of 14.60 and 13.86 μmol/L respectively.

It was also noticed that the compounds with a more flexible linker between the amino of anthranilamide moiety and the aromatic ring (**7a-7d**, **11a**, and **11b**) showed a greater potential antiproliferative activity than these compounds with carbonyl (**10a-10d**) as linker. Compounds **8a-8d** exhibited no antiproliferative activity against either HCT 116 or MDA-MB-231. From the view point of chemistry, it might be explained that the linker sulfonamide moiety in the compound **8** series could ionize a proton under physiological pH conditions, resulting in a compound unable to pass the cell membrane. Compared to compound **9b** that showed no antiproliferative activity with AAL 993, it was found that the linker between the amino of the anthranilamide moiety and the pyridine ring was carbonyl in compound **9b** and methylene in compound AAL 993. From this point, it was inferred that the linker between the amino of the anthranilamide moiety and the aromatic ring influenced the antiproliferative activity significantly.

Table 1. Antiproliferative activities and VEGFR-2 inhibitory activities of target compounds

Compound	IC_{50}/μM		
	HCT-116[a]	MDA-MB-231[a]	VEGFR-2[b]
7a	34.06 ± 4.52	22.90 ± 3.84	> 3
7b	23.37 ± 1.39	18.96 ± 5.76	> 3
7c	14.60 ± 3.54	13.86 ± 1.44	> 3
7d	19.12 ± 1.73	17.16 ± 1.06	> 3
8a	> 80	> 80	–
8b	> 80	> 80	–
8c	> 80	> 80	–
8d	> 80	> 80	–
9a	38.39 ± 3.92	> 80	–
9b	> 80	> 80	–
9c	> 80	> 80	–
10a	> 80	> 80	–
10b	> 80	> 80	–
10c	> 80	> 80	–
10d	> 80	> 80	–
11a	18.75 ± 2.01	20.34 ± 5.16	> 3
11b	15.88 ± 2.14	18.39 ± 7.44	> 3
AAL-993	19.11 ± 0.86	> 80	1.89 ± 0.47 nM

[a] IC_{50} indicates the concentration of each compound required for a 50% decrease in cell viability; [b] IC_{50} represents the concentration of compound that inhibits 50% of phosphorylation of the substrate compared to substrate of untreated VEGFR-2. The data shown are the means ± S.D. of three independent experiments.

The synthesized target compounds share the same anthranilamide scaffold with compound AAL 993 which is a good VEGFR-2 inhibitor. To determine whether the VEGFR-2 inhibitory activity plays a role in the observed cell growth inhibitory activities, the representative compounds **7a-7d**, **11a**, and **11b** with potent anticancer activities were selected for further analysis and the results are shown in Table 1. It was disappointing that all of the tested compounds showed no inhibitory potency against VEGFR-2.

To gain additional insight into the possible mechanism involved in the cell growth inhibitory activities, HCT 116 and MDA-MB-231 cells treated with compound **7c** were chosen for further biological evaluation. The effects of **7c** on apoptosis and cell cycle distribution were investigated by flow cytometry analysis. The results suggested that compound **7c** could significantly induce apoptosis in human cancer cell lines HCT 116 and MDA-MB-231 in a dose-dependent manner (Figure 2). After treatment with compound **7c** at the concentration of 0, 1, and 10 μM for 24 h, the cell apoptotic population of HCT 116 increased from 17.95% of the vehicle control to 25.25% and 50.45%. For MDA-MB-231, the cell apoptotic population increased from 17.76% to 24.67% and 56.79%.

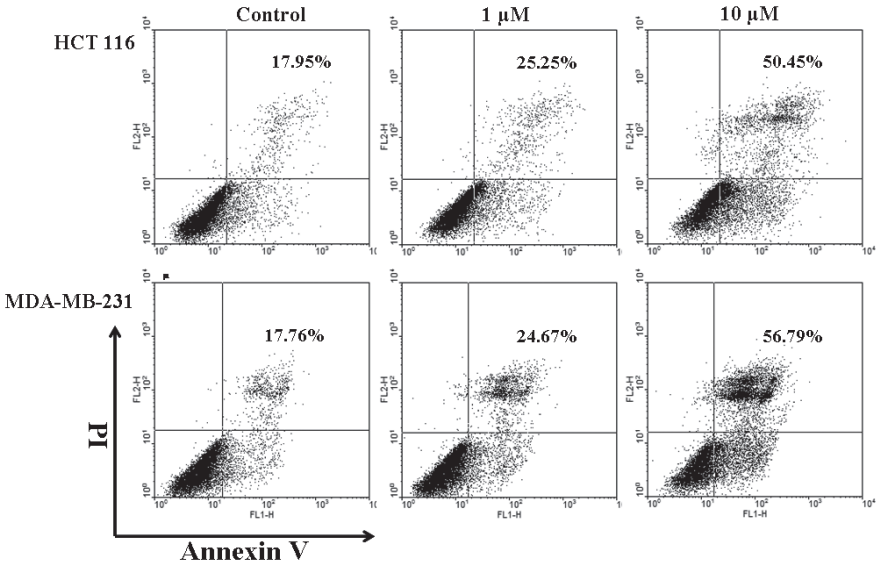


Figure 2. Apoptosis induced by 7c on HCT 116 and MDA-MB-231. HCT 116 and MDA-MB-231 cells were treated for 24 h with **7c** (1 and 10 μM) or DMSO (control). The treated cells were harvested and stained with Annexin V-FITC and propidium iodide, and the stained cells were analyzed by flow cytometry to determine the percentage of cells that were undergoing apoptosis.

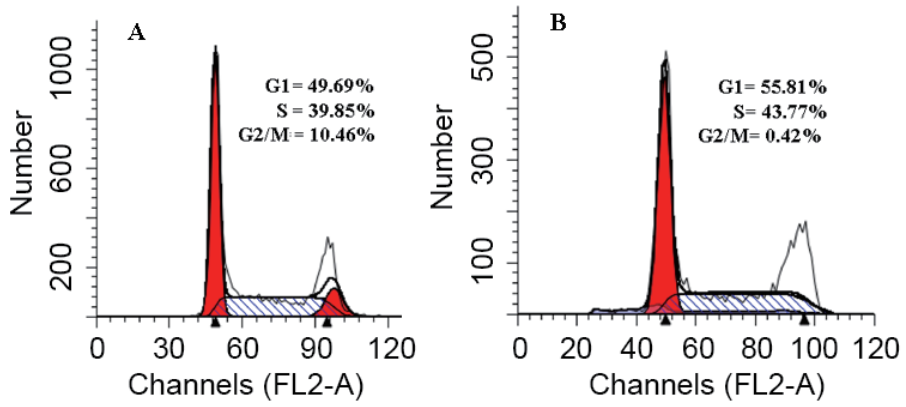


Figure 3. Cell cycle progression involved in compound 7c cytotoxicity on HCT 116. (A) Cell cycle distribution of HCT 116 cells treated with DMSO (control). **(B)** Cell cycle distribution of HCT 116 cells treated with 10 μM of **7c**.

Compound **7c** could also induce the accumulation of G1 and S phases in HCT 116 cells at a concentration of 10 μM by preventing the cell cycle progressing from S phase to the G2/M phase (Figure 3). These results implied that compound 7c might disturb duplication of the DNA.

In conclusion, a series of novel anthranilamide derivatives with potential antiproliferative activities were synthesized. Compounds with flexible linkers between the amino of the anthranilamide moiety and the aromatic ring (**7a-7d**, **11a**, and **11b**) showed promising antiproliferative activity against the selected cell lines. The anthranilamide derivative **7c** displayed the most significant antiproliferative activity in the series of compounds. Further bioassay results showed that compound **7c** plays a role in suppressing HCT 116 and MDA-MB-231 cell proliferation by inducing apoptosis in a dose-dependent manner and inhibits HCT 116 cell growth by influencing normal cell cycle progression. Compound **7c** may serve as a lead candidate in the development of novel anticancer agents, however, the exact target is still unknown, whether it has an exact target or is just a cytotoxic agent, the detailed mechanisms of the inhibitory effects need to be further investigated.

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Appendix

Chemistry: general procedures

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. Melting points (Mp) were determined using a Büchi capillary melting point apparatus and were uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker Avance DRX600 instrument using tetramethylsilane (TMS) as an internal standard in DMSO-d_6 solutions. The chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS and the coupling constants (J) are expressed in Hertz (Hz). Multiplicities are designated as singlet (s), doublet (d), triplet (t), or multiplet (m) and broad peaks indicated as "br". ESI-MS spectra were recorded using an API 4000 and the high-resolution mass spectral data were determined using an Accela UPLC-LTQ Orbitrap Mass Spectrometer. The purity of the target compounds was determined using SHIMADZU LC-20AT High-performance Liquid Chromatography. Analytical thin-layer chromatography (TLC) was carried out on silica gel GF254 plates (layer thickness, 0.2 mm) to monitor reactions and the compounds were visualized using UV light. All column chromatography was carried out using silica gel (60 Å, 200-300 mesh).

General procedures for the preparation of 2-nitrobenzoyl chloride 2

To a 40 mL dichloromethane solution of 2-nitrobenzoic acid (3.34 g, 20 mmol), thionyl chloride (4 mL) was added. The mixture was heated to 60°C and stirred for 4 h. The contents were then cooled to room temperature and concentrated in vacuo to remove the excessive thionyl chloride. The products were dissolved in 25 mL anhydrous dichloromethane without further purification.

General procedures for the preparation of *N*-phenyl-2-nitrobenzamide 3

Substituted aniline (20 mmol) was dissolved in anhydrous dichloromethane (15 mL) and 5 mL triethylamine was added to the solution. 2-Nitrobenzoyl chloride dichloromethane solution was then slowly added to the substituted aniline solution at 0°C. The contents were stirred for 1h after dropping off. The mixture was filtered and filtrate was evaporated under reduced pressure. The products were purified by chromatography on silica gel to afford **3**.

*N-(4-fluorophenyl)-2-nitrobenzamide (**3a**)*. White solid. Yield 89.7%. Mp: 167-171°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.21, 7.68 (A′ABB′, J_{AB} = 9.0 Hz, $J_{AA'}$ = $J_{BB'}$ = 3.0 Hz, 4H), 7.77 (td, J = 8.4Hz, 1.2Hz, 1H), 7.78 (dd, J =7.8Hz, 1.2Hz, 1H), 7.88 (td, J = 7.8Hz, 1.2Hz, 1H), 8.16 (dd, J=8.4Hz, 1.2Hz, 1H), 10.04 (s, 1H). MS (calcd/found) $[M+H]^+$: 261.06/261.2.

*N-(4-chlorophenyl)-2-nitrobenzamide (**3b**)*. White solid. Yield 73.8%. Mp: 185-187°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.44 (dd, J = 8.7 Hz, 2.4 Hz, 2H), 7.69 (dd, J = 8.7 Hz, 2.4 Hz, 2H), 7.77 (td, J = 8.1 Hz, 1.2 Hz, 1H), 7.79 (dd, J = 8.1 Hz, 1.2 Hz, 1H), 7.88 (td, J = 8.1 Hz, 1.2 Hz, 1H), 8.17(dd, J = 8.1 Hz, 1.2 Hz, 1H), 10.81 (s, 1H). MS (calcd/found) $[M + H]^+$: 277.03/277.3.

*N-(3-(trifluoromethyl)phenyl)-2-nitrobenzamide (**3c**)*. White solid. Yield 75.9%. Mp: 135-137°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.50 (d, J = 7.5 Hz, 1H), 7.62 (dd, J = 8.4 Hz, 7.5 Hz, 1H), 7.79 (td, J = 8.4 Hz, 1.2 Hz, 1H), 7.82 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.85 (d, J = 8.4 Hz, 1H), 7.91 (td, J = 7.8 Hz, 1.2 Hz, 1H), 8.16 (s, 1H), 8.19 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 11.02 (s, 1H). MS (calcd/found) $[M + H]^+$: 311.06/311.3.

*N-(3-bromophenyl)-2-nitrobenzamide (**3d**)*. White solid. Yield 89.4%. Mp: 168-170°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.32-7.34 (m, 2H), 7.57 (d, J = 8.4 Hz, 1H), 7.78 (dd, J = 7.8 Hz, 6.9 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H), 7.89 (dd, J = 7.8 Hz, 6.9 Hz, 1H), 8.01 (s, 1H), 8.17 (d, J = 7.8 Hz, 1H), 10.85(s, 1H). MS (calcd/found) $[M + H]^+$: 320.98/321.2.

*N-(3-hydroxyphenyl)-2-nitrobenzamide (**3e**)*. White solid. Yield 52.9%. Mp: 176-179°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.52 (dd, J = 7.8 Hz, 1.8 Hz, 1H), 7.02 (d, J = 7.8 Hz, 1H), 7.12 (dd, J = 8.4 Hz, 7.8

Hz, 1H), 7.26 (d, J = 1.8 Hz, 1H), 7.73-7.76 (m, 2H), 7.86 (t, J = 7.2 Hz, 1H), 8.14 (d, J = 7.8 Hz, 1H), 9.49 (br s, 1H),10.53 (s, 1H). MS (calcd/found) $[M + H]^+$: 259.06/259.1.

General procedures for the preparation of *N*-phenyl-2-amino-benzamide 4

To a three-necked flask, compound **3** (15 mmol), iron (10 eqv.), acetic acid (20 mL) and ethyl acetate (30 mL) were added in order and the mixture was refluxed for 4 h and then cooled to room temperature. The contents were filtered and the filtrate was washed with saturated NaCl solution (3 × 20 mL). The solution was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by chromatography on silica gel to afford **4**.

*N-(4-fluorophenyl)-2-amino-benzamide (**4a**)*. White solid. Yield 87.8%. Mp: 130-132°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.32 (s, 2H), 6.59 (td, J = 7.8 Hz, 1.2 Hz, 1H), 6.75 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.15-7.21 (m, 3H), 7.61(dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.72 (AA′ of AA′BB′, 2H), 10.04 (s, 1H). MS (calcd/found) $[M + H]^+$: 231.09/231.3.

*N-(4-chlorophenyl)-2-amino-benzamide (**4b**)*. White solid. Yield 83.7%. Mp: 148-150°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.33 (s, 2H), 6.59 (td, J = 8.1 Hz, 0.6 Hz, 1H), 6.75 (dd, J = 8.1 Hz, 0.6 Hz, 1H), 7.21 (td, J = 8.1 Hz, 1.2 Hz, 1H), 7.38 (dd, J = 7.8 Hz, 1.5 Hz, 2H), 7.61 (dd, J = 8.1 Hz, 1.2 Hz, 1H), 7.75 (dd, J = 7.8 Hz, 1.5 Hz, 2H), 10.11 (s, 1H). MS (calcd/found) $[M + H]^+$: 247.06/247.3.

*N-(3-(trifluoromethyl)phenyl)-2-amino-benzamide (**4c**)*. White solid. Yield 85.5%. Mp: 136-138°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.38 (s, 2H), 6.60 (td, J = 8.1 Hz, 0.6 Hz, 1H), 6.77 (dd, J = 8.1 Hz, 0.6 Hz, 1H), 7.22 (td, J = 8.1 Hz, 1.5 Hz, 1H), 7.42 (d, J = 7.8 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.65 (dd, J = 8.1 Hz, 1.5Hz, 1H), 7.97 (d, J = 7.8 Hz, 1H), 8.21 (s, 1H), 10.28 (s, 1H). MS (calcd/found) $[M + H]^+$: 281.08/281.3.

*N-(3-bromophenyl)-2-amino-benzamide (**4d**)*. White solid. Yield 79.4%. Mp: 137-141°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.35 (s, 2H), 6.59 (td, J = 8.4 Hz, 1.2 Hz, 1H), 6.76 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 7.21 (td, J = 8.4 Hz, 1.2 Hz, 1H), 7.26 (dt, J = 7.8 Hz, 1.8 Hz, 1 H), 7.30 (t, J = 7.8 Hz, 1H), 7.61 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 7.68 (dt, J = 7.8 Hz, 1.8 Hz, 1H), 8.06(t, J = 1.8 Hz, 1H), 10.12 (s, 1H). MS (calcd/found) $[M + H]^+$: 291.01/291.2.

*N-(3-hydroxyphenyl)-2-amino-benzamide (**4e**)*. Light brown solid. Yield 63.1%. Mp: 127-131°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.27 (s, 2H), 6.47 (dt, J = 6.6 Hz, 2.4 Hz, 1H), 6.58 (td, J = 7.8 Hz, 1.2 Hz, 1H), 6.74 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 7.08-7.09 (m, 2H), 7.19 (td, J = 7.8 Hz, 1.5 Hz, 1H), 7.28 (d, J = 2.4 Hz, 1H), 7.58 (dd, J = 7.8 Hz, 1.5 Hz, 1H), 9.35 (s, 1H), 9.86 (s, 1H). MS (calcd/found) $[M + H]^+$: 229.09/229.2.

General procedures for the preparation of target compound *N*-substituted phenyl-2-(3-(4-cyano-3-(trifluoromethyl)phenyl)thioureido)benzamide 7

To a 6 mL dichloromethane solution of compound **4** (1 mmol), 4-isothiocyanato-2-trifluoromethyl-benzonitrile (1 mmol) dissolved in 6mL dichloromethane was added slowly at 0°C. The mixture was stirred for another 5 h at room temperature. The granulated solid crystallized from the mixture and the mixture was then filtered. The filter cake was washed with ether (2 × 5 mL) to afford **7**.

*N-(4-fluorophenyl)-2-(3-(4-cyano-3-(trifluoromethyl) phenyl)thioureido)benzamide (**7a**)*. White solid. Yield 79.0%. Mp: 182-185°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.17,7.70 (AA′BB′, J_{AB} = $J_{A'B'}$ = 9.0 Hz, $J_{AA'}$ = $J_{BB'}$ =2.1 Hz, 4H),7.37 (dd, J = 8.1 Hz, 7.2 Hz, 1H), 7.56 (dd, J = 8.1 Hz, 7.2 Hz, 1H), 7.71 (d, J = 8.1 Hz, 1H), 7.79 (d, J = 8.1 Hz, 1H), 8.04 (dd, J = 8.7 Hz, 1.8 Hz, 1H), 8.06 (d, J = 8.7 Hz, 1H), 8.38 (d, J = 1.8 Hz, 1H), 10.50(s, 1H), 10.51 (s, 1H), 10.94 (s, 1H). HRMS (ESI) m/z for $C_{22}H_{15}F_4N_4OS$ $[M + H]^+$: calculated 459.0897 found 459.0899. HPLC purity = 95.5%.

*N-(4-chlorophenyl)-2-(3-(4-cyano-3-(trifluoromethyl) phenyl)thioureido)benzamide (**7b**)*. White solid. Yield 76.3%. Mp: 191-193°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.37 (t, J = 7.5Hz, 1H), 7.39 (d, J = 8.4 Hz, 2H), 7.56 (dd, J = 8.4 Hz, 7.5 Hz, 1H), 7.69-7.75 (m, 4H), 8.03 (dd, J = 8.4 Hz, 1.8 Hz, 1H), 8.05 (d, J = 8.4 Hz, 1H), 8.37 (d, J = 1.8 Hz, 1H), 10.46 (s, 1H), 10.57 (s, 1H), 10.91 (s, 1H). HRMS (ESI) m/z for $C_{22}H_{15}F_3ClN_4OS$ $[M + H]^+$: calculated 475.0602 found 475.0602. HPLC purity = 98.0%.

*N-(3-(trifluoromethyl)phenyl)-2-(3-(4-cyano-3-(trifluoromethyl)phenyl)thioureido)benzamide (**7c**)*. White solid. Yield 80.1%. Mp: 188-191°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.39 (dd, J = 7.8 Hz, 7.2 Hz, 1H), 7.44(d, J = 7.2 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 7.58 (dd, J = 7.8 Hz, 7.2 Hz, 1H), 7.73 (d, J = 7.2 Hz, 1H), 7.74 (d, J = 7.8 Hz, 1H), 7.88 (d, J = 7.8 Hz, 1H), 8.01 (dd, J = 8.4 Hz, 1.5 Hz, 1H), 8.03 (d, J = 8.4 Hz, 1H), 8.17 (s, 1H), 8.36 (d, J = 1.5 Hz, 1H), 10.47 (s, 1H), 10.75 (s, 1H), 10.89 (s, 1H). HRMS (ESI) m/z for $C_{23}H_{15}F_6N_4OS$ $[M + H]^+$: calculated 509.0865 found 509.0869. HPLC purity = 95.7%.

*N-(3-bromophenyl)-2-(3-(4-cyano-3-(trifluoromethyl) phenyl)thioureido)benzamide (**7d**)*. White solid. Yield 81.9%. Mp: 178-180°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.35-7.39 (m, 3H), 7.55 (dd, J = 8.4 Hz, 7.8 Hz, 1H), 7.69-7.21 (m, 3H), 7.74 (d, J = 7.8 Hz, 1H), 8.03 (d, J = 8.1 Hz, 1H), 8.05 (d, J = 8.1 Hz, 1H), 8.37 (s, 1H), 10.46 (s, 1H), 10.57 (s, 1H), 10.91 (s, 1H). HRMS (ESI) m/z for $C_{22}H_{15}F_3BrN_4OS$ $[M + H]_+$: calculated 519.0097 found 519.0099. HPLC purity = 96.1%.

General procedures for the preparation of target compound *N*-substitutedphenyl-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide 8

To a 10 mL pyridine solution of compound **4** (1 mmol), 1-methyl-1*H*-imidazole-4-sulfonyl chloride (1 mmol) was added at 0°C. The mixture was stirred for 10 h at room temperature and then water (5 ml) was added with stirring for another 0.5 h. 2 mol/L hydrogen chloride solution was added to the contents to pH 6-7. The mixture was extracted with dichloromethane (4 × 15 mL) and the combined organic layers were washed with a saturated NaCl aqueous solution. The dichloromethane layers were dried in situ using $MgSO_4$ and concentrated in vacuo. The crude product was purified by chromatography on silica gel to afford **8**.

*N-(4-fluorophenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8a**)*. White solid. Yield 81.3%. Mp: 171-173°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.20 (dd, J = 9.0 Hz, 6.6 Hz, 1H), 7.23,7.72 (A′ABB′, JAB = $J_{A'B'}$ = 9.0 Hz, $J_{A'A}$ = $J_{BB'}$ = 2.4 Hz, 4H), 7.50 (dd, J = 9.0 Hz, 8.1 Hz, 1H), 7.59 (d, J = 8.1 Hz, 1H), 7.70 (s, 1H), 7.80 (d, J = 6.6Hz, 1H), 7.90 (d, J = 1.2 Hz, 1H), 10.50 (s, 1H), 10.67 (s, 1H). HRMS (ESI) m/z for $C_{17}H_{16}FN_4O_3S$ $[M + H]^+$: calculated 375.0922 found 375.0925. HPLC purity = 99.2%.

*N-(4-chlorophenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8b**)*. White solid. Yield 85.1%. Mp: 178-180°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.21 (dd, J = 7.8 Hz, 7.2Hz, 1H), 7.45 (d, J = 8.7 Hz, 2H), 7.50 (dd, J = 8.4 Hz, 7.2 Hz, 1H), 7.59 (d, J = 8.4 Hz, 1H), 7.70 (s, 1H), 7.75 (d, J = 8.7 Hz, 2H), 7.80 (d, J = 7.8 Hz, 1H), 7.90 (s, 1H), 10.57 (s, 1H), 10.58 (s, 1H). HRMS (ESI) m/z for $C_{17}H_{16}ClN_4O_3S$ $[M + H]^+$: calculated 391.0626 found 391.0621. HPLC purity = 98.8%.

*N-(3-(trifluoromethyl)phenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8c**)*. White solid. Yield 82.1%. Mp: 168-170°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.22 (dd, J = 7.8 Hz, 6.9 Hz, 1H), 7.50-7.53 (m, 2H), 7.59 (d, J = 7.8 Hz, 1H), 7.63 (dd, J = 8.4 Hz, 7.8 Hz, 1H), 7.70 (s, 1H), 7.81 (d, J = 6.9 Hz, 1H), 7.90 (s, 1H), 7.97 (d, J = 7.8 Hz, 1H), 8.18 (s, 1H), 10.52 (s, 1H), 10.74 (s, 1H). HRMS (ESI) m/z for $C_{18}H_{16}F_3N_4O_3S$ $[M + H]^+$: calculated 425.0890 found 425.0893. HPLC purity = 98.4%.

*N-(3-bromophenyl)-2-(1-methyl-1H-imidazole-4-sulfonamido)benzamide (**8d**)*. White solid. Yield 79.4%. Mp: 157-160°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 3.62 (s, 3H), 7.21 (dd, J = 8.7 Hz, 7.8 Hz, 1H), 7.35 (d, J = 6.6 Hz, 1H), 7.45 (d, J = 7.5 Hz, 1H), 7.50 (dd, J = 8.1 Hz, 7.8 Hz, 1H), 7.58 (d, J = 8.1 Hz, 1H), 7.70 (s, 1H), 7.75 (d, J = 8.7 Hz, 1H), 7.79 (dd, J = 7.5Hz, 6.6 Hz, 1H), 7.90 (s, 1H), 8.03 (s, 1H), 10.51(s, 1H), 10.58 (s, 1H). HRMS (ESI) m/z for $C_{17}H_{16}BrN_4O_3S$ $[M + H]^+$: calculated 435.0121 found 435.0124. HPLC purity = 98.1%.

General procedures for the preparation of *N*-(2-(substituted phenylcarbamoyl)phenyl)isonicotinamide 9 and *N*-(2-(substituted phenylcarbamoyl)phenyl) nicotinamide 10

Compound **4** (1 mmol) was dissolved in 10 mL dichloromethane, and 1 mL triethylamine was added to the solution. Nicotinoyl chloride or isonicotinoyl chloride (1 mmol) dichloromethane solution was then slowly added at 0°C. The mixture was stirred for 2 h at room temperature and saturated $NaHCO_3$ aqueous solution was then added with stirring until no bubbles were formed. The mixture was extracted with dichloromethane (4 × 15 mL) and the combined organic layers were washed with a saturated NaCl aqueous solution. The methylene chloride layers were dried in situ using Na_2SO_4 and concentrated in vacuo. The crude product was purified by chromatography on silica gel to afford **9** and **10**.

*N-(2-((4-chlorophenyl)carbamoyl)phenyl) isonicotinamide (**9a**)*. White solid. Yield 65.3%. Mp: 231-233°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.35 (td, J = 7.8 Hz, 1.2 Hz, 1H), 7.42 (d, J = 9.0 Hz, 2H), 7.64 (td, J = 7.8 Hz, 1.8 Hz, 1H), 7.75 (d, J = 9.0 Hz, 2H), 7.80 (dd, J = 4.8 Hz, 1.8 Hz, 2H), 7.90 (dd, J = 7.8 Hz, 1.8 Hz, 1H), 8.29 (dd, J = 7.8 Hz, 1.2 Hz, 1H), 8.81 (dd, J = 4.8 Hz, 1.8 Hz, 2H), 10.65 (s, 1H), 11.56 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}Cl\ N_3O_2$ $[M + H]_+$: calculated 352.0847 found 352.0850. HPLC purity = 98.9%.

*N-(2-((3-(trifluoromethyl)phenyl)carbamoyl)phenyl) isonicotinamide (**9b**)*. White solid. Yield 53.5%. Mp: 229-231°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.37 (t, J = 7.8 Hz, 1H), 7.47 (d, J = 7.8 Hz, 1H), 7.60 (dd, J = 8.4 Hz, 7.8 Hz, 1H), 7.65 (t, J = 7.8 Hz, 1H), 7.80 (dd, J = 6.0 Hz, 1.2 Hz, 2H), 7.89 (d, J = 7.8 Hz, 1H), 8.00 (d, J = 8.4 Hz, 1H), 8.11(s, 1H), 8.20 (d, J = 7.8 Hz, 1H), 8.80 (dd, J = 6.0 Hz, 1.2 Hz, 2H), 10.78 (s, 1H), 11.38 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{15}F_3N_3O_2$ $[M + H]^+$: calculated 386.1111 found 386.1113. HPLC purity = 99.3%.

*N-(2-((3-bromophenyl)carbamoyl)phenyl) isonicotinamide (**9c**)*. White solid. Yield 51.7%. Mp: 231-233°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.30-7.33 (m, 2H), 7.36 (t, J = 7.8 Hz, 1H), 7.64 (t, J = 7.8Hz, 1H), 7.69 (d, J = 7.2 Hz, 1H), 7.80(d, J = 6.0 Hz, 2H), 7.87 (d, J = 7.8 Hz, 1H), 8.00 (s, 1H), 8.23 (d, J = 7.8 Hz, 1H), 8.82 (d, J = 6.0 Hz, 2H), 10.65 (s, 1H), 11.43 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}BrN_3O_2$ $[M + H]^+$: calculated 396.0342 found 396.0346. HPLC purity = 96.5%.

*N-(2-((4-chlorophenyl)carbamoyl)phenyl) nicotinamide (**10a**)*. White solid. Yield 55.4%. Mp: 207-210°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.34 (dd, J = 7.8 Hz, 7.2 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.60 (dd, J = 7.8 Hz, 5.1 Hz, 1H), 7.63 (dd, J = 8.4 Hz, 7.2 Hz, 1H), 7.76 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.8 Hz, 1H), 8.24 (dt, J = 7.8 Hz, 1.5 Hz, 1H), 8.27 (d, J = 8.4 Hz, 1H), 8.78 (dd, J = 5.1 Hz, 1.5 Hz, 1H), 9.07 (d, J = 1.5Hz, 1H), 10.63 (s, 1H), 11.47 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}ClN_3O_2$ $[M + H]^+$: calculated 352.0847 found 352.0849. HPLC purity = 99.3%.

*N-(2-((3-(trifluoromethyl)phenyl)carbamoyl)phenyl) nicotinamide (**10b**)*. White solid. Yield 63.9%. Mp: 218-220°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.36 (dd,

J = 7.8 Hz, 7.2 Hz, 1H), 7.47 (d, *J* = 7.8 Hz, 1H), 7.58-7.65 (m, 3H), 7.88 (d, *J* = 7.8 Hz, 1H), 8.00 (d, *J* = 8.4 Hz, 1H), 8.14 (s, 1H), 8.19 (d, *J* = 8.4 Hz, 1H), 8.24 (dt, *J* = 8.4 Hz, 1.8 Hz, 1H), 8.77 (dd, *J* = 5.4 Hz, 1.8 Hz, 1H), 9.08 (d, *J* = 1.8 Hz, 1H), 10.78 (s, 1H), 11.30 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{15}F_3N_3O_2$ $[M + H]^+$: calculated 386.1111 found 386.1115. HPLC purity = 99.3%.

*N-(2-((3-bromophenyl)carbamoyl)phenyl) nicotinamide (**10c**)*. White solid. Yield 51.3%. Mp: 199-201°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 7.30-7.36 (m, 3H), 7.60 (dd, *J* = 7.8 Hz, 4.8 Hz, 1H), 7.64 (td, *J* = 8.1 Hz, 1.8 Hz, 1H), 7.69 (dd, *J* = 7.2 Hz, 1.8 Hz, 1H), 7.86 (d, *J* = 8.1Hz, 1H), 8.02 (s, 1H), 8.21 (d, *J* = 8.1 Hz, 1H), 8.25 (dt, *J* = 7.8 Hz, 1.8 Hz, 1H), 8.78 (dd, *J* = 4.8 Hz, 1.8 Hz, 1H), 9.08 (d, *J* = 1.8 Hz, 1H), 10.64 (s, 1H), 11.53 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{15}BrN_3O_2$ $[M + H]^+$: calculated 396.0342 found 396.0348. HPLC purity = 95.1%.

*N-(2-((3-hydroxyphenyl)carbamoyl)phenyl) nicotinamide (**10d**)*. White solid. Yield 52.2%. Mp: 207-210°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 6.53 (dt, *J* = 7.2 Hz, 1.8 Hz, 1H), 7.09-7.14 (m, 2H), 7.30-7.34 (m, 2H), 7.60-7.64 (m, 2H), 7.88 (d, *J* = 7.8 Hz, 1H), 8.25 (dt, *J* = 7.8 Hz, 1.8 Hz, 1H), 8.33 (d, *J* = 7.8 Hz, 1H), 8.79 (dd, *J* = 4.8 Hz, 1.8 Hz, 1H), 9.08 (d, *J* = 1.8 Hz, 1H), 9.45 (s, 1H), 10.41(s, 1H), 11.61 (s, 1H). HRMS (ESI) m/z for $C_{19}H_{16}N_3O_3$ $[M + H]^+$: calculated 334.1186 found 334.1189. HPLC purity = 96.2%.

General procedures for the preparation of target compound *N*-substituted phenyl-2-((2-hydroxy-5-nitrobenzyl)amino)benzamide 11

To a solution of compound **4** (0.75 mmol) and acetic acid (50 µL, 2.9 mmol) in methanol (10 mL), 2-hydroxy-5-nitrobenzaldehyde (0.17 g, 1 mmol) was added and stirred at ambient temperature for 8 h. The resulting reaction mixtures were treated with 1.0 mL of a solution of $NaCNBH_3$ in methyl alcohol (10 mL) and stirred for a further 24 h at ambient temperature. The solvent was evaporated under reduced pressure and the products were diluted with dichloromethane (15 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 10 mL) followed by saturated NaCl (2 × 10 mL). The organic layer was dried over Na_2SO_4 and filtered. The crude product was purified by column chromatography to afford **11**.

*N-(4-chlorophenyl)-2-((2-hydroxy-5-nitrobenzyl) amino)benzamide (**11a**)*. Yellow solid. Yield 52.7%. Mp: 168-169°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 4.40 (d, *J* = 5.4 Hz, 2H), 6.63-6.67 (m, 2H), 7.01 (d, *J* = 8.7 Hz, 1H), 7.29 (dd, *J* = 8.7 Hz, 7.2 Hz, 1H), 7.40 (d, *J* = 8.7 Hz, 2H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.76(d, *J* = 8.7 Hz, 2H), 7.86 (br t, d = 5.4 Hz, 1H), 8.04 (dd, *J* = 9.0 Hz, 3.0 Hz, 1H), 8.08 (d, *J* = 3.0Hz, 1H), 10.28 (s, 1H), 11.41 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{17}ClN_3O_4$ $[M + H]^+$: calculated 398.0902 found 398.0904. HPLC purity = 98.0%.

*N-(3-bromophenyl)-2-((2-hydroxy-5-nitrobenzyl) amino)benzamide (**11b**)*. Yellow solid. Yield 55.6%. Mp: 146-149°C. ^{1}H-NMR (600 MHz, DMSO-d_6) δ 4.41 (d, *J* = 6.0 Hz, 2H), 6.63 (d, *J* = 8.7 Hz, H), 6.66 (dd, *J* = 7.8 Hz, 7.2 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 1H), 7.27-7.33 (m, 3H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.69 (d, *J* = 7.8 Hz, 1H), 7.87 (br t, *J* = 6.0 Hz, 1H), 8.05 (dd, *J* = 8.7 Hz, 2.4 Hz, 1H), 8.08 (d, *J* = 2.4 Hz, 1H), 8.10 (s, 1H), 10.30 (s, 1H), 11.41 (s, 1H). HRMS (ESI) m/z for $C_{20}H_{17}BrN_3O_4$ $[M + H]^+$: calculated 442.0397 found 442.0399. HPLC purity = 96.8%.

Original Article

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Use of silkworms to evaluate the pathogenicity of bacteria attached to cedar pollen

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ABSTRACT: Injection of a Japanese cedar pollen suspension into silkworm hemolymph kills the silkworms. A certain species of bacteria proliferated in the hemolymph of the dead silkworms. A 16S rDNA analysis demonstrated that the proliferating bacteria were *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacillus amyloliquefaciens*. Among them, *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* exhibited hemolysis against sheep red blood cells and were lethal to mice. A culture filtrate of *B. amyloliquefaciens* showed enzyme activity toward the pectic membrane of cedar pollen. These results suggest that silkworms as an animal model are useful for evaluating the pathogenicity of bacteria attached to cedar pollen.

Keywords: Silkworm, cedar pollen, bacillus, pathogenicity, cedar pollinosis

1. Introduction

Cedar pollinosis is one of the most prevalent causes of allergic rhinitis in Japan, causing many patients to suffer from runny nose, sneezing, and fatigue. Antigenic proteins inside cedar pollen, Cry j 1 and Cry j 2, are considered to cause pollinosis by promoting antigen-antibody reactions in the human nasal cavity (*1-3*). Immunoglobulin E antibody titers in the blood of patients with pollinosis are not related to the severity of clinical symptoms (*4*), suggesting that unknown factors determine the severity of pollinosis. These factors and their functions remain to be elucidated. We previously reported that cedar pollen undergoes morphologic changes in weak alkaline solution. Breaking down of the external walls is followed by expansion of the pectic membrane (*5*). Because the pH in the nasal cavity of patients with allergic rhinitis is higher than 8 (*6,7*), cedar pollen can be expected to undergo morphologic changes in the nasal cavity. The mechanism of destruction of the pectic membrane of cedar pollen and the release of antigenic proteins in the human nasal cavity, however, remain unclear. Bacteria and fungi are attached to pollen of various plants (*8,9*). Thus, we hypothesized the presence of bacteria on cedar pollen that may have a role in pollinosis.

We previously reported that silkworms were killed when injected with bacteria pathogenic to mammals such as *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Vibrio cholerae* (*10*). We also reported that pathogenicities of bacteria from soil and fish could be evaluated in silkworms (*11,12*). Silkworms as experimental model animals have several advantages: low rearing costs, fewer ethical issues regarding experimentation than vertebrate animals, and culturally established rearing method. For these reasons, we considered that silkworms were highly suitable for evaluating pathogenic microbes from the environment. In the present study, we attempted to isolate pathogenic bacteria from silkworms killed by the injection of cedar pollen into the hemolymph. This is the first report that pathogenic bacteria attached to pollen could be evaluated using silkworms.

2. Materials and Methods

2.1. *Animals*

Silkworms eggs (Hu•Yo × Tsukuba•Ne) were purchased from Ehime Sansyu (Ehime, Japan). The hatched larvae were raised to fourth-instar larvae with artificial diets and the fifth-instar larvae were fed antibiotic-free food for 1 day and then used for infection experiments. ICR mice (4 weeks old, female) were purchased from CLEA (Tokyo, Japan). All mouse protocols followed the Regulations for Animal Care and Use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (p24-49).

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2.2. *Cedar pollen injection into silkworms*

Japanese cedar pollen was collected from four different regions in Japan (Oita, Ibaraki, Chiba, and Tottori) in March in 2012 and 2013, dried, and preserved at 4˚C (ITEA, Tokyo, Japan). Cedar pollen (0.1 g) was suspended in 2 mL of 50 mM Tris/HCl (pH 8) buffer to promote the release of the external shell followed by expansion of the pectic membrane (*5*). Immediately or after 4 days of incubation of the cedar samples at 30˚C, aliquots of 0.05 mL were injected into silkworms, and the injected silkworms were reared at 27°C.

2.3. *Isolation of bacteria from silkworm*

Silkworms killed by the injection of bacteria were cut with dissecting scissors. The body fluid was spread onto Brain Heart Infusion (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) agar plates with an inoculating loop, and the plates were incubated at 30°C to isolate colonies.

2.4. *Colony count and determination of LD_{50} against silkworms*

Single colonies of bacteria isolated from silkworms were incubated in 1 mL of Brain Heart Infusion broth at 30°C. One hundred microliters of $10^{-4} \sim 10^{-7}$ diluted full growth samples were spread onto Brain Heart Infusion agar plates and the plates were incubated at 30°C for 12 h. The bacterial numbers in full growth were determined based on the number of colonies appearing on the plates. To determine the number of bacteria needed to kill 50% of the silkworms (LD_{50}), 0.05 mL of diluted sample was injected into the silkworm hemolymph, and silkworms were reared for 2 days.

2.5. *Determination of bacterial species*

Gram staining of the bacteria isolated from silkworms was performed using a Gram staining kit (Merck, Darmstadt, Germany) and the stained bacteria were observed under a microscope. The 16S rDNA regions were amplified by colony polymerase chain reaction (30 cycles; 94°C 15 sec, 50°C 30 sec, 68°C 1 min) using DNA polymerase KOD Fx Neo (Toyobo, Tokyo, Japan) and primer pairs U1(5'-CCAGCAGCCGCGGTAATACG-3'), U2(5'-ATCGGCTACCTTGTTACGACTTC-3'), and 9F(5'-GAGTTTGATCCTGGCTCAG-3'), 1541R(5'-AAGGAGGTGATCCAGCC-3'). The amplified DNA was separated by 1% agar gel electrophoresis, and extracted from gel using a DNA gel extraction kit (QIAGEN, The Netherlands). A cycle sequence reaction (25 cycles; 96°C 10 sec, 50°C 5 sec, 60°C 4 min) was conducted using a Big Dye Terminator (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and primers as mentioned above. DNA sequences were analyzed using a 3130xl Genetic Analyser (Applied Biosystems by Life Technologies). A BLAST search against 16S rDNA sequences of more than 400 bp was performed to identify the bacterial species. The bacterial species were identified based on sequences that matched with over 99% identity.

2.6. *Assay of digestion enzymes on pectic membrane*

Cedar pollen was suspended in 50 mM Tris/HCl (pH 8) buffer to promote the expansion of the pectic membrane. Bacteria were cultured in Brain Heart Infusion broth overnight, centrifuged to obtain supernatants, which were then filtered through a 0.22-µm Filter Unit (Millipore, Billerica, MA, USA). The filtrate (200 µL) was added to cedar pollen samples (200 µL). After incubation at 30°C for 12 h, the pectic membrane of the cedar pollen was observed under a microscope. Pectinase (11 units) from *Aspergillus niger* (Sigma-Aldrich, Steinheim, Germany) was used as a control.

2.7. *Hemolysin production assay*

Bacterial full growth was spread onto EHT agar plates (Kyokuto, Tokyo, Japan) and was incubated at 30°C for 12 h. When a transparent region appeared around colonies, the bacteria were judged to produce hemolysin.

2.8. *Pathogenicity against mice of bacteria obtained from cedar pollen*

Overnight cultures of the bacteria in Brain Heart Infusion broth at 30°C were washed with saline. Aliquots (500 µL) were injected into the mouse peritoneal cavity (n = 3). The number of mice alive after 5 days was monitored.

3. Results

3.1. *Killing of silkworms by cedar pollen injection*

Cedar pollen collected from four prefectures in Japan (Oita, Ibaraki, Chiba, Tottori) was suspended in a weak alkaline solution and injected into silkworms. Of 20 silkworms, 5 were killed in 3 days when injected with cedar pollen preserved at 4°C for 1 year after collection. Of 47 silkworms, 15 were killed when injected with cedar pollen stored for 2 months. In both cases, almost 30% of the silkworms were killed, suggesting that storage of the cedar pollen did not affect the silkworm killing effects. Furthermore, 35 of 36 silkworms were killed when injected with cedar pollen suspension incubated at 30°C for 4 days. These findings indicate that incubation of cedar pollen samples increases their lethality to silkworms.

Next, we attempted to isolate germs from the

silkworms killed by cedar pollen injection. We collected the body fluid from dead silkworms and spread it onto Brain Heart Infusion agar plates. After incubation at 30°C for 12 h, one or two species of numerous colonies appeared. Colonies were not observed when using body fluid from silkworms injected with control buffer without cedar pollen. All germ colonies reached full growth in Brain Heart Infusion broth after overnight incubation. All silkworms (n = 5) were killed within 20 h when the full growth (0.05 mL) was injected, suggesting that the microorganisms isolated on agar plates caused the killing effects on silkworms.

Aliquots of 100 µL of 0.05 g/mL cedar pollen suspension yielded 0 to 6 colonies when spread onto Brain Heart Infusion agar plates. In our protocol, the amount of cedar pollen injected to silkworms was 0.0025 g, thus the number of germs administered was less than 5, suggesting that a very small number of germs was sufficient to kill the silkworms. Therefore, we attempted to determine the LD_{50} of separated germs against silkworms. The LD_{50} of all separated germs against silkworms was less than 170 (cfu/larvae) (Table 1).

3.2. *Identification of isolated bacterium*

We attempted to identify the species of microorganisms that killed the silkworms. First, we performed ampicillin and ketoconazol sensitivity test to determine whether the germs were bacteria or fungi. All germs were sensitive to 12.5 µg/mL ampicillin and tolerant to 25 µg/mL ketoconazol. Therefore, all separated germs were considered to be bacteria. Furthermore, the results of the Gram staining demonstrated that all germs were Gram-positive, rod-shaped bacteria.

Next, we determined the 16S rDNA sequence to identify the species of the separated bacteria. These bacteria were identified as *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacillus amyloliquefaciens* (Table 1). Therefore, all bacteria that attached to cedar pollen and that showed high pathogenicity against silkworms belonged to *Bacillus* sp.

3.3. *Production of hemolysin and pathogenicity of separated bacteria against mice*

We previously reported that bacteria such as *Staphylococcus* sp., *Proteus* sp., and *Morganella* sp., which were isolated from soil and fish, exhibited pathogenicity against both silkworms and mice (*11,12*). Thus, we considered that the bacteria isolated from cedar pollen that showed high pathogenicity against silkworms would also exhibit pathogenicity against mice as well. To test this, we first examined whether the separated bacteria showed hemolytic activity on sheep red blood cells. *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* separated from cedar pollen all formed colonies on EHT agar plates with a transparent region around them. These findings suggest that these *Bacillus* sp. bacteria produce hemolysin against sheep red blood cells (Table 1).

Next, these germs were injected into the mouse peritoneal cavity to evaluate their pathogenicity against mammals. Three mice per group were injected with *B. cereus*, *B. thuringiensis*, or *B. weihenstephanensis*, and at least two mice died within 7 h in each group, whereas none of the mice injected with saline was killed. These results suggest that *B. cereus*, *B. thuringiensis*, and *B. weihenstephanensis* isolated from cedar pollen were all pathogenic against mice (Table 1).

Table 1. *Bacillus* sp. bacteria isolated from cedar pollen

Colony Number	Bacterial specieces	Locality	Date of collection	LD_{50} against silkworms (cells/larva)	Hemolysis against sheep red blood cells	Lethality in mice	Destructive activity against the pectic membrane
1	*Bacillus amyloliquefaciens*	Oita	March 2012	170	–	0/3	+
2	*Bacillus thuringiensis*	Ibaraki	March 2012	12	+	2/3	–
3	*Bacillus thuringiensis*	Chiba	March 2012	1.9	+	3/3	–
4	*Bacillus weihenstephanensis*	Totoori	March 2012	1.3	+	2/3	–
5	*Bacillus weihenstephanensis*	Totoori	March 2012	2.3	+	2/3	–
6	*Bacillus weihenstephanensis*	Totoori	March 2012	0.6	+	3/3	–
7	*Bacillus weihenstephanensis*	Totoori	March 2013	8.0	+	3/3	–
8	*Bacillus cereus*	Chiba	March 2012	26	+	3/3	–
9	*Bacillus cereus*	Oita	March 2012	0.9	+	2/3	–
10	*Bacillus cereus*	Oita	March 2013	0.5	+	3/3	–
11	*Bacillus cereus*	Ibaraki	March 2013	7.7	+	3/3	–
12	*Bacillus cereus*	Ibaraki	March 2013	2.5	+	3/3	–
13	*Bacillus cereus or Bacillus thuringiensis*	Chiba	March 2013	3.9	+	3/3	–
14	*Bacillus cereus or Bacillus thuringiensis*	Tottori	March 2013	2.6	+	3/3	–

Properties of *Bacillus* bacteria isolated from cedar pollen are presented. The species of bacteria were determined by 16S rDNA sequencing. The LD_{50} against silkworms, hemolysis against sheep red blood cells, lethality in mice, and destructive activity against the pectic membrane in culture filtrate are shown.

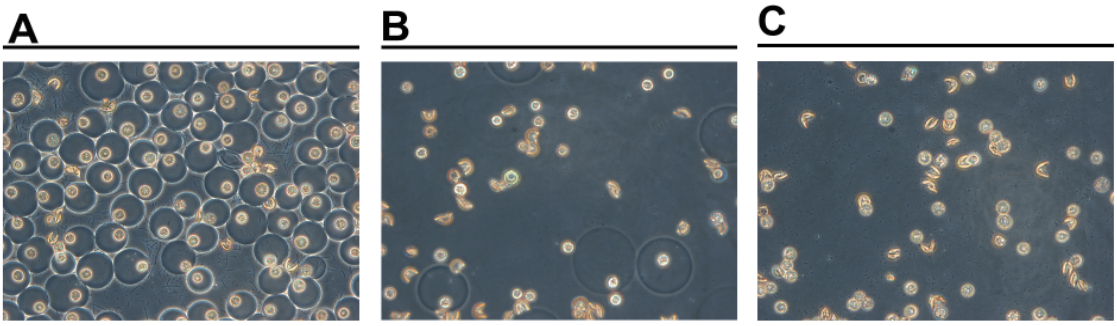

Figure 1. Phase-contrast microscope pictures of cedar pollen suspended in weak alkaline solution. Cedar pollen was incubated with BHI broth **(A)**, culture filtrate of *Bacillus amyloliquefaciens* **(B)**, or pectinase from *Aspergillus niger* **(C)** in a weak alkaline solution at 30°C for 12 h, and was observed under a microscope.

3.4. *Digestion activity in a bacterial culture against the pectic membrane of cedar pollen*

We examined whether bacteria isolated from cedar pollen secrete digestion enzymes against the pectic membrane of cedar pollen. Bacterial cultures were centrifuged and filtered, and each sample was incubated with cedar pollen suspended in a weak alkaline solution for 12 h at 30°C. The pectic membrane of cedar pollen disappeared after incubation with the sample from *B. amyloliquefaciens*, indicating that the cultural supernatant of *B. amiloliquefaciens* has enzymatic activity that digests the pectic membrane of cedar pollen (Figure 1).

4. Discussion

4.1. *Isolation of Bacillus sp. bacteria from cedar pollen*

Silkworms injected with cedar pollen were killed and four species of *Bacillus* sp. bacteria were isolated from the body fluid of the dead silkworms. Bacteria in genus *Bacillus* form spores, thus it is considered that *Bacillus* spores were attached to cedar pollen. The lack of difference in the mortality of cedar pollen preserved for 1 year or 2 months supports the notion that bacterial spores attached to cedar pollen killed the silkworms. Among many species of bacteria attached to cedar pollen, bacteria with the strongest pathogenicity might proliferate in the silkworm body fluid. Therefore, we conclude that *Bacillus* spores attached to cedar pollen proliferated in the body fluid of silkworms, resulting in the death of the silkworms.

It is uncertain when and how the *Bacillus* spores attach to the cedar pollen. Fungi proliferate in the stamens of cedar (*9*), thus it is highly conceivable that bacteria had already attached to the pollen before collection. The finding that all cedar pollen collected from four different prefectures in Japan contained the attached *Bacillus* spores supports the notion that the attachment of *Bacillus* spores to cedar pollen is a universal phenomenon. It is therefore likely that *Bacillus* spores are attached to cedar pollen flying from forest to urban areas.

4.2. *Evaluation of Bacillus pathogenicity using silkworms*

We previously reported that the LD_{50} against silkworms of bacteria separated from soil and fish was $10^4 \sim 10^8$ cfu (*11,12*). In contrast, the LD_{50} of bacteria separated from cedar pollen was much lower, suggesting high pathogenicities among environmental bacteria. Invertebrate animals such as silkworms possess no antibodies, and therefore, have no acquired immunity system. Invertebrate animals combat against invading pathogens by relying solely on their natural immunity systems (*13*). Innate immunity systems in invertebrates are highly conserved in mammals. Therefore, we speculate that pathogenic bacteria against silkworms isolated from cedar pollen can tolerate attacks by innate immune systems of mammalian hosts.

B. cereus, *B. thuringiensis*, and *B. weihenstephanensis* isolated from cedar pollen produce hemolycin on agar plates. Also, these *Bacillus* sp. bacteria are lethal in mice when injected into the mouse peritoneal cavities. The findings suggest that these bacteria are pathogenic to mammals as well as to silkworms. By monitoring pathogenicity against silkworms, we demonstrated that 3 of 4 bacterial species isolated from cedar pollen were pathogenic in mice. Therefore, silkworms can be considered a useful animal model for easy and efficient evaluation of the pathogenicity of environmental bacteria against mammals.

4.3. *Digestion of the pollen pectic membrane by pectinase produced by bacteria*

Here we demonstrated that the culture supernatant of *B. amyloliquefaciens* isolated from cedar pollen digested the pectic membrane of cedar pollen. This finding suggests that this bacterium secretes pectinase. We previously reported that cedar pollen undergoes morphologic changes, release of external walls, and expansion of pectic membrane when suspended in a weak alkaline solution (*5*). Nasal cavities of human patients with allergic rhinitis are reported to have an alkaline pH (*6,7*). Therefore, cedar pollen that invade the nasal cavity undergoes morphologic changes, as

mentioned above. Digestion of the pectic membrane by pectinase secreted from bacteria may cause the release of antigenic proteins Cry j 1 and Cry j 2, which cause allergic reactions. Thus, the removal of pectinase-producing bacteria or the administration of pectinase inhibitors can contribute to preventing cedar pollinosis.

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Pharmacokinetics and safety of single-dose ribavirin in patients with chronic renal impairment

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ABSTRACT: This open-label study assessed the pharmacokinetics of a single 400-mg oral dose of ribavirin in 6 healthy volunteers and 18 subjects with varying degrees of renal impairment (mild: creatinine clearance [CLcr] 61-90 mL/min/1.73m^2, moderate: CLcr 31-60 mL/min/1.73m^2, severe: CLcr 10-30 mL/min/1.73m^2, *n* = 6 in each group). Blood and urine samples were collected pre-dose and up to 168 hours post-dose for pharmacokinetic analyses. Compared with control subjects, ribavirin area under the plasma concentration-time curve from time zero to the time of the final quantifiable sample (AUC_{tf}) and maximum plasma concentration (C_{max}) values were increased, and apparent clearance (CL/F), clearance (CLr), and amount excreted (A_e) values were reduced in subjects with renal impairment. Mean ribavirin AUC_{tf} was increased 2- to 3-fold in patients with moderate-severe renal impairment compared with control subjects. Ribavirin CL/F and CLr were significantly correlated with CLcr. Single-dose ribavirin was safe and well tolerated in all subjects. The pharmacokinetics of ribavirin were substantially altered in subjects with stable chronic renal impairment, possibly reflecting changes in ribavirin metabolism associated with renal impairment.

Keywords: Creatinine clearance, bioavailability, excretion

1. Introduction

Ribavirin is a broad-spectrum antiviral agent that is active against a number of RNA and DNA viruses, including hepatitis C virus (HCV) (*1-6*). In patients with chronic hepatitis C, the addition of ribavirin to interferon (IFN) α or pegylated interferon (PEG-IFN) α therapies significantly improves efficacy compared with IFN-based monotherapy regimens, and sustained virologic response rates of 52-56% have been reported in patients receiving PEG-IFN α/ribavirin combination therapy (*7,8*). Ribavirin also forms a component of current protease inhibitor-base triple-therapy regimens with boceprevir or telaprevir (*9-12*).

The single-dose pharmacokinetics of ribavirin have been described in healthy volunteers and in patients with compensated liver disease (*13-17*). These studies reported that ribavirin elimination occurs by both renal and hepatic pathways, with renal processes accounting for only 5-15% of total elimination (*13,15,17,18*). Although renal excretion accounts for a low proportion of total elimination, ribavirin is known to accumulate in patients with renal failure and is not removed by hemodialysis (*19,20*).

Ribavirin treatment is associated with a well-described profile of adverse events, most notably hemolytic anemia, which necessitates hemoglobin monitoring and often results in ribavirin dose modification or use of erythropoietin (*21*). Given the potentially important effects that chronic renal insufficiency has on the pharmacokinetics of ribavirin, this study was conducted to determine the pharmacokinetics, safety, and tolerability of single oral doses of ribavirin in subjects with normal renal function and in those with varying degrees of stable chronic renal insufficiency.

2. Materials and Methods

This was an open-label, parallel-group, single-dose study, to assess the pharmacokinetic properties of a single oral 400-mg dose of ribavirin. All medication was provided by Schering-Plough (batch 36524-068; Kenilworth, NJ, USA). This study was conducted in accordance with Principles of Good Clinical Practice and the Declaration of Helsinki. All subjects provided written informed consent to participate in this study, and the protocol was approved by the Research Consultants Review Committee.

2.1. *Study population*

Subjects (both males and females), aged 18 to 65 years, with normal renal function or varying degrees of stable

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chronic renal insufficiency were eligible for enrollment. Subjects with normal renal function (creatinine clearance [CLcr] > 90 mL/min) were excluded if they had a history of cardiovascular, neurologic, hematologic, gastrointestinal, cerebrovascular, respiratory, hepatic, or renal disease, or any other disorder requiring physician care. Subjects with evidence of HIV or hepatitis B coinfection, or urinary traces of drugs of abuse, were also excluded. Subjects with compromised renal function (CLcr < 90 mL/min) were excluded if they had significant medical disorders unrelated to their renal disorder that would significantly interfere with their ability to participate in the study. None of the subjects with compromised renal function had chronic hepatitis C infection. For analysis, participants were divided into four groups according to CLcr (based on a 24-hour urinary collection): group I, CLcr > 90 mL/min/1.73 m^2 (normal), group II, CLcr 61-90 mL/min/1.73 m^2 (mild), group III, CLcr 31-60 mL/min/1.73 m^2 (moderate), and group IV, CLcr 10-30 mL/min/1.73 m^2 (severe). CLcr was determined using the Cockcroft and Gault equation (*22*).

2.2. *Study design*

Participants were fasted overnight and then received a single oral 400-mg dose of ribavirin (2 × 200 mg capsules) with 200 mL of water. Participants continued fasting until 4 hours post-dose. Subjects were discharged from the study center after 48 hours, and subsequent samples were collected on an outpatient basis. Blood samples for determination of plasma ribavirin concentrations were obtained immediately prior to drug administration, then at specified time intervals until 168 hours post-dose. Samples were collected into a syringe. The needle was removed and the contents of the syringe gently placed into a heparinized vacutainer tube that had its top removed. The sample was gently mixed and stored on ice until centrifuged at 4°C at 1,000 rpm for 15 minutes. The plasma was separated and frozen at –80°C until assayed. Block urine samples were collected just prior to drug administration and at 12- to 24-hour intervals until 168 hours post-dose to measure renal clearance of ribavirin. Samples obtained during each collection period were refrigerated. After measuring total volume, a 25-mL aliquot from each block sample was frozen at –80°C until assayed. Plasma concentrations of ribavirin were determined using a high-performance liquid chromatography/mass spectrometric method validated with respect to linearity, precision, accuracy, limit of quantification (LOQ; 50 ng/mL), and selectivity. Urine concentrations of ribavirin were determined using a high-performance liquid chromatographic-mass spectrometric method, validated with respect to linearity, precision, accuracy, LOQ (250 ng/mL), and selectivity. In brief, solid-phase extraction was performed on samples using phenylboronic acid columns, after addition of the internal standard ($^{13}C_3$-ribavirin) and di-ammonium hydrogen orthophosphate buffer. Columns were eluted with a solution of formic acid in 50/50 methanol/water (v/v). The resulting eluate was injected into a Hewlett-Packard 1090 Series II HPLC (Palo Alto, CA, USA) with a SCIEX API 300 MS/MS detector (Framingham, MA, USA), fitted with a hypersil 3.0 × 4.6 mm analytical column (3-µm particle size). The mobile phase consisted of acetonitrile (82%) and ammonium acetate (18%). Assay precision and bias were less than 11%, respectively, for all samples.

Safety was assessed based on the results of vital signs, which were measured at screening, immediately prior to dosing (0 hour), and then at regular intervals until 168 hours post-dose. Electrocardiograms and laboratory assessments were obtained at screening and 168 hours post-dosing. Adverse events were assessed according to the Common Toxicity Criteria (CTC) grading system.

2.3. *Pharmacokinetic analysis*

Plasma and urine ribavirin concentrations above the LOQ (plasma 50 ng/mL; urine 250 ng/mL) were used to calculate pharmacokinetic parameters using model-independent methods (*23*). The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were the observed values. The area under the plasma concentration-time curve from time zero to the time of the final quantifiable sample (AUC_{tf}) was calculated using the linear trapezoidal method. Individual terminal rate constants could not be determined with precision, therefore the elimination half-life ($t_{½}$) and AUC_I were not reported. Pharmacokinetic analysis is consequently limited to estimation of the individual AUC_{tf} value instead of the AUC_I. Apparent plasma clearance (CL/F) was calculated by dividing dose by the AUC_{tf}. Clearance (CLr) was calculated by dividing the amount excreted (A_e) in the urine from time 0 to 168 hours by the AUC_{tf}.

2.4. *Statistical analyses*

Summary statistics were determined for the pharmacokinetic parameters of each group. Analyses of variance were used to extract group effects for AUC and C_{max} in the original and log-transformed scales, and for T_{max} in the original scale. All pairwise contrasts were presented without adjustment for multiple comparisons, and were based on residual errors from the analyses of variance. Linear regression analyses were performed to evaluate the association between CL/F and CLr with CLcr.

3. Results

3.1. *Patient characteristics*

Twenty-four participants were enrolled. Six subjects had

Table 1. Demographic and baseline characteristics of 24 participants

	Group I (*n* = 6)	Group II (*n* = 6)	Group III (*n* = 6)	Group IV (*n* = 6)
Mean age, y (range)	35.5 (21-51)	49.3 (30-64)	46.0 (27-64)	39.3 (28-52)
Female/male (*n*)	3/3	2/4	4/2	2/4
Race (*n*)				
White	6	4	3	4
Black	0	1	1	1
Hispanic	0	1	2	1
Mean weight, kg (range)	77.5 (65-90)	78.8 (70-94)	78.7 (57-96)	74.7 (50-94)
Mean height, cm (range)	173.8 (160-186)	172.7 (168-180)	167.3 (157-187)	168.8 (157-180)

Table 2. Ribavirin pharmacokinetic parameters after a single oral 400-mg dose in patients with varying degrees of renal insufficiency

Parameter, mean (%CV)	Group I (*n* = 6)	Group II (*n* = 6)	Group III (*n* = 6)	Group IV (*n* = 6)
CLcr (mL/min)	142 (36)	74 (11)	50 (22)	18 (21)
C_{max} (ng/mL)	630 (64)	821 (48)	732 (63)	1,161 (29)
T_{max} (h)	1.5 (37)	2.0 (55)	1.2 (35)	2.2 (45)
AUC_{tf} (ng•h/mL)	9,646 (57)	17,451 (44)	20,413 (54)	31,687 (19)
CL/F (mL/min)	887 (50)	497 (71)	403 (49)	216 (17)
CLr (mL/min)	129 (33)	71.8 (55)	35.8 (58)	11.6 (31)
A_e (0-168 h; mg)	65.7 (40)	61.0 (21)	34.9 (23)	21.3 (18)

Data indicate mean (% coefficient of variation).
Abbreviations: AUC, area under the serum concentration-time curve from time zero to time of final quantifiable sample; A_e, amount excreted in urine; CL/F, total body clearance; CLr, renal clearance; CLcr, creatinine clearance; C_{max}, peak serum concentration; T_{max}, time of peak serum concentration.

normal renal function and 18 had varying degrees of renal impairment (Table 1). Data from all subjects were included in the pharmacokinetic and safety analyses.

3.2. *Pharmacokinetics*

Mean T_{max} values were similar between all four groups, ranging between 1.2 and 2.2 hours, indicating rapid absorption of ribavirin. There were trends for higher C_{max} and AUC_{tf} values in groups with more severe renal dysfunction (Table 2). AUC_{tf} was approximately 2-fold higher in subjects in group III, and approximately 3-fold higher in subjects in group IV, than in subjects with normal renal function (20,413 ng·hr/mL and 31,687 ng·hr/mL, respectively *vs*. 9,646 ng·hr/mL). The differences for AUC were significant in both the original and log-transformed scales ($p < 0.05$). However, there was considerable overlap between the individual AUC_{tf} values among groups I-III (Figure 1). Intergroup differences in C_{max} were not significant. Ribavirin concentration-time profiles are shown in Figure 2. There were prolonged terminal elimination phases in groups II-IV, with higher concentrations seen in groups with greater levels of renal dysfunction.

Both ribavirin CL/F and CLr decreased with declining renal function, with mean CL/F reduced by ~50% in group III and 75% in group IV compared with subjects with normal renal function. Regression of CL/F and CLcr showed the following statistically significant relationship: $CL/F = 153 + (4.89 \times CLcr)$ ($r^2 = 0.47$, $p < 0.05$). Regression of CLr against CLcr was also highly statistically significant ($CLr = 1.23 + (0.86 \times CLcr)$; $r^2 = 0.70$, $p < 0.001$).

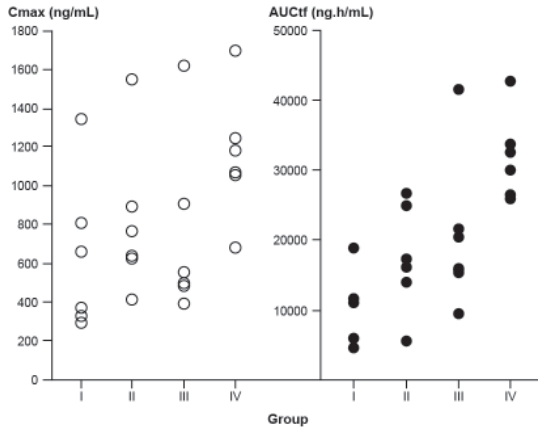

Figure 1. Individual patient ribavirin C_{max} and AUC_{tf} values for groups I-IV.

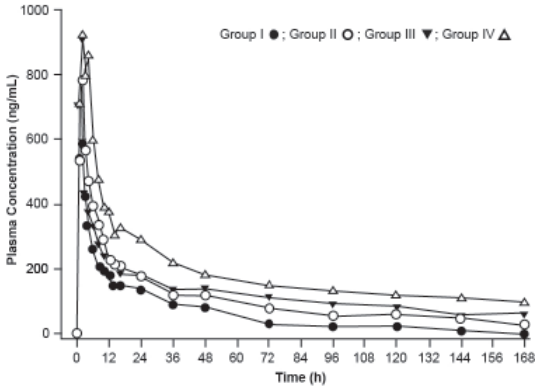

Figure 2. Mean ribavirin concentration-time profiles following administration of a single oral 400-mg dose. Group I (●); group II (○); group III (▼); group IV (△).

3.3. *Safety*

Ribavirin was safe and well tolerated when administered as a single oral 400-mg dose to subjects with renal impairment. Twenty-three adverse events were reported by 14 of 24 participants, the most common being headache (6 reports), nausea (3 reports), and fatigue, dizziness, and musculoskeletal pain (2 reports each). The majority of adverse events were mild and transient. Severe viral gastroenteritis and pneumonia were each reported in one patient; however, both were considered unrelated to study medication. Apart from expected laboratory test abnormalities in subjects with renal dysfunction in groups II to IV, there were no changes of clinical relevance noted during this study.

4. Discussion

The main finding of this study was that ribavirin exposure is significantly increased in patients with renal impairment. The safety and tolerability of a single oral dose of ribavirin were acceptable in all subjects.

Ribavirin is eliminated by both renal and hepatic routes, with gastrointestinal metabolism accounting for the majority of first-pass elimination of the parent molecule (*13,17*). Although renal excretion accounts for only 5-15% of total elimination of ribavirin (*13,18*), proportionally much greater changes in pharmacokinetic parameters were observed in patients with renal dysfunction compared with controls, and the magnitude of these changes increased with the severity of renal impairment. One possible explanation is that metabolism of ribavirin may be altered in patients with renal failure.

Although the mechanisms contributing to changes in nonrenal clearance in subjects with renal dysfunction are poorly understood, such changes are not uncommon findings with many drugs (*24,25*). Ribavirin is almost entirely absorbed after oral administration but undergoes significant first-pass metabolism, and absolute bioavailability is approximately 50% (*26*). The enzymes responsible for this process and their localization have not yet been identified; however, the site of metabolism is cytosolic and not ribosomal. Hydrolysis to form the carboxamide metabolite is one of the main metabolic pathways for ribavirin (*27*), and hydrolysis reactions are reportedly reduced in chronic renal failure (*28*).

There may be some similarities between the findings of the present study and the effect of renal impairment on didanosine pharmacokinetics (*29*). Didanosine and ribavirin are both purine nucleoside analogues, and both are substrates for the N1 nucleoside transporter. In subjects with normal function, renal clearance accounts for ~50% of didanosine's total clearance; however, in patients with end-stage renal disease, didanosine AUC was increased 4- to 5-fold (*29*). This difference was primarily due to changes in renal and nonrenal clearance estimates; it was not due to altered absolute bioavailability, and changes in volume of distribution were small (*29*). This report concluded that the pharmacokinetic changes were due to altered metabolism of didanosine associated with renal failure.

Although the present study has identified changes in ribavirin pharmacokinetics associated with reduced renal function, it has not been possible to use these data to develop dose reduction strategies for long-term use of ribavirin. The single-dose pharmacokinetics of ribavirin do not predict steady-state kinetics because of its extensive accumulation (*26*). A study in patients with chronic hepatitis C and renal impairment highlights the difficulties associated with ribavirin administration in this population (*30*). Of seven patients receiving ribavirin as a component of their antiviral medication, one had a stable ribavirin dose throughout the study, four were required to reduce their initial dose, and only two were able to increase their initial ribavirin dose. In this study, all but one patient completed treatment with ribavirin doses between 200 and 600 mg/day. In another study, Bruchfeld and colleagues examined the pharmacokinetics of ribavirin in patients undergoing treatment of HCV, either with normal renal function or with renal impairment (glomerular filtration rate (GFR) of 5-57 mL/min) (*31*). Most patients in this study were also receiving native interferon α, in line with standard HCV therapy at that time. Ribavirin pharmacokinetics were linearly dependent on renal function with a small nonrenal clearance dependent on body weight and age. Estimated GFR was a significantly better predictor of ribavirin clearance than body weight, although up to 40% of inter-individual variability in ribavirin total clearance was not explained by estimated GFR and body weight. Based on their data, the authors suggested that ribavirin dosage should be primarily based on renal function and proposed a dosing schedule based on GFR and body weight (*31*). Other groups have used pharmacokinetic monitoring of ribavirin (*32,33*), adjustment of ribavirin dose based on hemoglobin levels (*32,33*), dose titration strategies (*33,34*), or administration of erythropoietin (*34*) to titrate ribavirin levels within a therapeutic range. The prescribing information for ribavirin indicates that patients with creatinine clearance values < 50 mL/min were not included in phase 3 efficacy studies and, consequently, use of ribavirin is contraindicated in patients with CLcr < 50 mL/min (*35*). At this time it is not possible to recommend ribavirin doses for use in patients with CLcr < 50 mL/min.

Single 400-mg oral doses of ribavirin were generally safe and well tolerated in healthy volunteers with normal renal function and in patients with renal insufficiency. The most commonly reported adverse event, headache, has been reported previously following single- and multiple-dose administration of ribavirin (*14*), and no serious drug-related adverse events were reported.

In conclusion, ribavirin pharmacokinetics are substantially altered in subjects with stable chronic renal impairment compared with controls. According to prescribing information, ribavirin may be used in patients with CLcr > 50 mL/min; however, additional studies are needed to establish safe dosing regimens for patients with CLcr < 50 mL/min.

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Conflicts of interest

Drs. Gupta, Glue, and Kantesaria were employees of Schering Plough Corporation at the time this study was conducted. Dr. Glue is also an author on patent US6824768 B2. Dr. Gupta and Dr. Kantesaria have no other disclosures.

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Commentary

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Adjuvant systemic drug therapy and recurrence of hepatocellular carcinoma following curative resection

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ABSTRACT: Postoperative recurrence of hepatocellular carcinoma (HCC) has a negative impact on long-term survival. According to available evidence, many systemic untargeted agents are ineffective as adjuvant therapy to prevent the recurrence of HCC following curative resection. Interferon α has potential effectiveness as adjuvant therapy for HCC in the presence of underlying conditions such as HBV or HCV infection. Oral polyprenoic acid has also proven its effectiveness according to a prospective study; however, no other studies have reported polyprenoicacid (acyclic retinoid) to be effective. Sorafenib is the only systemic molecular targeted agent that has proven effectiveness as adjuvant therapy according to a pilot study. To date, 11 randomized clinical trials are underway with different agents as adjuvant systemic drug therapy to prevent the recurrence of HCC following curative resection according to *Clinicaltrial.gov*. Adjuvant systemic drugs may be the most promising of all adjuvant modalities in the near future since HCC may be a systemic disease rather than a local disease.

Keywords: Hepatocellular carcinoma, recurrence, adjuvant systemic drug

Hepatocellular carcinoma (HCC) is the fifth most common cancer leading to death worldwide and is estimated to cause half a million deaths annually. HCC is treated primarily by surgical curative resection. However, HCC frequently recurs postoperatively and has a negative impact on long-term survival. Most patients have intrahepatic recurrence, while a few have both intrahepatic and extrahepatic recurrence. The cumulative 5-year rate of intrahepatic recurrence is as high as 100% (*1*,*2*).

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Presumably, there are three causes of intrahepatic recurrence (*3*). Incomplete resection followed by residual tumor foci is responsible for early recurrence. Multicentric tumor may be responsible for late recurrence. Persistent viremiain dependently increases the recurrence of HCC in patients with underlying HBV and/or HCV infection, although the underlying mechanism remains unknown. Many attempts have been made to achieve better outcomes to prevent the recurrence of HCC following curative resection. In addition to surgical techniques, transcatheter arterial chemoembolization and immunotherapy have also been tried and tested, but their results were uncertain. Other approaches are systemic untargeted and molecularly targeted agents, though such agents are difficult to develop.

Unfortunately, many systemic untargeted agents are ineffective as adjuvant therapy to prevent the recurrence of HCC following curative resection according to available evidence. This is especially true for chemotherapy that was developed and tested mainly in 1990s and 2000s. For HCC with underlying conditions such as HBV or HCV infection, the potential effectiveness of interferon α as adjuvant therapy to prevent the recurrence of HCC has been proven in clinical trials and meta-analysis (*4*,*5*), although one multicenter clinical trial found it ineffective (*6*). Furthermore, a prospective clinical study and its updated analysis of the long-term follow-up data found that oral polyprenoic acid (acyclic retinoid) can prevent second primary HCC after surgical resection of original HCC since acyclic retinoid may delete malignant clones before such clones expand into detectable HCC (*7*,*8*) (Table 1). However, no other studies have reported polyprenoicacid (acyclic retinoid) to be effective.

According to currently available evidence, there is only one systemic molecular targeted agent, sorafenib (Table 1), that has proven effectiveness as adjuvant therapy for HCC to prevent early recurrence after hepatic resection (continuous sorafenib 400 mg *q.d.* for 4 months after hepatic resection). However, the study that yielded that finding was just a pilot study (*9*).

HCC is a genetically heterogeneous tumor, complicating its treatment with a single agent. Since

Table 1. Agents that are potentially effective as adjuvant systemic drugs according to current evidence

Items	Drug	Chemical formula
Untargeted agents	Interferon α	$C_{896}H_{1395}N_{245}O_{261}S_9$
	Acyclic retinoid	(2E,4E,6E,10E)-3,7,11,15-Tetramethylhexadeca-2,4,6,10,14-pentaenoic acid
Targeted agents	Sorafenib	4-[4-[[4-Chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-*N*-methyl-pyridine-2-carboxamide

Table 2. Ongoing clinical trials with adjuvant systemic drugs toprevent the recurrence of HCC according to *ClinicalTrials.gov*

Items	Drug	Trial phase	Current status	*ClinicalTrials.gov* identifier	Estimated primary completion date
Untargeted agents	Lamivudine or entecavir	IV	Unknown	NCT00768157	2009-09
	Adefovirdipivoxil and lamivudine	III	Ongoing, not recruiting	NCT00455091	2012-04
	Interferon-α-2b and ribavirin	IV	Ongoing, not recruiting	NCT00375661	2012-12
	Thymopentin	III	Unknown	NCT00460681	2012-02
	Ginsenoside Rg3 capsules	I-II	Recruiting	NCT01717066	2014-12
	Huaier granules	IV	Enrolling	NCT01770431	2014-10
Targeted agents	Sorafenib	III	Ongoing, not recruiting	NCT00692770	2014-05
	Gefitinib	II	Unknown	NCT00282100	2012-12
	PI-88	III	Recruiting	NCT01402908	2013-12
	Capecitabine	II-III	Unknown	NCT00561522	2012-07
	Tyroserleutide	III	Enrolling	NCT01489566	2013-03

sorafenib became the first agent to provide a significant improvement in overall survival and began serving as the standard treatment for patients with advanced HCC and Child-Pugh class A liver cirrhosis (*10*), several important signaling pathways of angiogenesis and proliferation have been identified in relation to hepatocarcinogenesis. These include Ras/Raf/MEK/ERK (MAPK), phosphoinositol-3 kinase (PI3k)/Akt/mTOR, hepatocyte growth factor (HGF)/c-mesenchymal epithelial transition factor (c-Met), insulin growth factor receptor, transforming growth factor-β, Wnt/β-catenin, Hedgehog and Notch (*11*). Several agents are being developed and tested in clinical trials. A phase III randomized, double-blind, placebo-controlled clinical trial (STORM) is underway to further evaluate sorafenib as adjuvant systemic drug therapy to prevent the recurrence of HCC following curative resection. This trial is active but not recruiting and is estimated to be completed by 2014-05.

Besides the STORM clinical trial, there are 10 other clinical trials with different agents as adjuvant systemic drug therapy to prevent the recurrence of HCC following curative resection (Table 2). Five agents can be categorized as systemic molecular targeted agents. One is heparanase inhibitor PI-88, which was tested in a phase III clinical trial that terminated. Another phase III clinical trial with heparanase inhibitor PI-88 is underway. Six agents can be categorized as systemic untargeted agents. Three of the six can be subcategorized as systemic antiviral agents, one can be subcategorized as a systemic immune agent, and the remaining two can be subcategorized as systemic herbal agents.

Since recurrence remains a key obstacle to a better prognosis for HCC following curative resection, a modality targeting recurrence would be crucial. To date, there are no globally accepted adjuvant systemic drugs with strong evidence of effectiveness, but adjuvant systemic drugs are being painstakingly developed and clinically tested. Adjuvant systemic drugs may be most promising of all modalities in the near future since HCC may be a systemic disease rather than a local disease.

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Commentary

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Intensive research on the prospective use of complementary and alternative medicine to treat systemic lupus erythematosus

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ABSTRACT: Traditional Chinese medicine has gained increasing acceptance worldwide as a form of complementary and alternative medicine and has been used to treat systemic lupus erythematosus (SLE) inside and outside of China. Herbal medicines are generally low in cost, plentiful, and cause very little toxicity or few adverse reactions in clinical practice. However, the mechanisms by which traditional Chinese medicine treats SLE remain unclear. The immunosuppressive properties of traditional Chinese medicines and/or immunomodulation by those medicines could play an important role in their treatment of SLE.

Keywords: Systemic lupus erythematosus, traditional Chinese medicine, immunity, integrative treatment

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease characterized by autoantibody production, complement activation, immune complex deposition, and lymphocyte proliferation that cause tissue and organ damage (*1*). SLE treatment is individualized and depends on manifestation of symptoms, organ involvement, and disease severity. Antimalarials and nonsteroidal anti-inflammatory drugs (NSAIDs) are useful in the treatment of mild symptoms. Oral corticosteroids and cytotoxic agents are used to treat more severe disease. Other medications (cyclophosphamide, immunosuppressive agents, and tacrolimus) may be used depending on the severity of disease and organ systems involved. Belimumab is approved for use in patients with mild to moderate disease currently taking standard therapy (*2*). Even so, the treatment of SLE remains a challenge today, particularly in terms of controlling the underlying disease process while at the same time preventing adverse reactions to therapy.

Western medicines such as glucocorticoids and immunosuppressants are used to suppress active immune responses, though this is only a temporary solution. However, long-term use of glucocorticoids and/or high-dose pulse therapy with immunosuppressants often leads to adverse reactions. Traditional Chinese medicine (TCM) focuses on the overall regulation of immune function by reconstructing a stable state, and it seeks to regulate Yin and Yang, Qi and blood and the function of Zang Fu internal organs by enhancing the body's defensive capabilities, improving immune function, and limiting adverse reactions. The other major principle of TCM is an emphasis on individual therapy. The diagnosis and treatment strategy may different substantially for different patients with the same type of SLE. This is called the principle of "treatment based on differentiation of symptom patterns".

2. SLE from the viewpoint of TCM

TCM views SLE as a systemic disease associated with the state of the entire body. According to TCM theory, SLE is caused by imbalances between endogenous physical conditions within the body and exogenous pathogenic factors. Those pathogenic factors for SLE, in Chinese medicine terms, include exuberant heat and toxins, a yin deficiency and interior heat, a yang deficiency in the spleen and kidney, and a qi-yin deficiency. These factors strike when a person is in a weak physical condition, without the strength to resist.

3. Possible mechanisms by which TCM treats SLE

SLE is an autoimmune disease. The immunological indicators differ due to different symptoms in patients (*3*). Many different types of herbal medicines are used to treat SLE, but few of those medicines have undergone randomized controlled trials. Each therapeutic strategy differs based on the differentiation of symptom patterns. Table 1 shows examples of compounds or extracts derived from traditional Chinese herbal medicines used to treat

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Table 1. Examples of compounds or extracts derived from traditional Chinese medicine being used to treat SLE

Main TCM herbs	Active component	*In vitro* and/or *in vivo* system	Target molecules and pathways	*Ref.*
Moutan Cortex	Paeonol	Rat	Reduces levels of TNF-α, IL-1β, and IL-6.	*9*
Moutan Cortex	Extracts	Human basophils (KU812 cells)	Suppresses the expression of ICAM-1 and the release of CCL2, CCL5, CXCL8, and IL-6.	*10*
Moutan Cortex	Extract containing paeonol and paeoniflorin	Human gingival fibroblasts	Inhibits activation of various inflammation-related genes.	*11*
Moutan Cortex	Extracts	Mouse model of type II collagen-induced arthritis (CIA)	Improves the clinical arthritis index, ameliorates the histological deformation of joints, decreases serum levels of rheumatoid arthritis biomarkers, and attenuates Th1-related responses. Suppresses the production of MMPs, TNF-α, IL-1β, IL-6, and chemokines. Suppresses the activation of NF-κB and AP-1.	*12*
Moutan Cortex	Spray-dried moutan cortex extract	Mouse peritoneal macrophage	Inhibits the expression of iNOS and TNF-α release. Blocks the activation of NF-κB.	*13*
Radix Rehmanniae	Extracts	NC/Nga mice	Reduces the total number of mast cells, CCR3(+) eosinophil immunoreaction, and total serum levels of IgE, IL-2, and IL-4.	*14*
Radix Rehmanniae	Rehmanniae polysaccharides	Ultraviolet B (UVB) ray treated mice	Increases serum levels of IL-2, IL-4, and IL-10, increases skin GSH, SOD, CAT, and GSH-Px activity, and decreases skin MDA levels.	*15*
Radix Rehmanniae	NF3, which comprises of Astragali Radix and Rehmanniae Radix in the ratio of 2:1(w/w)	Human skin fibroblast cell line Hs27	Up-regulates TGF-β1, BMP-6 synthesis, expression of type I and III collagens, fibronectin, and TIMP-1 and down-regulates MMP-9 expression in skin fibroblast cells. Regulates gene transcription for extracellular matrix synthesis *via* the Smad pathway and gene transcription for cell motility *via* the Ras/MAPK (non-Smad) pathway.	*16*
Radix Rehmanniae	2,5-Dihydroxyacetophenone (DHAP)	Mouse macrophages (RAW264.7)	Inhibits iNOS expression and NO production. Decreases levels of TNF-α and IL-6. Inhibits the phosphorylation of ERK1/2 and NF-κBp65.	*17*
Radix Glycyrrhizae	Extract	Mouse macrophages (RAW264.7)	Inhibits NO, TNF-α, IFN-γ, and IL-10 production.	*18*
Radix Glycyrrhizae	Liquiritigenin	Raw264.7 cells; rats (carrageenan-induced paw oedema)	Inhibits NF-κB DNA binding activity and iNOS expression. Suppresses the production of TNF-α, IL-1β, and IL-6. Inhibits the formation of paw edema induced by carrageenan.	*19*
Radix Glycyrrhizae	Radix Glycyrrhizae polysaccharide	Mice	Down-regulates the population of Treg cells and Foxp3 expression in Treg cells. Decreases IL-10 and TGF-β levels and increases IL-2 and IL-12p70 levels in serum.	*20*
Radix Astragali	Aqueous extract	BALB/c mice	Reduces the production of IgG2a and IgM and suppresses IL-6 production in spleen cells.	*21*
Radix Astragali	Aqueous extract	Zymosan air-pouch mice; Raw 264.7 cells	Reduces the expression of iNOS, COX-2, IL-6, IL-1β, and TNF-α. Decreases the production of NO. Attenuates the activity of p38 and Erk1/2 and stimulates MKP-1. Interferes with the translocation of NF-κB to the nucleus, subsequently results in NF-κB-dependent transcriptional repression.	*22*
Poria Cocos	PCP (an immunomodulatory protein purified from the dried sclerotium of *Poria cocos*)	RAW 264.7 macrophages cells	A potential immune stimulator. Induces TNF-α and IL-1β. Regulates NF-κB-related gene expression. Activates peritoneal cavity macrophages to induce Toll-like receptor 4 (TLR4)-mediated myeloid differentiation factor 88 (MyD88)-dependent signaling.	*23*

Table 1. continued

Main TCM herbs	Active component	*In vitro* and/or *in vivo* system	Target molecules and pathways	*Ref.*
Chinese Yam	Diosgenin	BALB/c mice	Enhances OVA-specific serum IgG2a. Increases IFN-γ secretion and mRNA expression.	*24*
Fructus Corni	Aqueous extract	RAW 264.7 macrophage cells; mice	Inhibits the expression of COX-2 and iNOS and suppresses PGE2 synthesis and NO production. Down-regulates NF-κB. Suppresses the acetic acid-induced writhing response in mice.	*25*
Fructus Corni	7-*O*-galloyl-D-sedoheptulose	Type 2 diabetic db/db mice	Modulates protein expression of NF-κBp 65, COX-2, iNOS, JNK, phospho-JNK, AP-1, TGF-β1, and fibronectin.	*26*
Artemisia Annua	Ethanol extract	Mice	Suppresses splenocyte proliferation and reduces serum IgG, IgG1, and IgG2b antibody levels.	*27*
Artemisia Annua	Artemisinin	Lupus nephritis (LN) mice	Increases the expression of GRα mRNA and transcriptional coactivator P300 300/CBP protein	*28*
Artemisia Annua	Artemisinin	RAW264.7 macrophage cells	Induces the production of IL-12p40 by inhibiting JNK activity.	*29*

Abbreviations: AP-1, activating protein-1; BMP-6, bone morphogenetic protein-6; CAT, catalase; GSH-Px, glutathione peroxidase; CC, CXC, CCL, CXCL: chemokine; CCR, chemokine receptor; COX-2, cyclooxygenase-2; GR, glucocorticoid receptor; GSH, glutathione; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDA, malonaldehyde; MKP-1, MAP kinase phosphatase-1; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; IL, interleukin; NO, nitric oxide; SOD, superoxide dismutase; TIMP-1, tissue inhibitor of metalloproteinases-1; TGF-β, transforming growth factor-β.

SLE with their mechanism of action on immunologic functions.

Many physicians are aware of the potential for better treatment of SLE by combining TCM and Western medicine. This approach to the treatment of SLE can lead to an enhanced synergistic effect and also draw on unique advantages of each form of medicine, such as preventing infection, stabilizing a patient's condition, reducing recurrence, and greatly improving the quality of life in patients with SLE. Over the past few years, TCM has made progress in treating SLE. Typically, Chinese herbal compounds are often combined with Western medicines such as corticosteroids and immunosuppressive agents in the integrative treatment of SLE in China. Several studies have examined such integrative treatment of SLE in patients. Combining the Lang-Chuang Medicinal Decoction with prednisone to treat SLE resulted in an efficacy of 93.33% (56/60) compared to 80% in the control group (40/50) (*4*). A double-blind, placebo-controlled study found that Ziyin Lupus Capsules combined with hormones were superior to hormones alone (*5*). Combining the Qingyang Toujie Mixture with prednisone tablets effectively improved the balance of Th1/Th2 cytokines and alleviated toxic and adverse reactions to hormone or immune inhibitors (*6*). Combining Qubanyangyin granules with conventional (Western) treatment improved clinical efficacy, reduced the toxic effects of conventional treatment, and decreased the rate of recurrence (*7*). Results of a double-blind, randomized controlled trial of the Dan-Chi-Liu-Wei combination (DCLWC) and conventional therapy to taper the steroid dose and prevent disease flare-ups suggested that combining DCLWC with conventional therapy to treat SLE was safe and may have a marginal effect on decreasing disease activity (*8*). Tapering of the steroid dose was not possible during the 6-month duration of the trial, and a long-term follow-up and large-scale studies are needed to confirm the effects of DCLWC.

4. Conclusion

TCM has gained increasing acceptance worldwide. Herbal medicines are generally low in cost, plentiful, and cause very little toxicity or few adverse reactions in clinical practice. Despite the vast interest and ever-increasing demand, the absence of strong evidence-based research and the lack of standardization of herbal products are the main obstacles toward the global adoption of TCM. A prescription for Chinese medicine may have multiple active ingredients delivering a comprehensive, integrated treatment of SLE *via* multiple targets and their associated pathways. If treatments are effective, then there must be underlying mechanisms that can be investigated and verified scientifically. Understanding these mechanisms can help to increase the efficacy of Chinese medicines in a logical and rational manner. Therefore, prospective randomized studies (or randomized controlled trials) in patients with SLE are needed to substantiate the use of TCM and an evidentiary basis for TCM also needs to be established as well.

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