Compound-based Chinese medicine formula: From discovery to compatibility mechanism

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Abstract

*Ethnopharmacological relevan*ce: Chinese medicine formula (CMF) has a long history of clinical use in the treatment of various diseases under the guidance of traditional Chinese medicine (TCM) theory. The application of CMF can be divided into three levels, crude extracts, homologous compounds mixture, and specific compounds. However, the modern scientific connotation of the CMF theory has not been clarified.

Aim of the review: To critically evaluate the research strategy for the investigation of compound-based CMF (CCMF).

Materials and methods: The related information was collected from the scientific databases, including CNKI, Elsevier, ScienceDirect, PubMed, SpringerLink, Web of Science, and Wiley Online.

Results: The research design including discovery, screening, optimization, pharmacodynamics models, and target research techniques including the targets for compatibility compounds were evaluated. Essentially it has been evaluated that the *in vitro* multicellular three-dimensional culture or organoid model has been proposed for the optimization model for compatibility research of CCMF. Based on these, the traditional compatibility theory of CMF, such as Monarch-Minister-Assistant-Guide (Jun-Chen-Zuo-Shi in Chinese), can probably be elucidated by the CCMF research.

Conclusions: CCMF has the clear advantage of providing the exact composition and controllable quality of modern medicines, in addition to having the characteristics of multi-ingredients and multi-targets synergistic effects of TCM. However, CCMF is still associated with challenges which need to be addressed for its future use.

Keywords: Chinese medicine formula; compound; target; compatibility; methodology

Abbreviations

Chinese medicine formula (CMF), traditional Chinese medicine (TCM), homologous compounds mixture-based CMF (HCMF), compound-based Chinese medicine formula (CCMF), traditional Chinese medicine formula (TCMF), Shexiang-Baoxin Pill (SXBXP), Feedback System Control (FSC), Cellular thermal shift assay (CETSA), microscale thermophoresis (MST), Drug affinity responsive target stability (DARTS), high-throughput screening model based on cellular thermal shift assay (HTS-CETSA)

1. Introduction

As a long-standing science and culture, Chinese medicine formula (CMF) has a long history of clinical use in the treatment of various diseases for thousands of years, and has contributed to the prosperity and civilization of the China and the surrounding countries including Japan and Korea.

In recent years, acceptance of CMF has gradually increased due to its clinical effect (Liu et al., 2015; Zhu et al., 2018). The proper compatibility of CMF requires strict guidance of traditional Chinese medicine (TCM) theory instead of the use of simply aggregated medicinal plants or compounds. "Monarch-Minister-Assistant-Guide" (Jun-Chen-Zuo-Shi in Chinese) is one of the most typical and important theories, which vividly defines the different roles of the constituents in CMF. In this theory, "Mornarch drug" stands for an essential ingredient in a prescription and its leading curative role is aimed at the cause or the main syndrome of a disease. "Minister drug" stands for the ingredient which can strengthen the curative effect of the monarch drug. "Assistant drug" mainly refers to the ingredient which can cooperate with the monarch and minister drugs and inhibit their possible side effects or toxicities. "Guiding drug" mainly refers to the ingredient which can guide other constitutents to the pathogenic sites. However, the modern scientific connotation of this theory needs to be further explored and explained.

As outlined above, CMF refers to a systemic constitution of Chinese medicines under the guidance of TCM theory for specific types of diseases. The earliest form of CMF is the compatibility of crude extracts, such as Xiao-Chaihu Dcoction composed of pieces of Chaihu (*Bupleurum chinense* DC.), Banxia (*Pinellia ternate* (Thunb.) Breit.), Renshen (*Panax ginseng* C. A. Mey.), Gancao (*Glycyrrhiza uralensis* Fisch.), Huangqin (Scutellaria baicalensis Georgi), Shengjiang (*Zingiber officinale* Rosc.), and Dazhao (*Ziziphus jujuba* Mill.), which was recorded in the book of Shanghan-Zabing-Lun written by Zhongjing Zhang 1800 year ago. Even now, the crude extracts remain the most common method of CMF application due to its several advantages, including definite and long-term proven effect (China Pharmacopoeia Committee, 2015; Liu, et al., 2015; Zhu et al., 2013; Zhu et al., 2018), relatively easier obtainment, simpler processing and lower cost than chemical agents that cannot be obtained by complete synthesis. However, the unclear chemical composition (active, ineffective and toxic ingredients), unverified functional targets, and indistinct molecular mechanisms hinder the extensive use of CMF globally. To overcome the defects of crude extracts, such as

the quality control, safety and inconvenient storage, further research of CMF needs to be undertaken.

Zhang and Wang (2005) proposed the concept of homologous compounds mixture-based CMF (HCMF), also known as component-based CMF, such as total saponins from Huangqi (*Astragalus membranaceus* (Fisch.) Bge.), which propelled the CMF research to a new level (). HCMF refers to the partially identified homologous compounds mixture derived from Chinese medicine under the compatibility principles of TCM theory (Zhang et al., 2015). Although HCMF has relatively controllable quality, safety and effectiveness, such as approved Qishen-Yiqi-Diwan, Shouwu-Danshen-Diwan, Zhigan-qin Capsule, and Sanye-Tangzhi Tablet, there still exists unascertained chemical constituents, and unclear targets or mechanisms, which result in the obstacles for successful illustration of the scientific connotation of the compatibility theory at the molecular level.

Recently, the emerging technology of protein structure elucidation and target validation methods have helped to further push the pharmacological research of CMF, especially its new form which is composed of specific compounds with clear targets and mechanisms (Wang et al., 2008; Zhang et al., 2010). In view of this, we presented the viewpoint of the promising compound-based Chinese medicine formula (CCMF). This refers to a mixture composed of specific natural compounds derived from Chinese medicines or chemical drugs, under the principles of prescription compatibility of TCM theory. CCMF not only possesses the probable advantages of enhanced efficacy and reduced toxicity in traditional Chinese medicine formula (TCMF), but also has the improved safety, reliable therapeutic effects and clear chemical composition-based stable quality. Furthermore, the therapeutic target and mechanism are most likely to be clarified at the molecular level (Wang et al., 2008; Zhang et al., 2010), and the scientific connotation may be truly revealed for the compatibility theory, hence leading to the modernization of research of TCM. Although TCMF and HCMF also exhibit the probable synergism and attenuation effects similar to CCMF, their effective constituents are yet to be identified and mechanism of action needs to be elucidated (Table 1).

Table 1 Comparison among TCMF and HCMF and CCMF

	TCMF	HCMF	CCMF
Effect	Synergism and attenuation		
Effective constituent	Unclear	Partially clear	Completely clear
Inactive substance	Inclusion	Inclusion	Exclusion
Drug target	Indeterminate	Indeterminate	Determinable
Action Mechanism	Animal level	Animal and cellular levels	Animal, cellular and molecular levels
Elucidation of scientific connotation	No	Difficult	Yes

2. Discovery of CCMF

CCMF can be obtained from the following five main sources: classic prescriptions, classic Chinese medicine pair, single Chinese medicine, HCMF, and synergistic active compounds.

2.1. Classic Prescriptions

Classic prescriptions have shown good clinical effect (Liu et al., 2015; Zhu et al., 2013; Zhu et al., 2018). These provide abundance of compounds with potential activity that can be used to comprise CCMF, such as Four-Gentlemen Decoction (Si-Jun-Zi Decoction in Chinese) with the effect of improving digestive system function, Si-Wu Decoction with the effect of improving erythropoiesis, Liuwei-Dihuang Pill traditionally used as a tonic to improve fatigue, lower back pain, menstrual symptoms and night sweats, Jingui-Shenqi Pill used as a warming enery tonic and used to treat various health conditions such as poor circulation, oedema, heart failuire and osteoporosis. The information of chemical constituents of TCMF can be obtained from the constituent analysis and related databases. Furthermore, the initial basic composition of CCMF can be obtained from high content screening of these compounds according to the original therapeutic activities of TCMF.

Similar to the classic prescriptions, Chinese patent medicines approved by the Chinse Food and Drug Administration (CFDA) for their clinical application with

satisfactory effect is another source of CCMF. Chinese patent medicine is the novel Chinese medicine formulation developed through the modern pharmaceutical technology under the TCM principle of syndrome differentiation and treatment, and thus diagnosis and treatment is based on the analysis of the illness and the patient's condition. The advantages of Chinese patent medicine are convenient use, rapid effect, and reduced side effects. For example, Shexiang-Baoxin Pill (SXBXP, China Pharmacopoeia Committee, 2015), consisting of Shexiang (*Moschus berezovskii* Flerov.), Chansu (*Bufo bufo gargarizans* cantor), Renshen (*Panax ginseng* C. A. Mey.), Suhexiang (*Liquidambar orientalis* Mill.), Niuhuang (*Bos taurus domesticus* Gmelin), Rougui (*Cinnamomum cassia* Presl) and Bingpian (borneol), has been successfully used to treat angina pectoris and myocardial infarction. Modern pharmacological studies show that SXBXP can relax blood vessels, reduce myocardial infarction, inhibit vascular calcification and promote angiogenesis (al., 2018). We previously investigated the chemical constituents of SXBXP, 57 non-volatile components were detected by liquid chromatography with diode array detection and electrospray ionisation mass spectrometry (LC-DAD-ESI-MS) and 47 of these were identified. Among them, 20 are triterpene saponins from ginseng, 18 are bufadienolides from toad venom, 5 are cholic acids from bezoar, 1 is bilirubin from bezoar including 3 other compounds (Peng et al., 2009). 49 volatile compounds were identified by gas chromatograph-mass spectrometer (GC-MS) analysis and compared with NIST05 online database. These findings provide a series of compounds which can be applied in the CCMF research based on SXBXP. Arsenic sulfide, indirubin and tanshinone IIA are the main active compounds of Realgar-Indigo naturalis formula (Zhu et al., 2018), which is a famous Chinese patent medicine and is composed of realgar, indigo naturalis, Danshen (*Salvia miltiorrhiza* Bge.) and Taizishen (*Pseudostellaria heterophylla* (Miq.) Pax ex Pax et Hoffm.). The combination use of these three compounds significantly prolongs the life span of tumor bearing mice compared to their mono- or bi-treatment, displaying probable synergistic effects (Wang et al., 2008).

2.2. Classic Chinese Medicine Pair

Chinese medicine pair is the fixed drug combination of two commonly used medicines and is the smallest unit of the TCM compatibility. Chinese medicine pair usually exhibits better effect or reduced toxicity compared to their use alone and is the

accumulation of clinical experience from ancient times (Yue et al., 2017; Ma et al., 2019). For example, Huangqi (*Astragalus membranaceus* (Fisch.) Bunge) and Danggui (*Angelica sinensis* (Oliv.) Diels) can form a classic Chinese medicine pair, which is commonly used to significantly enhance the effect of reducing oxygen consumption and resisting fatigue (Chang et al., 2018). The main active compounds in this pair includes astragaloside, formononetin, calycosin, calycosin glycoside, and ferulic acid, etc. Zhu et al. (2019) found that the combination of ferulic acid, astragaloside and formononetin can significantly improve proliferation and aging of hematopoietic stem cells, when compared with the single drug. Another example of classic Chinese medicine pair of Fuzi (*Aconitum carmichaelii* Debeaux) and Gancao (*Glycyrrhiza uralensis* Fisch.) is from the classic treatise: Shanghan-Zabing-Lun written by Zhongjing Zhang. Hypaconitine and glycyrrhetinic acid have been identified as the main effective compounds of Fuzi and Gancao, respectively, and their combination can significantly improve the injured morphology and injury-related indexes of rat myocardial H9c2 cells caused by hypoxia and glucose deficiency at a ratio 1:1 (Wang et al., 2016). These compounds from classic Chinese medicine pair can form the basic composition of CCMF.

2.3. Single Chinese Medicine

A single Chinese medicine often contains complex chemical compounds, some of which display significant compatible therapeutic effects. Yimucao (*Leonurus artemisia* (Laur.) S. Y. Hu) has heat clearing properties that may help to regulate menstruation, promote diuresis and clear toxins from the body (China Pharmacopoeia Committee, 2015) and stachydrine hydrochloride and leonurine hydrochloride are the main active constituents of Yimucao. Li et al. (2019) found that the combination of stachydrine hydrochloride (30, 45 mg/kg) and leonurine hydrochloride (15, 30 mg/kg) can significantly reduce the levels of aspartate transaminase (AST), creatine kinase (CK), CK isoenzyme-MB (CK-MB), lactate dehydrogenase (LDH), hydroxybutyrate dehydrogenase (HBDH), cardiac troponin I (cTnI), and malondialdehyde (MDA) which are serum biomarkers for myocardial injury during ischemia-reperfusion injury in rats. Furthermore the diastolic function of the heart is improved and offered protection against myocardial injury during ischemia-reperfusion, which is similar to the effects of Simvastatin tablets. Danshen (*Salvia miltiorrhiza* Bunge) can improve

blood circulation and disperse stasis, and it is also widely used to relieve menalgia. Traditionally, it is used to “clear heart-fire” and may also be used in mental-emotional conditions (China Pharmacopoeia Committee, 2015). The water soluble compounds present include protocatechuic aldehyde, protocatechuic acid, rosmarinic acid, caffeic acid, sodium danshensu, salvianolic acid A, and salvianolic acid B, etc. Tian et al. (2014) found that the combination of these seven compounds can significantly improve the memory impairment caused by cerebral ischemia-reperfusion injury in mice, enhance the hypoxia tolerance, increase the activity of superoxide dismutase (SOD) and catalase (CAT) in brain, and reduce the activity of acetylcholinesterase (AChE) and the level of malonaldehyde. The mechanism of memory protection involved may be related to scavenging of free radicals, apoptosis inhibition of nerve cells, and nerve regeneration in brain tissue. These active compounds can be conducive to form the novel CCMF.

2.4. Homologous compounds mixture-based CMF (HCMF)

Homologous compounds mixture-based CMF (HCMF), also known as components based-CMF, are composition groups with defined proportion or high homogeneity. The chemical components can be relatively recognized, and the proportion of each component is fixed. However, HCMF still contains a large number of unknown chemical constituents since it is difficult to purify the effective fractions, such as total alkaloids and total saponins. Hence it is necessary to further isolate, identify and screen the compounds contained in these preparations, which may contribute to the novel CCMF optimization.

Fan et al. (2017) studied the compatibility law of anti-cancer compounds from total flavonoids of Jingjie (*Schizonepeta tenuifolia* Briq.) by microfluidic chip technology in a well-distributed experiment design. The results shows that the combination of luteolin, luteoloside, quercitrin, hesperidin, apigenin and genistein at a ratio of 3.06: 2.90: 2.04: 4.17: 0.12: 2.75 has better effect of apoptosis induction on lung cancer cell line A549 compared to the same dosage of total flavonoids, and the drug potency order of sequence is luteoloside, quercitrin, luteolin, apigenin, genistein and hesperidin. Cao and Deng (2016) isolated effective glycosides from the original extract of Buyang Huanwu Decocotion by acid-base precipitation and ion exchange chromatography. The mass fractions of astragaloside IV, amygdalin and paeoniflorin in the effective components are 37.98 mg/g, 5.48 mg/g and 103.6 mg/g, respectively.

The results suggest that the combination of these three compounds significantly inhibits the proliferation of rat aortic vascular smooth muscle cells (VSMC) induced by platelet derived growth factor (PDGF), and the IC_{50} is lower than the single compounds or the glycoside component.

2.5. Synergistic Active Compounds

A TCM formula usually consists of many natural products, such as herbs, animal based products and minerals, each of which contains large number of compounds. One of the biggest challenges in the modernization of TCM is to identify the active compounds that contribute to the therapeutic effects. Therefore, the identification of active compounds is necessary to elucidate the underlying mechanisms and scientific basis of TCM formula. Currently, the pharmacological effects of numerous compounds isolated from Chinese medicines and natural medicines have been studied, such as artemisinin (Bhattacharjee et al., 2018) and arsenic trioxide (Jiang et al., 2019). These compounds can comprise formula according to different key links of etiological and pathological of diseases. Ischemic stroke, also known as cerebral infarction/death, refers to ischemic necrosis or encephalomalacia caused by the ischemia and hypoxia of local brain tissue resulting from cerebral blood supply disorders. Cerebral infarction is characterized by high morbidity, disability, recurrence and mortality. The major treatment measures include thrombolysis, anti-platelet aggregation, anti-inflammatory and neuroprotection. Paeonol, a natural compound, can reduce the levels of inflammatory factors in rat brain tissue and serum after reperfusion injury of ischemic stroke, such as TNF-α, IL-1β and IL-6, which may offer a protective role by reduction of local excessive inflammation response through inhibition of the production of inflammatory factors (Yang et al., 2010). Paeonol can also significantly decrease the protein and mRNA expressions of ICAM21 and VCAM21 in ischemic brain tissue of rats, which may offer a protective role by reduction of the expression of adhesion molecules in ischemic brain tissue (Zhang et al., 2008). Paeoniflorin can significantly reduce the volume of infarction caused by middle cerebral artery occlusion and improve the MCAO-caused functional deficiencies of the nervous system in rats (Xiao et al., 2005). It also alleviates the free radicals-elicited oxidative damage in rats after reperfusion injury of local ischemic stroke by improving the elimination of free radicals and increasing the expression of Nrf2 (He et al., 2014). In mice hippocampal CA1

neurons, paeoniflorin can not only inhibit Na^+ current in a concentration- and frequency- dependent manner, but also control the gated properties of Na^+ current, thus antagonizing intracellular Ca^{2+} overload (Zhang et al., 2003). Luteolin has a significant anti-inflammatory effect and can reduce LPS-induced increase of IL-6 by interfering with JNK signaling pathway and activating activator protein-1 (AP-1) in microglial cells (Saebyeol et al., 2008), and inhibiting the NF-κB, MAPK and Akt signaling pathways in activated microglia cells (Zhu et al., 2014). These compounds target different links of stroke and can be prepared by using different chemical mixtures to provide novel CCMF.

A compound library is a collection of entity compounds with specific structures or functions and their related information under certain specific standards. PubChem is an open access biological information platform and database supported by the US National Institutes of Health (NIH) with the structure information of more than 30 million compounds and 1 million biochemical experimental data. The compound database China Natural Products supported by Ministry of Science and Technology of China has collected more than 10,000 natural compounds. These compound library and database have provided the information for the compatibility formulation of CCMF.

3. Screening approach for compatibility compound

3.1. Network Pharmacology

TCM network pharmacology is a rising interdisciplinary science and effectively integrates the study of TCM pharmacology with network science, systems biology, computational science and bioinformatics (Li and Zhang, 2013; Zhao et al., 2019). Currently the chemical compounds included in TCM formula can be obtained from several databases, such as TCMSP (Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform) (Ru et al., 2014), TCM Database@Taiwan (Traditional Chinese Medicine Database@Taiwan) (Chen, 2011), TCMID (Traditional Chinese Medicine Integrated Database) (Xue et al., 2013), NPACT (Naturally occurring Plant based Anticancerous Compound-activity-Target database) (Mangal et al., 2013), CancerHSP (Anticancer Herbs database of Systems Pharmacology) (Tao et al., 2015), and NPASS (Natural Product Activity and Species Source Database) (Zeng et al., 2018). The active compounds can be selected according to the ADME-related parameters (Ru et al., 2014).

Kushen is the dry root of *Sophora flavescens* Aiton and Chen et al. (2017) virtually screened the anti-angiogenesis flavonoids from Kushen by molecular docking technology. A ligand database was established by collecting 126 flavonoids compounds which have been separated and identified. A receptor database is composed of 6 targets closely related to angiogenesis such as vascular endothelial growth factor α (VEGF-α), TEK receptor tyrosine kinase (TEK), vascular endothelial growth factor receptor 2 (KDR), etc. The small molecule drugs which have inhibitory effect on each target are listed in the DrugBank as a control and the lowest scoring of the listed small molecule drugs to each target is set as the threshold. The Discovery Studio 2.5 (DS 2.5) software is used for molecular docking, and a total of 37 compounds with scores higher than the threshold were identified and top 10% were investigated.

3.2. Biochromatography

Biochromatography is a new chromatographic technique that combines bioactive materials such as receptors, carrier proteins, cell membranes and carriers as stationary phases, and utilizes the specific binding of stationary phase and compounds to screen the drug candidates. Due to the biological activity of the bioactive materials on the stationary phases, the screening compounds can be bound to the stationary phases through the hydrophobic force, van der Waals force, electrostatic action and binding sites of specific ligand, which reflects the pharmacological potential and significance of screening compounds. The biochromatography technology can eliminate the interference of inactive compounds, narrow down the research focus, and identify the structure of leading compounds when combined with HPLC and mass spectrometry.

Wu et al. (2019) has established a cell membrane chromatography/ultra-high-performance liquid chromatography-time of flight mass spectrometry (CMC/UPLC-TOF/MS) analysis method for water extracts of Liuwei-Dihuang Decoction and completed rapid identification of 16 potential active compounds including catalpol, paeonol, oleanolic acid, etc. The isolated catalpol displays high affinity and was further proven to induce the growth of mouse osteoblasts and skull mineral area in osteoporotic zebrafish.

3.3. High throughput screening

High throughput screening (HTS) technology is a powerful platform for drug screening with the characteristics of automatic operation, rapid detection, molecular/cellular level, computer analyses and use of micro-sample volumes. Marciano et al. (2019) compared the effect of small molecular compounds on MCF-7 breast cancer cell lines with or without sugar in the cell medium on a 384-well microplate. Out of 7,000 compounds, 67 have been successfully identified with the effects of increased sensitivity of MCF7 to the sugar-free culture. Yuliantie et al. (2018) established a high-throughput screening model at the cellular level using secretory embryonic alkaline phosphatase (SEPA) as a reporter gene on 384-well plates, and 25 positive compounds were identified from 32,000 compounds. The cytotoxicity, phosphorylation effect on STAT protein and the transcription of IFN regulatory factor (IRF) of these compounds were further investigated.

3.4. Serum pharmacochemistry of Chinese medicine

Serum pharmacochemistry of Chinese medicine uses modern separation and multi-dimensional combination techniques to analyze, identify or characterize the compounds in human/animal serum after administration of Chinese medicines, to reveal the correlation between these compounds and their efficacy (Wang, 2010). In serum pharmacochemistry of Chinese medicine, only the compounds distributed in serum are treated as the research object, which may significantly simplify the number of compounds to be optimized.

The common analytical techniques for serum pharmacochemistry include gas chromatography, gas chromatography-mass spectrometry, high performance liquid chromatography, liquid-mass spectrometry, capillary electrochromatography, thin-layer scanning, spectrophotometry, atomic absorption spectrometry, etc. HPLC method can be established for the determination of characteristic components of Chinese medicine and CMF extracts. HPLC-DAD, HPLC-DAD-MS/MS and other analytical techniques are used for classification, analysis of the structure and identification of the migrating components contained in serum. To obtain the accurate results, the fingerprints of standard substance, drug-containing serum and blank serum should be determined, analyzed and compared under the same conditions. Of particular note the serum-containing components may change with time due to the different administration methods, physical and chemical properties, absorption sites and rates of Chinese

medicine ingredients (An et al., 2013). By analyzing the serum fingerprints of the blood samples collected at different time points, the time-varying process of the whole blood composition spectrum *in vivo* can be found. It is necessary to establish a dynamic serum pharmacochemical profile to reflect the changes of chemical composition *in vivo* and the correlation between the prototype drug and metabolic components. The application of ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) can be used to identify the trace components on line (Wang et al., 2011), and pharmacodynamics can be determined (Yan et al., 2012).

In our previous work, the compounds distributed in blood of SXBXP have been successfully investigated, and 17 prototype compounds and 3 metabolites were identified from rat plasma by HPLC-ESI-MS/MS (Jiang et al., 2010). 10 volatile compounds including 6 prototypes and 4 metabolites have been identified by GC/MS and NIST05 database comparison (Guo et al., 2012). Although a total of 96 compounds were identified from SXBXP *in vitro*, only 30 compounds were detected in blood which may simplify the difficulty of SXBXP compatibility research.

There are also some limitations in the use of serum pharmacochemistry for Chinese medicine to determine its therapeutic effects. For example, Chinese medicine and formula contain complex chemical constituents, with different physical and chemical properties with different rates of absorption and distribution, therefore, it is difficult to fully determine the dynamic changes of compounds (Chen et al., 2016). The experimental animal species and their physiological and pathological conditions also have great influences on the analysis of results, such as the obvious differences in the absorption under physiological and pathological conditions in rats (Liu et al., 2014; Shi et al., 2014). There are great differences in the pharmacokinetic characteristics of components distributed in blood from Danggui-Buxue Decoction between ischemia rats and normal rats (Shi et al., 2014). In spite of the increased difficulties in animal modeling, the application of pathological animal models has better practical significance compared with the commonly used normal animal models.

4. Optimization method of compatibility compounds

Due to the complex chemical constituents of traditional Chinese medicine, the research on its compatibility lacks relevant experience at the molecular level. The information on how to obtain the optimal combination of these active compounds is a

crucial step in the study of CCMF. Therefore, it is necessary to optimize the compatibility of effective compounds according to the corresponding clinical diseases and syndromes, so as to screen out the best compatibility formula. Several commonly used optimization methods are summarized below (Table 2).

Table 2 Comparison of common optimization methods

	Advantages	Disadvantages	Range of application
Feedback System Control	Efficient and rapid	The dispersion degree of drug dose has great influence	Focuses on integrative system responses; broad selection of drugs
Orthogonal Design	The data analysis program is simple	Low accuracy	Low level test
Uniform design	The number of trials is greatly reduced	Can not estimate the main and interaction effects in the anova model	The test points are evenly distributed within the test range
Increase-decrease design	Good reliability, large information processing space, and less experiments	Narrow scope of application	The effect is clear and there are only two kinds of TCM compounds
ED-NM-MO trigeminy method	Multi-components optimization ratio	Calculation is complicated	Multi-component compounds

4.1. Feedback System Control

Generally, the combination use of drugs can reduce the dosage and side effects or enhance therapeutic effect compared to the single drug (Ding et al., 2017). As a novel screening technique for drug combination therapy, Feedback System Control (FSC) was firstly proposed by Wong et al. (2008). FSC is based on combination of experimental results under the guidance of differential evolution (DE) algorithm. It uses parabolic reaction surface (PRS) to define the transfer function of phenotype output and drug dose input, thereby achieving fast and precise optimization of multiple drug or

dose combinations and greatly improving the speed and efficiency. Compared to traditional methods, FSC is more efficient, which significantly reduces the efforts, cost, time and number of experimental subjects (Nowaksliwinska et al., 2016; Wong et al., 2008; Silva et al., 2016). Tsutsui and the co-authors used FSC to screen out an optimal composition, consisting of three small molecule inhibitors, which enabled the single cell culture system of maintaining embryonic stem cells through single cell passage on a fibronectin-coated surface (Tsutsui et al., 2011). Yu et al. (2013) identified the potential best combination of four flavonoids and conducted a preliminary evaluation based on the optimization of Huangqi-derived flavonoids by using an engineering approach of the FSC scheme. The optimal combination of flavonoids to maximize hypoxia response element (HRE) -mediated transcriptional activity was quickly acquired. Ding et al. has applied FSC technique to screen out the best combination of four drugs for the treatment of intestinal aphids, so as to effectively alleviate clinical drug resistance (Ding et al., 2017). A very effective "cocktail" program with lower dosage and improved effect has been found only after 4 rounds and 32 combined tests. Therefore, FSC can undertake a significant role in screening of drug combination, which is also suitable to screen for CCMF compatibility.

4.2. Orthogonal Design

Orthogonal design is a method of designing multi-factor and multi-level tests through orthogonal test tables with the characteristics of uniform dispersion, uniformity and comparability. It is suitable for the compatibility optimization of TCM with limited scope and was performed at three factors and four levels for three components (polysaccharides, saponins and phenols) isolated from Banxia-Baizhu-Tianma Decoction (Xu et al., 2019). The fatty and salty diet hypertensive rats with phlegm-dampness congestion symptoms were used as screening models and the parameters of glucolipid metabolism were converted into integrated efficiency according to the equal weight index. The optimal combination dosages of saponins, phenols and polysaccharides are 9.00 mg/kg, 14.50 mg/kg, and 12.95 mg/kg, respectively.

4.3. Uniform design

Uniform design is a method based on the principle of uniformity and can select some representative compounds and uniformly distributed points from the whole range

reflecting the major characteristics of the research object. Uniform design can reduce the scope and number of the experiments to a great extent, especially for the multi-factors and multi-levels research, which is more suitable for experiments at tissue level or at whole animal level. However, it lacks the symmetrical comparability compared with the orthogonal design, and the regression analysis method should be used to process the results.

Wu et al. (2016) used uniform design method to optimize the compatibility of four main compounds included in Evodia rutaecarpa decoction, namely ginsenoside-Rg1 (Rg1), ginsenoside-Rb1 (Rb1), evodiamine (EV) and evodiamine (RU) for the mouse disease model of migraine. The results show that Rb1 and EV significantly increase the pharmacodynamics indicators and improve the efficacy, while Rg1 and RU contribute little to the overall formula.

4.4. Other experimental designs

In addition, other experimental designs like increase-decrease design and ED-NM-MO trigeminy method may also be used in CCMF study. Shang et al. (2003) established this method of experiment with a baseline geometric proportion property for optimization and screening of the proportion of small formula. The advantages are the comprehensive and reliable information analysis, the large information processing space and the relatively small number of experiments. However, the increase-decrease design is used only in small CMF with clear therapeutic effects, such as formula with only two ingredients.

The ED-NM-MO trigeminy method is proposed on the basis of experimental design (ED)-nonlinear modeling (NM)-multi-objective optimization (MO). It uses three related links to achieve nonlinear multi-objective optimization of prescription dose ratio. The ED-NM-MO method is suitable for the investigation of medicine properties and can be used to optimize the dosage and ratio of CMF. The compatibility composition of Danshen and Sanqi was optimized by this method, and the optimal ratio is obtained for 7 pharmacodynamic indicators and 6 pharmacodynamic indicators, respectively (Wang et al., 2006).

5. Therapeutic evaluation model

Drug screening can be conducted at the levels of molecules, cells, tissues and animals. However, the use of tissues and animals are not suitable for the composition screening for CCMF owing to their low throughput. The screening at molecular level is also inappropriate for CCMF due to its characteristics of single-target. Hence, some common models for cancer research, including patient-derived cancer cell lines (PDX), patient-derived xenografts (PDC), multicellular three-dimensional (3D) cultures and organoids, are introduced. Especially, the cell 3D-culture and organoid models have tremendous potential for CCMF research.

5.1. Multicellular three-Dimensional culture model

In recent years, cell 3D-culture has attracted considerable attention as it can simulate physiology and pathology state better than traditional two-dimensional (2D) culture. It has a higher degree of interaction between cells and maintains more orderly tissue processing of tiny structures of organs in the body (Nath and Devi, 2016). The multicellular tumor spheroids (MCTS) is a type of models most commonly used in tumor research since its 3D structure can simulate the angiogenic tumor region composed of proliferating cells and necrotic cells (Froehlich et al., 2016). There are two construction methods for MCTS, including material assisted and mechanical device assisted construction. The low melting point agarose and Matrigel with appropriate growth factors or extracellular matrix are the commonly used materials for 3D-culture (Matte et al., 2016). The mechanical means are used to construct a physical environment conducive to cell aggregation into pellets, including suspension drop method, rotating culture system method, and so on.

The formation of cell spheroids is a cell-dependent manner, although not all kinds of cells can be used in 3D-culture model. Froehlich et al. (2016) has studied the ellipsoid formations of three breast cancer cell lines MCF-7, MDA-MB-231 and SK-BR-3 under different culture conditions, including hanging drop, liquid overlay and suspension culture and 25% methocel was recommended as the most reliable and effective condition. MCF-7 cells can form spheroids under nearly all of analytical conditions. MDA-MB-231 cells form spheroids under only one scheme (liquid overlay technique, 3.5% Matrigel), but SK-BR-3 cells are not spherical under any condition. Galateanu et al. (2016) used human colorectal cancer multi-cells to establish a 3D spheroid model which was applied to evaluate the effect of the clinical drug combination of folinic acid,

oxaliplatin and 5-fluorouracil. These results demonstrate the effect of carrying liposomes loaded with drugs on multicellular tumor spheres. However, the dose range needs to be confirmed through *in vivo* studies for combinational use of three drugs and their subsequent encapsulation into liposomes.

The MCTS establishes a stereoscopic information exchange system similar to the real tumor, which plays a great role in the connection between cells and clinical trials, and enhances the early prediction of the potential of candidate drugs (Luo and Gao, 2018). However, there are still some limitations for this out-of-body model. For example, not all immortal cell lines spontaneously form MCTS, which requires extensive optimization and validation, limiting the use of this model (Juergen et al., 2009). To address this problem, more complex *in vitro* systems are currently being evaluated, including co-culture with other cell types, such as fibroblasts, to promote the formation of MCTS and the replication of complex signals that occur *in vivo* (Cui et al., 2017; Park J et al., 2016). On the other hand, single cell type model cannot reflect the multi-target effect characteristics of CCMF, establishment of the 3D model of multi-type cells co-culture is the development direction in future.

As a powerful *in vitro* drug evaluation model, 3D cell culture has made many gratifying achievements since its emergence. Zhang et al. (2014) found that the pathological changes in the redistribution of phosphorylated p21 activated kinase (pPAK) and other related proteins in nerve cells could only be observed under the condition of 3D culture, which could not be found in the conventional 2D cell culture model. APP and PSEN1 mutations are expressed successfully in ReNcell VM cell lines, generating FAD ReNcell lines, and then obtaining cell model that could accelerate neuronal cell differentiation to form neural network through 3D cell culture method (Choi et al., 2014; 2016). This research successfully reproduces the pathological process of Aβ deposit and drive tau protein extracellular accumulation, which provides more effective methods and techniques for the treatment of alzheimer's disease.

Cell culture is an indispensable experimental technique during drug development process. 3D cell culture has become a valuable tool, which is closer to the real situations *in vivo*, helping to bridge the gap between the *in vitro* and *in vivo* models with reduced animal number used in the early stage of experiments and the possiblilities of error (Yu and Zhou, 2019). However, most 3D cell cultures rely on gel substrates, and the applications are limited by their solids and opacity, as well as the inconsistencies of the

cells' exposure to environmental stimulus. Therefore, before 3D cell cultures can be widely accepted and regarded as stardard in the drug development and precision medicine related research, there are still many difficulties and obstacles which need to be tackled and solved.

5.2. Organoid Model

Organoid is a 3D "micro-organ model" prepared by self-organization of different types of stem cells and can mimic the structure and function of original organs (Fatehullah et al., 2016). Such *in vitro* culture systems include a self-renewing stem cell population that can differentiate into multiple organ-specific cell types with similar spatial tissue to the corresponding organ and be able to reproduce some of its functions thus providing highly physiologically relevant systems (Lancaster and Knoblich, 2014). In recent years, investigations on organoid have mainly focused on *in vitro* models of diseases and *in vitro* organ reconstruction, which will open up new approaches for biological research. The development and application of organoid models will be one of the hot topics in tumor and stem cell research in future (Xu et al., 2018).

Organoid can be prepared using somatic cells, adult stem cells (including progenitor cells) or pluripotent stem cells. In 2009, the intestinal organ simulation technology made the technological breakthroughs. Toshiro found that adult intestinal stem cells could proliferate and spontaneously organize *in vitro* (Toshiro et al., 2009), hence the researchers developed a 3D culture system capable of reconstructing the appropriate environment for intestinal stem cells *in vitro* and differentiating from intestinal epithelial cells or single LGR5+ stem cells into organoids with self-renewal ability and maintaining the villous structure of epithelial tissues. Huch et al. optimized the condition of the mouse liver organ culture system and successfully obtained liver organs using liver duct cells derived from humans (Huch et al., 2015). This type of organ maintains the stability of the genome during long-term culture and can be converted into functional hepatocytes under conditions of *in vitro* culture and transplantation. After the differentiation of ESCs into the endoderm induced by activin A, Noggin and transforming growth factor-β (TGF-β) inhibitors, the expression levels of SOX2 and FOXA2 both increased, which activated the HH pathway and inhibited the FGF pathway respectively, and then the quasi-organs of the lung were obtained (Dye et al., 2015). Fong et al. (2016) co-cultured tumor cells derived from PDX model

of prostate cancer with osteoblasts, and the 3D model formed in this culture system could well maintain the proliferation activity of cells and the state of osteogenesis, which is consistent with the phenotype of bone metastasis of prostate cancer. Therefore, this organ model can be used to study the interaction between tumor cells and microenvironment including stromal cells.

Compared with traditional 2D culture models, the organoid represents an innovative technique that summarizes the physiological processes of the entire organism, with such advantages as closer proximity to physiological cell composition and behavior, more stable genome, and more suitable for biological transfection and high-throughput screening. Compared with animal model, the organoid model is simpler to operate and can be used to study the mechanisms of disease occurrence and development. At present, although organoids show the ability of self-renewal and differentiation *in vitro* experiments, but its passage times are limited. However, the major challenge is the improvement of passage ability and the maintenance of proliferation and undifferentiation in patients. On the other hand, the effects of matrix and the vascular system have not been considered in the present organoid culture. Although organoid technology is still in its infancy, it has great potential to study a wide range of subjects, including developmental biology, disease pathology, cell biology, reproductive mechanisms, precision medicine, drug toxicity and efficacy trials. It is believed that with the continuous innovation of technology, quasi-organs as an ideal model will play an increasingly important role in human disease research.

5.3. PDC and PDX Models

Since the successful establishment of the HeLa cell line in 1951, a new field of cancer research has been opened, making PDC a major model for cancer research. Tumor cells need only simple culture conditions, can proliferate indefinitely *in vitro*, and are suitable for large-scale drug screening. However, the tumor cell lines also have serious defects in that the heterogeneity and the characteristics of tumor cells *in vivo* are lost during *in vitro* culture. The PDX model refers that the tumor tissue derived from patients is cut into small pieces and then transplanted into immune-deficient mice (Lai et al., 2017). Some tumor cell lines originated from mice can also be transplanted into mice to form tumor. Although the PDX model can maintain the heterogeneity of tumors to a large extent and has been applied to drug screening, it still faces many

problems, such as low success rate of transplantation, low screening throughput, large sample size of tumors and long experimental period (Gao and Chen, 2015; Xu et al., 2018).

6. Research methods of molecular targets

Many publications have systematically introduced the techniques for active targets of natural products (Zeng and Peng, 2018; Zhou and Xiao, 2018), including affinity-based protein profiling (A*f*BPP), drug affinity responsive target stability (DARTS), cellular thermal shift assay (CETSA), stability of proteins from rates of oxidation (SPROX), target identification by chromatographic co-elution (TICC), microscale thermophoresis (MST), chemical genomics screening, chemical genomics bioinformatics prediction, differential genomics screening, differential proteomics screening, and so on. Among these, CETSA and DARTS as the validation methods can be applied to screen drug targets when combined with other assistant techniques such as quantitative mass spectrometry. We briefly introduce the frontier techniques in CCMF research below, such as CETSA, DARTS and MST.

6.1. Cellular Thermal Shift Assay

Cellular thermal shift assay (CETSA) was first proposed in 2013 (Daniel et al., 2013). The basic principle is that when a small molecule compound binds to a protein, the thermal stability of the protein molecule is significantly improved. This kind of binding protein needs higher temperature to be aggregated and degenerated. When heated to a certain temperature, the binding protein and the unbound protein can be separated by high-speed centrifugation. The known proteins can be validated by western-blot analysis. This unique technique can directly measure drug-protein binding, and has been widely used to verify conjugation (Dai et al., 2018; Jafari et al., 2014). Currently, more than 98.3% of 558 known small molecular drug targets belong to proteins (Santos et al., 2017), and more than 3000 potential target proteins can be combined with small molecule compounds to exert pharmacological effects (Yue et al., 2012). However, CETSA can only be applied to validate the known proteins of target due to the limitation of western-blot technique, and it is not suitable for high-throughput screening.

Currently, there are two kinds of high-throughput methods for drug targets screening based on improved CETSA technique. The first is to screen drugs according to the target and isolate specific compounds that bind to target proteins from candidate compounds. The novel techniques can be used to increase the throughput of screening, such as ALPHAScreen based on the interaction between donor and receptor microbeads (Jafari et al., 2014) and ligand-stabilized soluble target protein detection based on enzyme fragment chemiluminescence quantification (Martinez et al., 2018). Another is to screen targets by known drugs. Some proteins combined with a small molecule compound are obtained through high-throughput screening, which is very suitable for the target screening of CMF compounds. However, it is difficult to construct high-throughput screening models based on cellular thermal shift assay (HTS-CETSA) for screening target proteins owing to the large number of candidate target proteins, their physical and chemical properties and the difficulty of biotin labeling of small molecule compounds.

6.2. Drug affinity responsive target stability (DARTS)

Drug affinity responsive target stability (DARTS) analysis technique was proposed by Brett et al. (2009). The basic principle is that the binding of natural small molecule compounds to target proteins can prevent proteases from digesting the target proteins. The antienzymatic stability changes of the target proteins before or after the binding is detected by electrophoresis, and then the target proteins directly bound to small molecule compounds can be analyzed by bio-mass spectrometry. The advantages of this technique are easy operation without drug modification, independence of the drugs' action mechanism, and the technique is especially suitable for validation of protein targets with poor affinity but high abundance. For example, this technique was used to discover the binding target proteins eIF4A for resveratrol (Brett et al., 2009). However, these results may be disturbed by non-direct target proteins, especially in living cells. DARTS can also be used to identify the binding target proteins of small molecule compounds when combined with western-blot analysis (Park Y et al., 2016). Nitazoxanide, an anti-parasite drug, was proved to effectively inhibit the Wnt signaling pathway independent of adenomatous polyposis coli by targeting peptidyl arginine deiminase 2 through DARTS and western-blot analyses, suggesting its potential in the treatment of Wnt-pathway mutations in cancer patients (Qu et al., 2018). On the other

hand, the binding targets of plant extracts could also be identified by DARTS. With the combined DARTS and bio-mass spectrum analysis, the grape seed extract was found to down-regulate the whole proteins involved in translation process through the endoplasmic reticulum (ER) stress response protein of human colon cancer cells, resulting in the modification of oxidized protein, especially the amino acid residues of the protein target (Derry et al., 2014).

6.3. Microscale thermophoresis

Microscale Thermophoresis (MST) refers to the directional motion of molecules in the microscopic temperature gradient field. The change of biomolecular structure or conformation will cause the differences of its hydration layer, molecular weight and surface charges, which will influence the molecular distribution in the temperature gradient field. This kind of changes can be used to analyze molecular interactions and various stoichiometric parameters. MST can directly measure molecular interactions in similarly natural environments (serum and cell lysate) and in many biological solutions. MST can not only analyze the interaction between proteins, but also measure the combination of small molecular compounds and proteins. The affinity of small molecule inhibitor quercetin with kinase PKA was measured in buffer solution and human serum respectively. The affinity in serum was reduced by 400 times, indicating that the biological matrix can affect the combination of drugs with proteins, thereby interfering with the drug function (Wienken et al., 2011). Hexokinase 2 (HK2) is the rate-limiting enzyme in the first step of the glycolytic pathway, and is highly expressed in cancer cells and plays a key role in oncogenesis and metastasis. Bao et al. has verified that the target of a compound isolated from *Ganoderma lucidum* is HK2 protein by MST analysis (Bao et al., 2018). This compound significantly inhibits the activity of HK2, suggesting that it may be a potential drug for cancer therapy. However, MST analysis can only provide the indirect evidence of binding between small molecules and proteins through the affinity value, hence it still needs to be confirmed by other methods.

7. Discussion

Unclear modern scientific connotation of TCM is a major scientific issue limiting the breakthrough of TCM modernization and internationalization. The compatibility application of CMF is an important feature for TCM (Fan et al., 2014), but it is difficult

to clearly elucidate the compatibility mechanism at the levels of decoction pieces and components.

Hence, we proposed that compound-based Chinese medicine formula (CCMF) (Figure 1), which can be obtained from optimized screening of compounds from decoction pieces, components-based Chinese medicine or activity compounds with synergistic effects, can become the future direction of TCM. The complex and unclear composition of TCM is difficult to control its safety, effectiveness and quality, which limits its further clinical application. For example, TCM injections probably have shown good clinical therapeutic effects, such as the antiviral effect and improvement of myocardial ischemia. However, due to the complex constitution of unknown and ineffective materials which has been injected directly into circulation system, TCM injections have caused series of adverse drug reactions and concerns. According to the 2017 Annual Report for National Adverse Drug Reaction Monitoring by CFDA, TCM injections alone accounts for 84.1% of reported TCM averse dug reactions which leads to the restriction and banning of some effective TCM injections. The CCMF research based on TCM injections can help to solve this safety issues.

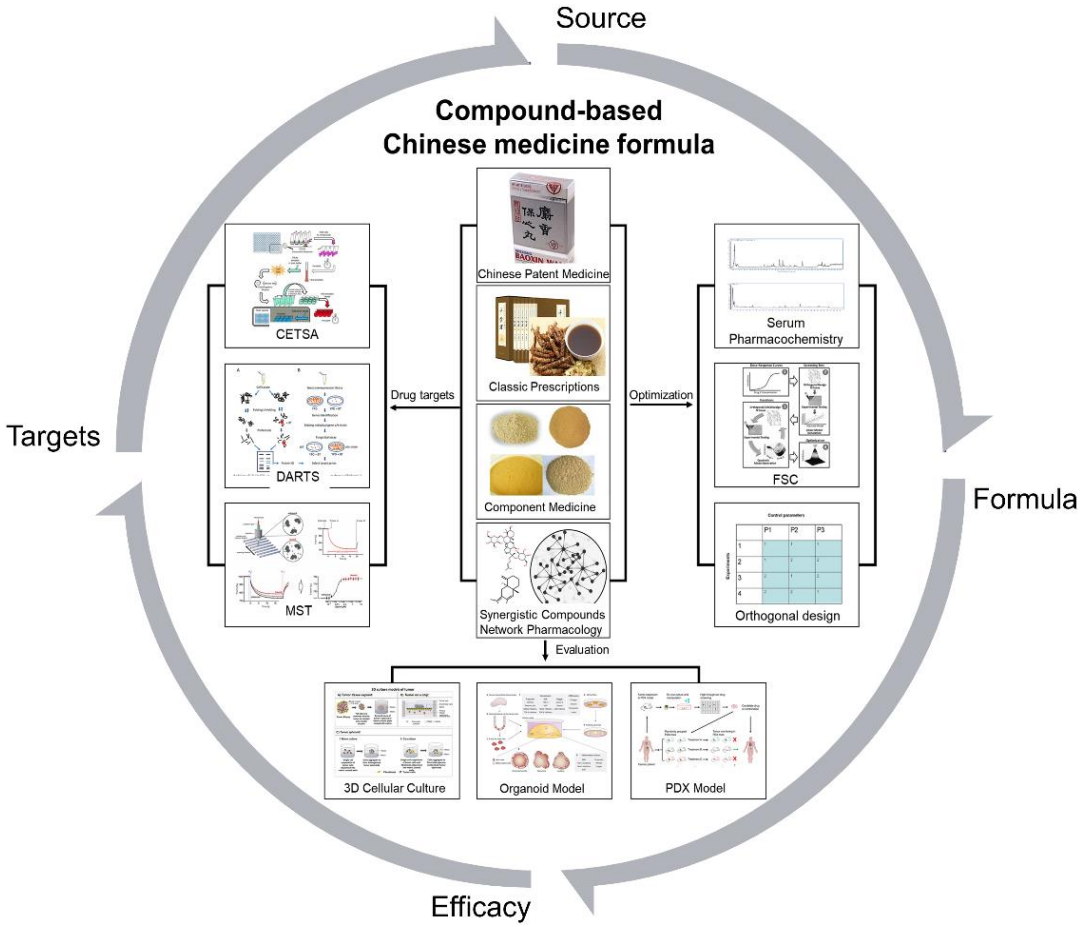

Figure 1. Key study contents involved in the research of compound-based Chinese medicine formula. In this review, Chinese patent medicine, classic prescriptions, component medicine and synergistic compounds are introduced as the common sources of compound-based Chinese medicine formula (CCMF). Serum pharmacochemistry, feedback system control (FSC) (Liu et al., 2014) and orthogonal design are the recommended optimization method for CCMF. The cellular thermal shift assay (CETSA) (Matte et al., 2016), drug affinity responsive target stability (DARTS) (Luo and Gao, 2018) and microscale thermophoresis (MST) (Cui et al., 2017) has been introduced as the screen method for drug targets. The three-dimensional (3D) cellular culture (Xu et al., 2018), organoid model (Daniel et al., 2013) and PDX model (Park J et al., 2016) has been summarized as the promising method for the efficacy evaluation.

CCMF not only has the advantages of clear chemical composition and controllable quality of modern medicines, but also has the characteristics of synergistic effect of TCM. Furthermore, the compatibility mechanism of its Monarch-Minister-Assistant-Guide (Jun-Chen-Zuo-Shi in Chinese) can be investigated on the basis of the therapeutic targets to elucidate the scientific connotation. For example, the Realgar-Indigo naturalis formula (RIF) has been proven clinically to show good therapeutic effects on acute promyelocytic leukemia in combination with all-trans retinoic acid

(Zhu et al., 2013; 2018). To further investigate molecular mechanism, a systematic study of RIF at compounds-level was carried out by Wang et al. (2008). Arsenic sulfide, indirubin and tanshinone IIA have been identified as the main active ingredients of the formula. Arsenic sulfide alone can prolong the survival time of the acute promyelocytic leukemia mice, but the combination with indirubin and tanshinone IIA shows more signigicant therapeutic effect through the enhanced degradation of PML-RARα oncoprotein caused by arsenic sulfide which serves as "monarch drug", increased expression of genes related to the leukocyte differentiation and maturation which caused by tanshinone IIA as "minister drug", and decreased expression of protein promoted cell cycle by indirubin as "assistant drug". As the "guiding drugs", tanshinone IIA and indirubin can also increase the expression aquaglyceroporin 9 which is responsible for arsenic sulfide transportation and cell uptake. Wang et al. (2008) has clearly elucidated the synergistic molecular mechanisms of RIF, and provided an excellent example for CCMF research which should be the future direction for TCM modernization.

There are three core contents in the compatibility study of CCMF, including the screening model, compatibility optimization, and targets validation. Although the complex disease environments can be better simulated in animals, they are not suitable for compatibility optimization of formula because of the limited throughput. The *in vitro* model of single type of cells can usually neither conform to the multifactor-caused disease progression, nor can it reflect the characteristics of multi-component and multi-target synergism of CMF. The multicellular co-culture and organoid *in vitro* could be the ideal models for formula screening, which can not only simulate multi-factor disease environments, but also have characteristics of high screening throughput. The FSC introduced in this review also can be combined with multicellular co-culture for screening of compatibility formula of CCMF.

8. Conclusions

Compatibility is the advantage of Chinese medicine formula in the treatment of diseases, but its scientific connotation has not been elucidated owing to unclear active components and binding target. Although TCMF has the advantages of easier obtainment, simpler processing and lower cost, it is difficult to control its quality, safety and effectiveness due to the presence of numerous ineffective and unknown

components. HCMF still contains partially inactive and unknown substance, which is responsible for the indeterminate binding target. The quality, safety and effectiveness of CCMF can be controlled by the use of well defined compounds. Moreover, its target and mechanism of action can also be clarified further. Although there are many approaches to investigate the action targets, including CETSA, DARTS, and MST, it still lacks the high throughput screening methods for binding targets of CCMF. The combination of mass spectrometry with CETSA or DARTS has improved the throughput in a certain extent, but there still exist some defects such as isotope labeling, complicated operation, high technical requirement and cost. Multicellular culture and organoid are better *vitro* models for investigation of CCMF, but are currently limited in their use. There is an urgent need to establish screening models of compatibility and screening targets for CCMF.

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Conflicts of interest

The authors have declared that there is no conflict of interests.

Author contributions

Xin Luan wrote and revised the manuscript. Li-Jun Zhang collected the literature and wrote part of the manuscript. Xiao-Qin Li collected the literature. Khalid Rahman revised the manuscript. Hong Zhang, Hong-Zhuan Chen and Wei-Dong Zhang presented the research ideas and revised the manuscript.

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