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Copyright
The progress in adjuvant therapy after curative resection of liver metastasis from colorectal cancer

Wei Zhang, Tianqiang Song*

Department of Hepatobiliary Surgery, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin, China.

Summary

Colorectal cancer liver metastases (CRLM) are common and found in almost 50% of patients with colorectal cancer. Surgical resection has proved to be the most effective therapy for metastatic colorectal cancer isolated to the liver and has yielded long term survival. However, recurrence frequently occurs within the remaining liver as well as at extra-hepatic sites. The role of adjuvant therapy has been investigated in many studies but has still been controversial until now. This review examines the incorporation of adjuvant systemic chemotherapy, regional chemotherapy with hepatic arterial infusion and molecular targeted therapy following liver resection for patients with CRLM, and summarized the advantage and adverse effects for these treatments. Finally, we propose the prospective of future adjuvant treatments to further improve prognosis.

Keywords: Colorectal cancer liver metastases (CRLM), adjuvant treatment, systemic chemotherapy, molecular-targeted therapy, hepatic arterial infusion (HAI)

1. Introduction

The liver is the most common site of colorectal cancer metastasis, with 15% of patients presenting with liver metastases at the time of diagnosis and up to 60% of patients developing liver metastases during the course of their disease (1). Surgical resection has proved to be the most effective therapy for metastatic colorectal cancer isolated to the liver and has yielded 5-year overall survival (OS) rates of 28% to 44% (2-7) and 10-year survival of over 20% (5,6,8) (Table 1). However, the risk of postoperative recurrence, especially in the remnant liver, remains high, occurring in approximately 75% of patients. Furthermore, there is the view that it is the liver metastatic disease, rather than the primary cancer, that gives rise to systemic metastatic disease (9). So it is of great importance to achieve long-term survival by suppressing the liver metastasis after resection for liver metastasis from colorectal cancer (CRLM).

Chemotherapy and molecular targeted therapy have made great progress in treatment of advanced colorectal cancer, and adjuvant chemotherapy has proved to prolong survival after resection of primary colon cancer, especially with the development of modern chemotherapeutic medications (10-13). However, there is no standard treatment in the adjuvant setting after resection of liver metastasis from colorectal cancer. In the current review, we summarize chemotherapy, molecular targeted therapy as well as regional chemotherapy with hepatic arterial infusion (HAI) for patients with curatively resected liver metastasis from colorectal cancer in an adjuvant setting. We propose a future perspective potential strategy of adjuvant therapy for patients with CRLM.

2. Systemic chemotherapy after curative resection of colorectal cancer liver metastasis.

The efficacy of adjuvant chemotherapy for liver metastasis of CRLM is still controversial. A retrospective review of 792 patients supported the importance of adjuvant chemotherapy in terms of significantly prolonging overall survival (8). Two phase III trials (Federation Francophone de Cancerologie Digestive (FFCD) Trial 9002 and the European Organization for
Research and Treatment of Cancer/National Cancer Institute of Canada Clinical Trials Group/Gruppo Italiano di Valutazione Interventi in Oncologia (ENG) trial) used a similar design and showed a trend favoring adjuvant chemotherapy with a fluorouracil (FU) bolus-based regimen, but both trials had to close prematurely because of slow accrual, thus lacking the statistical power to demonstrate the predefined difference in survival. A pooled analysis of individual data from these two trials shows a marginal statistical significance in favor of adjuvant chemotherapy after complete resection of colorectal cancer metastases (14) (Table 2).

The most convincing evidence comes from a phase III trial, EORTC trial 40983. The EORTC intergroup trial 40983 showed that perioperative chemotherapy with FOLFOX4 (folinic acid, fluorouracil, and oxaliplatin) increases progression-free survival (PFS) compared with surgery alone for patients with initially resectable liver metastases from colorectal cancer (15), with 3-year PFS improved in eligible patients ($p = 0.041$) and resected patients ($p = 0.025$). However, at a median follow-up of 8.5 years (16), there is still no difference in overall survival with the addition of perioperative FOLFOX4 compared with surgery alone (5 year OS: 51.2% vs. 47.8%). It showed that 107 (59%) patients in the perioperative chemotherapy group had died versus 114 (63%) in the surgery-only group (HR 0.88, $p = 0.34$), and median overall survival was 61.3 months in the

### Table 1. Long-term survival after liver resection for colorectal cancer liver metastasis

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>No. cases</th>
<th>5-year OS</th>
<th>10-year OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nordlinger-France</td>
<td>1996</td>
<td>4</td>
<td>1,568</td>
<td>28%</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fong-US-MSKCC</td>
<td>1999</td>
<td>5</td>
<td>1,001</td>
<td>37%</td>
<td>22%</td>
</tr>
<tr>
<td>Rees-UK</td>
<td>2008</td>
<td>6</td>
<td>929</td>
<td>36%*</td>
<td>23%*</td>
</tr>
</tbody>
</table>

* Cancer-specific overall survival; * Synchronous liver metastasis with multi-institutional data. n.a., not available.

### Table 2. Systemic chemotherapy after resection of CRLM

<table>
<thead>
<tr>
<th>Author</th>
<th>Ref.</th>
<th>Study types</th>
<th>No. cases</th>
<th>Regimen for chemotherapy</th>
<th>Survival benefic for chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parks (2007)</td>
<td>8</td>
<td>Large cohort</td>
<td>792 (374 vs. 518)</td>
<td>5-FU-based adjuvant chemotherapy</td>
<td>Improved survival ($p = 0.007$, log-rank test)</td>
</tr>
<tr>
<td>Mity (2008)</td>
<td>14</td>
<td>Pooled analysis of two phase III RCTs</td>
<td>278, CT: 138, S: 140</td>
<td>FU 400 mg/m² administered i.v. once daily plus DL-LV 200 mg/m² [FFCD] for 5 days or FU 370 mg/m² plus L-LV 100 mg/m² IV for 5 days [ENG] for six cycles at 28-day intervals</td>
<td>Benefit was statistically marginal, with median DFS: 27.9 vs. 18.8 ($p = 0.058$) and median OS: 62.2 vs. 49.3 ($p = 0.095$).</td>
</tr>
<tr>
<td>Nordlinger (2008)</td>
<td>15</td>
<td>Phase III</td>
<td>364(171 vs. 152)</td>
<td>Perioperative FOLFOX4 (EORTC-40983; short-term results)</td>
<td>3-year PFS improved in eligible patients ($p = 0.041$) and resected patients ($p = 0.025$).</td>
</tr>
<tr>
<td>Nordlinger (2013)</td>
<td>16</td>
<td>Phase III</td>
<td>364(171 vs. 152)</td>
<td>Perioperative FOLFOX4 (EORTC-40983; long-term results)</td>
<td>No benefit in 5 year OS: 51.2% (95% CI: 43.6-58.3) vs. 47.8% (95% CI: 40.3-55.0).</td>
</tr>
<tr>
<td>Sorbye (2012)</td>
<td>17</td>
<td>Phase III</td>
<td>342</td>
<td>Perioperative FOLFOX4 (EORTC-40983 inter-group study)</td>
<td>3-year PFS (35% vs. 20%) better for patients with moderately (5.1-30 ng/mL, $p = 0.018$) and highly (&gt;30 ng/mL, $p = 0.0075$) elevated CEA.</td>
</tr>
<tr>
<td>Ychou (2009)</td>
<td>18</td>
<td>Phase III</td>
<td>306</td>
<td>FOLFIRI vs. LV5FUs</td>
<td>For DFS, FOLFIRI not better than LV5FUs.</td>
</tr>
<tr>
<td>Kim (2011)</td>
<td>19</td>
<td>Single armed</td>
<td>60</td>
<td>mFOLFOX6 (oxaliplatin 130 mg/m² d1) or 5-FU (1,000 mg/m² d1–3, continuous infusion) for 6 months.</td>
<td>Increased OS and RFS (compared to historical control).</td>
</tr>
<tr>
<td>Hirokawa (2014)</td>
<td>20</td>
<td>Retrospective</td>
<td>110</td>
<td>77 patients (70%) received chemotherapy (5-FU ± LV, tegafur/uracil ± folinate, oteracil (TS-1), etc.). 25 patients received FOLFOX, FOLFIRI or HAI with 5-FU/cisplatin.</td>
<td>Risk factors: H2-classification, pT4 and LN+, &lt; 2 factors: no benefit for OS and RFS; ≥ 2 factors: OS better.</td>
</tr>
</tbody>
</table>

Notes: IV, intravenously; FU, fluorouracil; FOLFOX, FOLFIRI, CEA, carcinogen embryo antigen, OS, overall survival, PFS, progression-free survival; CT, chemotherapy, S, surgery alone.
perioperative chemotherapy group and 54.3 months in the surgery alone group.

Some scholars made comments about the results. First, the PFS increase in resected patients suggested that FOLFOX delays progression of disease but does not improve long term survival compared with surgery alone. Second, the chemotherapy in EORTC trial 40983 perioperative rather than adjuvant, because chemotherapy was given for 6 cycles before and 6 cycles after surgery, so the perioperative chemotherapy may select patients most likely to benefit from hepatic resection.

Interestingly, Sorbye et al. (17) analyzed the predictive factors for the benefit of a subgroup of patients in the EORTC 40983 study and found that for patients with moderately or highly elevated CEA (> 5 ng/mL), the 3-year PFS was 35% with perioperative chemotherapy compared to 20% with surgery alone, and performance status (PS) 0 and BMI lower than 30 were also predictive for the benefit of perioperative chemotherapy (interaction p = 0.04 and p = 0.02), suggesting that application of adjuvant FOLFOX4 maybe only be justified in subgroup patients with specific features.

Chemotherapy with Irinotecan is not justified in the adjuvant setting. A phase III clinical trial conducted by Ychou et al. (18) showed that FOLFIRI in the adjuvant treatment of CRLM showed no significant improvement in DFS compared with LV5FUs.

Many retrospective studies with small sample sizes provided insufficient evidence (Table 2). Kim et al. (19) showed that oxaliplatin-based adjuvant chemotherapy (mFOLFOX6) after radical resection resulted in increased OS and RFS compared to historical controls. Hirokawa et al. (20) found benefit for OS and RFS only in patients with more than 2 risk factors (including H2-classification, invasion depth pT4, and lymph node positive).

In conclusion, the application of adjuvant systemic chemotherapy in CRLM is still controversial but promising, with FOLFOX as the main regimen for chemotherapy. FOLFIRI should not be recommended as adjuvant chemotherapy because of a negative phase III trial. Further studies are urgently needed to clarify the effectiveness of adjuvant systemic chemotherapy by stratification of patients with risk factors that are more predictive of advanced disease, and by identification of patients who will more probably benefit from systemic chemotherapy.

3. Molecular targeted therapy

Although molecular targeted therapy has been widely used for advanced colorectal cancer, bevacizumab (BV), cetuximab, and panitumumab were not allowed to be used in the adjuvant setting for patients with stage II or III colon cancer outside the setting of a clinical trial as suggested by the National Comprehensive Cancer Network (NCCN) guideline. As for stage IV colorectal cancer with liver metastasis, there is no evidence supporting the rationale of using adjuvant molecular targeted therapy. For bevacizumab, Kemeny et al. (21) conducted a randomized phase II trial of adjuvant hepatic arterial infusion and systemic chemotherapy with or without bevacizumab in patients with resected hepatic metastases from colorectal cancer. With a median follow-up of 30 months, 4-year survival was 85% and 81% (p = 0.5), 4-year RFS was 46% versus 37% and 1-year RFS was 83% and 71% (p = 0.4) for No BEV versus BEV arms. So it was obvious that the addition of BEV to adjuvant HAI plus systemic therapy after liver resection did not increase RFS or survival. Furthermore, the combination with BEV appeared to increase biliary toxicity. Meanwhile, Turan et al. (22) also showed that addition of BEV to chemotherapy had no impact on both RFS and OS, with median RFS (p = 0.375) and OS (p = 0.251) similar in BEV and NoBEV arms. Until now, the evidence level of combination of bevacizumab with systemic therapy was not high; the only randomized two-arm phase III study (23) is ongoing in patients after radical resection of CRLM to investigate bevacizumab in combination with capcitabine plus oxaliplatin (CAPOX) versus CAPOX alone as adjuvant treatment (Table 3).

The story with cetuximab was even worse, in a newly released result from a randomised phase-III clinical trial (New EPOC study) (24), addition of cetuximab to chemotherapy and surgery for operable colorectal liver metastases in KRAS exon 2 wild-type patients results in shorter progression-free survival. However, resectable hepatic metastases have been identified as an ideal setting for the development of such targeted approaches because of the availability of pre-and post-treatment tumor tissue for the identification of molecular biomarkers. In addition, resected stage IV disease could serve as a model for micro-metastatic disease for the development of novel adjuvant therapies for earlier stage colorectal cancer (CRC).

In conclusion, there is no evidence supporting the combination of BEV and chemotherapy to benefit patients with resected CRLM, and a randomized phase III study is ongoing to reveal the answer more thoroughly. The addition of cetuximab to chemotherapy after surgery should not be recommended. Other molecular targeted therapy should be tested in phase III clinical trials.

4. Hepatic artery infusion (HAI) in combination with systemic chemotherapy

The most common site of recurrence develops in the remnant liver or other organs after hepatic resection for patients with CRLM. Recognition of differences in the blood supply to metastases compared to normal liver parenchyma has allowed for the development of hepatic arterial delivery of systemic chemotherapeutic...
agents. Roughly, 20-25% of blood entering the liver is supplied by the hepatic arteries, and 75-80% is supplied by the portal vein. However, experimental studies have demonstrated that hepatic tumors 0.5-3.0 cm or greater in diameter are fed mainly from the hepatic arteries. Hepatic arterial administration can therefore deliver high concentrations of drugs to metastatic tumors in the liver. Furthermore, drugs used for HAI have a short half-life and are primarily metabolized in the liver, allowing extremely low drug concentrations to be maintained in the peripheral blood, thereby minimizing the risk of systemic adverse events. HAI chemotherapy provides much better local control of liver metastases from colorectal cancer than systemic chemotherapy (25). The most commonly used agent in HAI is floxuridine (FUDR), a pyrimidine antimetabolite that is converted to 5-fluorouracil in the liver. Floxuridine has a very high rate of hepatic extraction and a short half-life, making it optimal for hepatic infusion (26). Other chemotherapeutic drugs have also been used in HAI, Kemeny was the first to apply HAI with oxaliplatin in 2001 (27), and HAI with irinotecan in 2005 (28).

HAI with FU/DXM in combination with intravenous FU, with or without LV has yielded clinical benefits in CRLM (Table 4). Kemeny et al. (29) from MSKCC conducted a randomized controlled phase-III clinical trial; 74 patients were randomized to combined HAI and systemic chemotherapy and 82 to systemic chemotherapy alone. A significant benefit was seen in patients receiving combined therapy. The median survival in the group receiving combined therapy was 68.4 months compared with 58.8 months for those receiving systemic chemotherapy alone. At 2 years the rate of survival free of hepatic recurrence was 90% in the combined-therapy group compared with 60% in the systemic chemotherapy-only group \((p < 0.001)\). However recurrence outside the liver appeared similar in both groups. Recently, Kemeny et al. (30) re-analyzed patients in the same trial with a median follow-up of 10.3 years and found that overall PFS is significantly greater in the combined-therapy group than in the monotherapy group \((14.1 \text{ vs. } 20.5 \text{ months}, HR \text{ 1.48, } p = 0.030)\). The median survival free of hepatic progression has not yet been reached in the combined therapy group, whereas it has reached 32.5 months in the monotherapy group \((p < 0.01)\). However, the benefit of overall survival was only marginally significant, with a median OS of 68.4 months versus 58.8 months in the combined and monotherapy group, respectively \((p = 0.10)\). Furthermore, patients with a high risk of recurrence (a score of 3 to 5) as evaluated by a clinical risk score had a median survival of 60.0 months in the combined therapy group and 38.3 months in the monotherapy group \((p = 0.13)\), while patients with a lower risk of recurrence (a score of 0-2) had a similar median survival (83.3 months vs. 82.8 months), indicating that the effect of postoperative HAI may be more potent in patients with residual disease.

Three cohort studies (31-33) showed a significant PFS benefit with HAI therapy but a significant OS.

### Table 3. Adjuvant molecular targeted therapy after curative resection of synchronous/metachronous CRLM

<table>
<thead>
<tr>
<th>Author</th>
<th>Ref.</th>
<th>Study types</th>
<th>No. cases</th>
<th>Regimen</th>
<th>Survival benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kemeny (2011)</td>
<td>21</td>
<td>Phase II (two-armed) (MSKCC)</td>
<td>156</td>
<td>HAI + systemic chemotherapy (fluorodeoxyuridine/DXM) with or without BEV. Systemic therapy and BEV 5 mg/kg was delivered on days 15 and 29; oxaliplatin 85 mg/m^2 (or irinotecan 150 mg/m^2), LV 400 mg/m^2, and fluorouracil 2,000 mg/m^2 infusion for 2 days</td>
<td>BV-no additive benefit to chemotherapy. 4-year OS: 85% vs. 81% ((p = 0.5)). 4-year RFS: 46% vs. 37%. 1-year RFS: 83% vs. 71% ((p = 0.4)) for no BEV versus BEV arms</td>
</tr>
<tr>
<td>Turan (2013)</td>
<td>22</td>
<td>Cohort study. (Turkey)</td>
<td>204</td>
<td>Chemotherapy with fluoropyrimidine-based ((n = 27)), irinotecan-based ((n = 84)) and oxaliplatin-based ((n = 93)) combinations. 87 received BEV while 117 did not (No BEV).</td>
<td>Chemotherapy type and addition of BEV have no impact on both RFS and OS; Median RFS ((p = 0.375)) and OS ((p = 0.251)) were similar.</td>
</tr>
<tr>
<td>Primrose (2014)</td>
<td>24</td>
<td>Phase III RCT (New EPOC study) (Southampton, UK)</td>
<td>236 (117 vs. 119)</td>
<td>CapeOx regimen and Cetuximab (regimen one: 500 mg/m^2 every 2 weeks; regimen two: a loading dose of 400 mg/m^2 followed by a weekly infusion of 250 mg/m^2)</td>
<td>PFS significantly shorter in chemotherapy plus cetuximab group than chemotherapy group ((14.1 \text{ vs. } 20.5 \text{ months}, HR \text{ 1.48, } p = 0.030)).</td>
</tr>
<tr>
<td>Snoeren (2010)</td>
<td>23</td>
<td>Phase III RCT</td>
<td>n.a.</td>
<td>Bevacizumab in combination with capecitabine plus oxaliplatin (CapeOx) vs. CapeOx alone</td>
<td>ongoing</td>
</tr>
</tbody>
</table>

Notes: DFS, disease-free survival; OS, overall survival; PFS, progression-free survival; DXM, dexamethasone; HAI, hepatic arterial infusion; BV, bevacizumab; CapeOx, Capecitabin and Oxaliplatin; RCT, randomized clinical trial; HR, hazard ratio.
benefit was only reported in one study (33), the other two studies only revealed a marginal survival benefit (31, 32) (Table 4). So in conclusion, HAI therapy has shown better improvement of PFS but to a less extent in prolonging OS, suggesting a better local control of liver metastasis after resection of CRLM. There is a clue that HAI may be more effective in patients with higher risk scores for recurrence, indicating that HAI may be applied to patients with residual disease in the era of individualized medicine.

5. Adverse effect

Pathologic lesions of the background liver may be encountered among patients who undergo chemotherapy, with sinusoidal dilatation tending to occur if oxaliplatin is used in combination chemotherapy (34), and steatohepatitis if CPT-11 (irinotecan) is used (35), neurotoxicity is also very common from oxaliplatin (36). A phase II study which compared systemic chemotherapy without BV or with BV showed that the combination of systemic chemotherapy with BV resulted in incidences of biliary complications, in which 4 out of 5 patients with an elevated total bilirubin level received a biliary stent due to chemotherapy and BV (27).

For HAI, biliary sclerosis after hepatic arterial infusion pump chemotherapy for patients with colorectal cancer liver metastasis should be kept in mind (37). In patients who received a combination of mitomycin (MMC), the biliary sclerosis happened in 4.6%-13.4% of patients and the adverse effect was more likely to happen in patients with adjuvant therapy than in patients with an advanced stage (5.5% vs 2.0%) (26). It has been reported that the incidence of biliary sclerosis can be reduced by DXM and be effectively managed if detected early. Even though, the implantation and maintenance of HAI pumps is challenging and only a few large-volume centers have the expertise, and complication rates specifically attributable to HAI pumps have ranged from 22-41% (38, 39). The liver injury of preoperative chemotherapy with modern chemotherapeutic medications and major liver resection also adds more risk for adjuvant HAI (40).

Table 4. Hepatic artery infusion (HAI) in combination with systemic chemotherapy

<table>
<thead>
<tr>
<th>Author</th>
<th>Ref.</th>
<th>Study types</th>
<th>No. cases</th>
<th>Regimen for chemotherapy</th>
<th>Survival benefit for chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kemeny (1999)</td>
<td>29</td>
<td>Phase III (MSKCC, US)</td>
<td>156 (74 vs. 82)</td>
<td>6 Cycles of HAI with fluorouridine/DXM + intravenous FU, with or without LV, or six weeks of similar systemic therapy alone</td>
<td>The median OS: 72.1 m vs. 59.3 m; 2-year hepatic RFS 90% vs. 60%; 2-year RFS rate 57% vs. 42% (p = 0.07).</td>
</tr>
<tr>
<td>Kemeny (2005)</td>
<td>30</td>
<td>Sam as above</td>
<td>156 (74 vs. 82)</td>
<td>Sam as above</td>
<td>Overall PFS is greater in the combined-therapy group (31.3 vs. 17.2 months, p = 0.02). Median hepatic RFS is greater (not reached vs. 32.5 months (p &lt; 0.01). Clinical-risk scoring system predicted survival. Patients with score 3-5 had OS of 60 m to 38.3 m, and patients with score 0-2 had of 83.3 m vs. 82.8 m.</td>
</tr>
<tr>
<td>Go’er’e (2013)</td>
<td>31</td>
<td>Cohort study (France)</td>
<td>98 (44 vs. 54)</td>
<td>HAI: oxaliplatin 100 mg/m²; IV: modified LV5-FU2 or the de Gramont regimen.</td>
<td>3-Year DFS significantly longer for HAI-OXA + IV than IV (33% vs. 5%, p &lt; 0.0001). 3-year OS slightly higher for HAI + IV group (75% vs. 62%, p = 0.17).</td>
</tr>
<tr>
<td>House (2011)</td>
<td>32</td>
<td>Cohort study (MSKCC, US)</td>
<td>250 (125 vs. 125)</td>
<td>HAI-FUDR/DXM + systemic chemotherapy. Systemic chemotherapy: FU/LV plus oxaliplatin or irinotecan.</td>
<td>Combination of HAI-FUDR improved 5-year liver RFS, overall RFS, and DSS compared to systemic chemotherapy alone.</td>
</tr>
<tr>
<td>Ota (2004)</td>
<td>33</td>
<td>Cohort study (Yokohama, Japan)</td>
<td>84 (37 vs. 47)</td>
<td>HAIC: 1,500 mg of 5-FU, 24-h continuous infusion once a week for 8 weeks.</td>
<td>5-Year liver RFS were 72.6% in the HAIC group and 29.8% in the control group (p = 0.0005). 5-year OS: 61.4% vs. 28.0% (p = 0.0069).</td>
</tr>
<tr>
<td>Alberts (2010)</td>
<td>41</td>
<td>PHASE-II single armed (Mayo Clinic, US)</td>
<td>76</td>
<td>HAI-FUDR/DXM/Heparin Systemic: CapeOX (OXA:130mg/m² + Cape 1,700 mg/m²/d)</td>
<td>2-Year survival rate 86%. 88% alive at 2 years after operation. 30 patients had disease recurrence.</td>
</tr>
</tbody>
</table>

DFS, disease-free survival; OS, overall survival; DSS, disease-specific survival; RFS, recurrence-free survival; FU/LV, 5-fluorouracil/leucovorin; OXA, oxaliplatin; HAI-FUDR: hepatic arterial infusion with FUDR; DXM, dexamethasone
6. Conclusions

In conclusion, CRLM with resectable liver metastasis benefits from adjuvant chemotherapy either with systemic chemotherapy to control systemic metastasis or hepatic arterial infusion to control liver-specific recurrence. The combination of molecular targeted therapy should be withdrawn except in the settings of clinical trials, as it not only has no benefit but also may impair survival. Further studies should be emphasized on personalized therapy by identifying patients with residual disease and with higher-risk of distant metastasis; molecular targeted therapy should be given to patients with residual disease and with activation of specific pathways.

Acknowledgements

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References


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Chemical constituents and bioactivities of *Colla corii asini*

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**Summary**

In China, *Colla corii asini* is a health-care food and traditional Chinese medicine widely used in life-nourishing and clinical hematic antanemic therapy for more than 2,000 years. In this paper we compiled the chemical constituents isolated and detected from *Colla corii asini* including amino acids, proteins/gelatins, polysaccharides, volatile substances, inorganic substances, etc. Meanwhile we investigated the biological activities of *Colla corii asini*, which have been reported over the past few decades, including, hematologic diseases inhibitory activities, anti-aging activity, antitumor activity, immunomodulatory activity, bone repair activity, anti-inflammatory activity, antifatigue activity, etc. However, few reports on the relationships between the chemical constituents and bioactivities have been found, further studies of *Colla corii asini* are still necessary to facilitate research and development in the future.

**Keywords:** *Colla corii asini*, chemical constituents, biological activities

1. **Introduction**

*Equus asinus* Linnaeus (*Equus asinus* L, Figure 1A), commonly known as domesticated ass or donkey, is widely distributed in the northeast, north and northwest of China. The skin of *Equus asinus* L. has long been used as the key raw material to prepare *Colla corii asini* (*E'jiao, A'jiao*). *Colla corii asini* (Figure 1B), a gelatin-like block shaped preparation, belongs to the minority of top-grade traditional Chinese medicine (TCM) which should be obtained through a refining process after water extraction from *Equus asinus* L. skin (1). In China, *Colla corii asini* is a health-care food and TCM widely used in life nourishing and clinical hematic antanemic therapy for more than 2,000 years (2,3). In 2013, the sales of *Colla corii asini* have reached nearly 2 billion Yuan.

Studying active compounds is important for the development of TCM. These compounds could be meaningful for the understanding of mechanisms of action, and could constitute a promising bio-resource for the development of potential drugs and value-added products. Although little study to date has addressed the pharmacological action of the chemical compounds from *Colla corii asini*, the chemical and pharmacological properties of *Colla corii asini* have been investigated (especially in China) since the 1980s (4,5). Several bioactive natural products, mainly gelatins and amino acids, have been reported in *Colla corii asini*. In this review, we compiled the chemical constituents isolated from *Colla corii asini* over the past few decades. The biological activities of *Colla corii asini* and its constituents are also discussed.

2. **Chemical constituents**

Several classes of compounds have been isolated from *Colla corii asini*, including amino acids, proteins/gelatins, polysaccharides, volatile substances, inorganic substances, etc. Some of their names, 1-58, are collected in Table 1, and some of their structures, 1-24, are shown in Figure 2.

2.1. **Amino acids**

Amino acids are the most abundant components of *Colla corii asini*. From the 1980s, the amino acid
composition in Colla corii asini has been determined repeatedly using automatic amino acid analyzers (1,4,6-8). It has been reported that 18 types of amino acids were detected in hydrolyzed Colla corii asini, and the total content was from 51.94% to 82.03%. Their names and respective content are collected in Table 2.

In addition, Cheng et al. determined 4 amino acids using a pre-column derivatization high performance liquid chromatography (HPLC) method. Besides glycine, alanine and proline that have been listed, the content of hydroxyproline was determined to be 8.99%–11.23% (9).

2.2. Proteins/gelatins

The total protein content of Colla corii asini was determined to be 74.56% to 84.94% using the Kjeldahl nitrogen determination method (4). From the refined preparation of the skin of Equus asinus L., the constituent proteins of Equus asinus L. skin could support a clue to expose the constituents of Colla corii asini. In 2006, three majority proteins, collagen α1 (I), collagen α2 (I) and donkey serum albumin were determined from the skin of Equus asinus L. Their respective content was about 12.6%, 11.67% and 19.6% in the total proteins (10). Then, a citric-soluble collagen and a pepsin-soluble collagen were successfully extracted from Equus asinus L. skin, and both were identified as type I collagen, containing two different α chains (α1 and α2) (11).

Gelatin is commonly considered to be the most abundant and biologically active component of Colla corii asini, and has been determined (4,6-11). Then, a citric-soluble collagen and a pepsin-soluble collagen were successfully extracted from Equus asinus L. skin, and both were identified as type I collagen, containing two different α chains (α1 and α2) (11).

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corii asini (1-3). It is a mixture of peptides and proteins produced by partial hydrolysis of collagen (12-14). The content of hydroxyproline which marked the composition of gelatin was determined to be 8.99-11.23% in Colla corii asini (9). In 2012, in order to distinguish Colla corii asini from other animal skin glue, the marker for Colla corii asini gelatin was identified as the fragment GEAGPAGPAGPGPVGAR by an ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC/Q-TOF-MS) sample profiling method coupled with principal component analysis (PCA) (15).

2.3. Polysaccharides

Polysaccharides are important components of the skin of Equus asinus L. However, so far, only dermatan sulfate (DS), 1, was isolated from Colla corii asini (16,17). DS is a glycosaminoglycan (GAG) that is distinguished from chondroitin sulfate (CS) by the

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Content (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>3.37-5.14</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.11-1.31</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Serine</td>
<td>1.25-2.86</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>6.27-9.01</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Glycine</td>
<td>13.36-23.63</td>
<td>(1,4,6,7,9)</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.33-9.22</td>
<td>(1,4,6,7,9)</td>
</tr>
<tr>
<td>Valine</td>
<td>1.71-2.31</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.29-1.56</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.46-1.38</td>
<td>(1,4,6,7,8)</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.19-3.45</td>
<td>(1,4,6,7,8)</td>
</tr>
<tr>
<td>Tyronine</td>
<td>0-2.27</td>
<td>(1,4,6,7,8)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.35-2.44</td>
<td>(1,4,6,7,8)</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.42-3.57</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.26-0.30</td>
<td>(1,4,6)</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.53-0.88</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.54-6.76</td>
<td>(1,4,6,7)</td>
</tr>
<tr>
<td>Proline</td>
<td>6.52-13.50</td>
<td>(1,4,6,7,9)</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>0.50</td>
<td>(1)</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>8.99-11.23</td>
<td>(9)</td>
</tr>
<tr>
<td>NH3</td>
<td>0.28-3.27</td>
<td>(1,4)</td>
</tr>
<tr>
<td>Total</td>
<td>51.94-82.03</td>
<td>(1,4,6,7)</td>
</tr>
</tbody>
</table>
presence of iduronic acid (IdoA), the C-5 epimer of D-glucuronic acid (GlcA) (18-20).

2.4. Volatile substances

In 2010, a total of 23 volatile substances, 2-24, have been detected from Colla corii asini using a gas chromatograph-mass spectrometer (GC-MS). These volatile substances mainly included esters (2-3), ketones (4-6), halogenated hydrocarbons (7-13), heterocyclic compounds (14-18) and others (19-24) (21).

2.5. Inorganic substances

The contents of 8 inorganic oxides (25-32) and 26 inorganic elements (33-58) of Colla corii asini were qualitative and quantitative determined using atomic absorption spectroscopy (AAS), emission spectra (ES), and an inductively-coupled plasma emission spectrometer (ICP-AES). Calcium oxide (0.18%) and sodium (0.35%) respectively represent the highest content of inorganic substances (4,6).

3. Biological activities

3.1. Hematologic diseases inhibitory activities

Colla corii asini was reported to treat various hematologic diseases, including anemia, aleucocytosis, thrombopenia, etc.

3.1.1. Anti-anemia Activity

As a TCM, Colla corii asini has been widely used in clinical hematic antanemic therapy in China for more than a thousand years (1). However, until recent years, little study had addressed the effect of Colla corii asini on the anti-anemia process using modern pharmacological methods. From 2007 to 2011, Wu and co-workers investigated the hematopoietic effect and mechanism of fractions from enzyme-digested Colla corii asini on anemic mice separately induced by 5-fluorouracil, γ-rays, or cyclophosphamide, etc. (3,22-24). The results suggested that fractions from the enzyme-digested Colla corii asini promoted hematopoiesis by activating immature granulocyte and erythroid cells, partly by stimulating granulocyte-macrophage colony stimulating factor (GM-CSF) in all mice separately induced by 5-fluorouracil, γ-rays, or cyclophosphamide (CTX), etc. Dissimilarly, fractions promoted hematopoiesis partly by stimulating erythropoietin (EPO) secretion and suppressing serum transforming growth factor (TGF-β) release in 5-fluorouracil induced mice, partly by stimulating interleukin-6 (IL-6) secretion and elevating the reactive oxygen species (ROS) scavenging ability in γ-ray induced mice, and partly by stimulating CD34 secretion and increasing the ratio of S-phase-cells in CTX induced mice.

In 2011, Song et al. identified the curative effect of Colla corii asini on anemic mice induced by phenylhydrazine hydrochloride (25). In 2012, Peng et al. suggested that Radix Angelica Sinensis combined with Colla corii asini could improve hypoferric anemia in vivo in rats induced by low iron feed (26).

3.1.2. Thrombocytopenia therapeutic activity

Hemostasis has been recorded to be another important activity of Colla corii asini for a thousand years. In modern pharmacology research theory, platelets (PLT) are considered to be a key factor in hemostasis. From 2002 to 2006, Wei and co-workers observed the clinical curative effect of Colla corii asini in treating peripheral thrombocytopenia patients with malignant tumors (including lung cancer, esophageal cancer, liver cancer, gastric cancer, breast cancer and lymphoma) after radiotherapy or chemotherapy. The results showed that a large dose of Colla corii asini could significantly increase PLT levels ($p < 0.05$) and stimulate the activity of bone marrow stem cells (particularly the megakaryocytic cells) in these radio- or chemotherapeutic cancer patients (27-29).

3.1.3. Leukocyte increasing activity

In 2002, Zhang et al. reported that Colla corii asini could exert clinical curative effects on leukopenia patients caused by clozapine (30). Since then the effects and mechanisms of Colla corii asini to increase leukocytes gradually attracted attention from many researchers. In 2005 and 2009, studies separately performed by Zheng et al. and Xu et al. showed that Colla corii asini could increase leukocytes in vivo in CTX-induced leukopenia in mice. In the model mice, Colla corii asini could improve thymus index (TI) and spleen index (SI), and bone marrow cells through recovering the life cycle of bone marrow karyocyte cell, raise the contents of CD34+ cells and red blood cells (RBC), and increase the level of hemoglobin (HB), interleukin-3 (IL-3) and GM-CSF (31,32). In 2011, Ying et al. identified that Colla corii asini could also improve leukopenia symptoms in rats induced by CTX (33).

3.2. Anti-aging activity

As a life-nourishing food in China, Colla corii asini has always been considered to have an anti-aging effect (1). In 2001, Li et al. proved that Colla corii asini could improve the damage to learning and memory in lead-induced rats. In addition, the total antioxidant capacity of the hippocampus was detected to be increased significantly in Colla corii asini treated model rats.
The free radical theory of aging was conceived by Harman in 1956 (35). Abundant evidence suggests that oxidative stress plays a central role in the process of biological aging (36). In 2012, the potential anti-aging effect of Colla corii asini and related mechanisms was systematically investigated by Wang et al. using D-galactose (gal) induced aged model mice. Results indicated that Colla corii asini might have an effect to suppress the aging process through enhancing the antioxidative activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), scavenging free radicals such as malondialdehyde (MDA), and modulating aged-related gene expression (p16, p21) (1).

3.3. Antitumor activity

In 2005, Liu and co-workers performed serial research on the antitumor activity of Colla corii asini using modern pharmacological experiments. Their studies revealed that Colla corii asini could exhibit inhibitory effects on the growth of cancer cells in vitro, such as leukemia K562 cells and lung cancer PG cells. The growth-inhibitory effects were associated with apoptosis regulated by p53 and telomerase expression (37-40). Furthermore, their study also proved that Colla corii asini could suppress tumor growth in vivo in S180 sarcoma-bearing-mice, and prolong survival time of these model mice (41).

3.4. Immunomodulatory activity

In 2005, Zheng et al. reported that Colla corii asini could improve the proliferation and activation of depressed lymphocytes taken from radio-therapy cancer patients in vitro, as well as raise the ratio of Th1/Th2 cells, and the proportion of T cells and NK cells (42). Furthermore, the studies of Zhang et al. and Song et al. showed that Colla corii asini could enhance nonspecific and cellular immunity in vivo in hypo-immune mice induced by hydrocortisone. In these Colla corii asini treated model mice, delayed-type hypersensitivity (DTH), carbon clearance index, and paw swelling index were improved; SI and TI were increased; transformation ability of spleen lymphocytes and phagocytosis ability of celiac macrophages were enhanced; and the level of hemolysin, IL-3, and interferon-γ (IFN-γ) were improved. All the indexes were improved obviously compared to the model mice (p < 0.05) (43,44). Furthermore, Huang et al. proved the positive immunomodulatory activity of Colla corii asini in vivo in chickens using the erythrocyte rosette test (45).

3.5. Bone repair activity

In 2004, Gao et al. first reported that Colla corii asini could improve bone repair in vivo in the early- and meta-phase of bone repair in tibial drilled SD rats. The improvement might be associated with the proliferation of chondrocytes and osteoblasts regulated by expression of pro-collagen mRNA type I, II, III, and TGF-β1 mRNA. On the other hand, genes related to blood vessel formation, such as bone morphogenetic protein (BMP-2mRNA) and vascular endothelial growth factor (VEGF-mRNA) were influenced little by Colla corii asini (46). In 2009, a study by Chang et al. showed that Colla corii asini had no effect on the multiplication of Wistar rats' osteoblasts, but a positive effect on the differentiation of osteoblasts through promoting synthesis of alkaline phosphatase (ALP) in vitro (47).

3.6. Anti-inflammatory activity

In 2006, the effect of Colla corii asini to inhibit airway inflammation was first reported by Zhao et al. in asthmatic rats. The result showed that Colla corii asini could regulate the ratio of Th1/Th2 by decreasing Th2, and inhibit the shift of eosinophils from peripheral blood to the lungs (48). The clinical curative effects of Colla corii asini on digestive system inflammation such as ulcerative colitis, chronic atrophic gastritis, and peptic ulcers were demonstrated by Wu et al. and Chen respectively (49,50).

3.7. Antifatigue activity

In 2011, Colla corii asini was proved to have antifatigue activity simultaneously by Song et al. and Li et al. in mice using a weight-loaded swimming test. The results showed that Colla corii asini could increase liver index, promote the synthesis of liver glycogen and HB, and decrease the product of blood lactic acid and blood urea nitrogen in weight-loaded swimming mice (25,51).

3.8. Other activities

In 2009, Su et al. explored the clinical effect of Colla corii asini to improve uterine receptivity in controlled ovarian stimulation. The result suggested that Colla corii asini could improve the blood supply of the uterus, resulting in the improvement of endometrial thickness (32). Besides the above mentioned activities, the clinical therapeutic effect of Colla corii asini used to treat postoperative incision fat liquefaction and malignant hematuria were also reported in recent years (53,54).

4. Discussion

Colla corii asini (E’jiao, A’jiao), a gelatin-like preparation obtained through stewing and concentrating material from Equus asinus L. has been used as traditional Chinese medicine for more than 2000 years. Other activities include improving hemato-biochemical indexes, improving uterine receptivity in controlled ovarian stimulation, promoting angiogenesis by regulating BMP-2mRNA and vascular endothelial growth factor (VEGF-mRNA), and inhibiting airway inflammation. However, more studies are needed to fully understand the mechanism and long-term effects of Colla corii asini.
years. About 58 compounds were isolated or detected from *Colla corii asini* including mainly amino acids, proteins/gelatins, polysaccharides, volatile substances, inorganic substances, etc. As a health-care food and TCM, *Colla corii asini* showed a broad range of biological activities. Nevertheless, few reports on the relationships between the chemical constituents and bioactivities have been found, further studies to exploit other kinds of constituents, new biological activities and the relationships between chemical constituents and bioactivities are still necessary to facilitate further research and development.

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**References**


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Isotryptoquivaline F, a new quinazolinone derivative with anti TNF-α activity from Aspergillus sp. CM9a

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Summary Isotryptoquivaline F (1) was isolated from Aspergillus sp. CM9a, an endophytic fungus of Cephalotaxus mannii. The structure was elucidated by extensively 1D and 2D NMR and HR ESI MS spectroscopy. It has good TNF-α antagonistic effect, and can be used for anti-inflammatory drugs or other bioactive leading drugs.

Keywords: Cephalotaxus mannii, TNF-α antagonistic effect, anti-inflammatory drugs

1. Introduction

Endophytes are a group of microorganisms living within plant internal tissues or organs without causing any apparent symptoms or diseases in the hosts. They can serve as important sources of bioactive compounds, presumably due to the symbiotic relationship with their hosts (1). More recently, endophytes have been considered to be a prolific source of pharmacologically active natural products with potential medicinal or agrochemical applications (2,3). And we have started to investigate endophytic fungi as a source for biologically active natural products, and isolated a series of new compounds from endophytic microorganisms (4-9).

As part of our continuous screening for more active secondary metabolites from endophytic microorganisms, 11 compounds have been identified from strain Aspergillus sp. CM9a (9), and this time, isotryptoquivaline F (1) (Figure 1) was obtained from the fermentation extracts of A. sp. CM9a and it showed good anti TNF-α activity.

2. Materials and Methods

2.1. General experimental procedures

Mass spectra were measured using a Bruker DRX-600 NMR spectrometer; NMR spectra were measured on Bruker Bio TOF-Q spectrometer; NMR spectra were measured on Bruker DRX-600 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. Reversed-phase (RP) C18 silica gel for column chromatography (CC) was obtained from Merck and Sephadex LH-20 was obtained from Amersham Biosciences. Silica gel (200-300 mesh) for CC and silica gel GF254 for TLC were purchased from Qingdao Marine Chemical Ltd., Qingdao, Shandong, China. DMEM culture media was purchased from Gibco BRL. TNF-α was purchased from Sigma. And Cell Counting Kit-8 (CCK-8) was obtained from Dojindo, Japan.

2.2. Microorganism specimens

The fungal stain Aspergillus sp. CM9a was isolated from the current-year stems of Cephalotaxus mannii collected from Xishuangbanna, Yunnan, China (9). It was deposited at China Center for Type Culture Collection (CCTCC No: M2011006).

2.3. Fermentation and isolation of compound 1

The strain was incubated for 14 d at 28°C on potato-dextrose-agar (PDA) medium. The fermentation culture was extracted with EtOAc/MeOH/AcOH (80:15:5), and the extract partitioned between H2O/EtOAc. The EtOAc extract (4.2 g) was separated to nine fractions (Fr. A-H) by column chromatography (RP-18, 80 g), eluted with MeOH/H2O (0:100, 40:60, 60:40, and 100:0). These fractions were further purified by repeated column chromatography on Sephadex LH-20, RP-18...
silica gel and silica gel.

Fr. C (699 mg) was separated by CC (RP-18, 80 g, MeOH/H₂O 30:70; 40:60; 50:50) to give four fractions (Fr. C₁-C₄). Fr. C₂ was separated to four fractions (Fr. C₂a-C₂d) by CC (Sephadex LH-20, MeOH). Fr. C₂d was subjected to CC (Sephadex LH-20, acetone) to afford Fr. C₂d₃ (13 mg). Compound 1 (5 mg) was finally purified by Sephadex LH-20 eluted with acetone from Fr. C₂d₃.

2.4 Biological study

The anti TNF-α (Tumor necrosis factor-α) activity was evaluated against mouse fibroblast cell line L929 with TNF-α at 3 ng/mL for 24 h by WST-8 colorimetric assay (Cell Counting Kit, Dojindo, Japan).

The trypsin-dispersed cells L929 in 100 μL of DMEM culture medium containing 10% FBS were plated in each well of 96-well plates (Falcon, CA) at density of 10⁴ cells/mL. After growing for 24 h, the cells were washed with fresh culture media and then treated in triplicate with various concentrations of compound 1 (95 μL DME and 3 μL TNF-α and 2 μL compound in DMSO, and 97 μL DME and 3 μL DMSO as negative control; and 97 μL DME and 3 μL TNF-α as blank control) for 24 h at 37°C. Then 90 μL fresh DEME media and 10 μL CCK-8 (cell counting kit-8) solution were added directly to all wells and incubated for 2 h at 37°C.

The optical density of each well was measured with a microplate reader (M-3350, Bio-Rad) at 450 nm. Cell survival rate was calculated by the following equation: cell survival rate = (ODcontrol − ODtreated)/ODcontrol × 100%.

3. Results and Discussion

3.1. Elucidation of structure

Compound 1, [α]D²⁰ = −27.9 (c 0.43, MeOH), was obtained as white powder, and was determined to have the molecular formula C₂₂H₁₈N₄O₄ by HR-ESI-MS (403.1251 [M + H]⁺, 425.1031 [M + Na⁺]) and ¹³C-NMR.

Its ¹H-NMR spectrum exhibited one methyl doublet at δ 1.73 (3H, d, J = 6.9 Hz), one methylene signals at δ 2.64 (dd, J = 7.0, 6.4 Hz), 5.60 (s), nine aromatic protons at δ 7.71 (1H, d, J = 8.1 Hz), 7.43 (1H, t, J = 7.6 Hz), 7.49 (1H, d, J = 1.6 Hz), 7.58 (1H, m), 7.60 (1H, m), 7.71 (1H, d, J = 8.1 Hz), 7.86 (1H, t, J = 8.1 Hz), 8.24 (1H, t, J = 7.2 Hz) and 8.23 (s). The ¹³C-NMR and DEPT spectra (Table 1) displayed signals of three carbonyls (δ 173.6, 167.5, 140.4), five quaternary sp³ (δ 167.5, 148.6, 140.4, 137.4, 122.7), nine methine sp² (δ 148.6, 136.0, 131.3, 128.7, 128.0, 127.7, 127.0, 116.2, 116.2), one quaternary sp² (δ 78.1, 2 methine sp³ (δ 84.6 and 61.4), one methylene sp³ (δ 39.5) and one methyl carbons (δ 13.3).

The coupling system of the aromatic protons observed in the COSY spectrum (Table 1) revealed the presence of two 1, 2-disubstituted benzene rings. Analysis of the HMBC spectrum (Table 1) indicated that one of the 1, 2-disubstituted rings was part of the quinazolin-4(3H)-one moiety while another belonged to the indole portion of the molecule. The HMBC correlations between the signals of H-26 (δ 8.23, s) and C-24 (δ 148.6) as well as between the signals of H-20 (δ 7.71, d, 8.1,) and C-18 (δ 162.4), C-24 (δ 148.6), C-19 (δ 122.7) permitted identification of the N-substituted quinazolin-4-one and a 6-5-5 gem-dimethyl imidazoindolone ring system was
evidenced by the HMBC correlations between the signals of H-2 and C-3, C-9; the signals of H-13 and C-2, C-3, C-11 as well as between the signals of H-27 and C-15, and H-15 and C-14 and C-27. Above data suggested that compound 1 could correspond to the previously reported tryptoquivaline F or its C-12 epimer, tryptoquivaline J. (10,11). The main difference is that the five-membered spirolactone in tryptoquivaline F turned into an olefin alcohol because of keto-enolic tautomerism, which further confirmed by the chemical shifts of C-11 and 12 (δ 170.8 and 57.0 in tryptoquivaline F (Figure 1); 167.5 and 167.5 in compound 1). The NOESY spectrum exhibited clearly correlations between the signals of H-2 and H-15. Whereas, the orientations of H-2 and H-15 are opposite in tryptoquivaline F (11).

Therefore compound 1 was identified as isotryptoquivaline F because of the difference of the relative configuration of C-2 and C-15.

3.2. Biological study

The TNF-α inhibitory activity of 1 was dose-dependent manner (Figure 2), the survival rate of L929 cell lines rose from about 16.7% to 63.9% when the concentration of 1 changed from zero to 10 μg/mL (EC_{50} = 8.7 μM), which indicated that 1 had good activity against the necrotic cell death induced by TNF-α.

TNF-α is a pleiotropic cytokine that mediates biological activities in many immune-mediated inflammatory diseases such as rheumatoid arthritis, psoriasis, septic shock and inflammatory bowel disease (12). Blockage of the effect of TNF-α has been proved efficient for treating these diseases (13). However, the current clinically approved protein-based TNF-α inhibitors are capable of reducing TNF-α activity, but can have serious side effects (12).

Many natural compounds belonging to various classes such as phenolics, terpenes and alkaloids and cytochalsan have been found to inhibit the upstream signaling pathways to inhibit the expression of TNF-α (14,15), but there is no lead compound that can inhibit the excessive TNF-α or its downstream pathways. Here, we reported a new quinazolinone derivatives Isotryptoquivaline F, that was prepared from an endophytic strain A. sp. CM9a and exhibited good anti-TNF-α activity.

This is the first report that quinazolinone derivative compound exhibit TNF-α inhibitory activity, while the detailed biological activity and identified target of 1 are on the way to elucidate.

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References


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**Brief Report**

**Human mediator subunit MED15 promotes transcriptional activation**

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1. Introduction

The Mediator complex (Mediator) is a transcriptional cofactor that is highly conserved among eukaryotes, has up to 30 subunits and consists of four modules: head, middle, tail, and CDK/Cyclin (1-3). Of these, the tail module has been the least extensively studied to date. The tail module, which recruits various transcriptional regulators to the transcription machinery, consists of eight subunits: MED2 (also known as MED29), MED3 (also known as MED27), MED5 (also known as MED24), MED14, MED15, MED16, MED23, and MED25 (1,4). One of the subunits, MED15, was originally identified as Gal11 in budding yeast, where it plays an essential role in the transcriptional regulation of galactose metabolism with the potent transcriptional activator Gal4. For this reason, we investigated the function of the human MED15 subunit (hMED15) in transcriptional activation. First, we measured the effect of hMED15 knockdown on cell growth in HeLa cells. The growth rate was greatly reduced. By immunostaining, we observed the colocalization of hMED15 with the general transcription factors TFIIE and TFIIH in the nucleus. We measured the effects of siRNA-mediated knockdown of hMED15 on transcriptional activation using two different transcriptional activators, VP16 and SREBP1a. Treatment with siRNAs reduced transcriptional activation, and this reduction could be rescued by overexpression of HA/Flag-tagged, wild-type hMED15. To investigate hMED15 localization, we treated human MCF-7 cells with the MDM2 inhibitor Nutlin-3, thus inducing p21 transcription. We found that hMED15 localized to both the p53 binding site and the p21 promoter region, along with TFIIE and TFIIH. These results indicate that hMED15 promotes transcriptional activation.

**Keywords:** Mediator complex, transcriptional activation, MED15, RNA polymerase II, TFIIE, TFIIH

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although their structures share a striking resemblance, their binding specificities differ (7,9,10).

In eukaryotes, the expression of protein-coding genes is strictly regulated at the level of transcription by RNA polymerase II (Pol II) (11-13). Pol II requires five general transcription factors, TFIIB, TFIID, TFIIE, TFIIF, and TFIIF. TFIIE consists of two subunits, α and β, and functions in transcription initiation, as well as in the transition from initiation to elongation (14). Human TFIIE (hTFIIE) consists of ten subunits (XPB, XPD, p62, p52, p44, p34, Cdk7, Cyclin H, MAT1, and p8), which possess multiple catalytic activities that are required not only for transcription by Pol II but also for nucleotide excision repair (NER) (15). Human TFIIE (hTFIIE) recruits hTFIIH through the interaction between the acidic domain of hTFIIEα and the pleckstrin-homology domain of hp62. This may be an essential step for Pol II activation upon its C-terminal domain (CTD) phosphorylation (16).

Since yeast MED15 (Gal11) binds TFIIE (17), it is possible that hMED15 carries out some essential roles by interacting with hTFIIE. Thus, we studied the function of hMED15 in transcriptional activation. For the siRNA knockdown of hMED15, we designed three different siRNAs, all of which reduced hMED15 expression at the protein level. Knockdown of hMED15 caused slow growth and reduced transcriptional activation. With immunofluorescence microscopy, we observed hMED15 primarily in the HeLa cell nucleus, mostly co-localized with hTFIIE and hTFIIH. We investigated the localization of hMED15, TFIIE, and TFIIF on the p53 target gene p21 by chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR); these experiments revealed that all three proteins primarily localized to the upstream p53 binding site (p53BS1) and the core promoter region. These results suggest that hMED15 promotes transcriptional activation in collaboration with the general transcription factors TFIIE and TFIIF.

2. Materials and Methods

2.1. Plasmids

Full-length human Med15 cDNA (transcript variant 2, NCBI Accession No. NM_015889) was cloned into the pOTB7 mammalian expression vector (Life Technologies Japan). The VP16 (amino acids 413–490) and SREBP1a (amino acids 1–50) activation domains were fused to the Ga4 N-terminal DNA binding domain (amino acids 1–147) and cloned into the pM mammalian expression vector (Clontech Laboratories, Mountain View, CA, USA).

2.2. Antibodies

Anti-hMED15 (11566-1-AP, Proteintech group, Chicago, IL, USA), anti-hTFIIEα (C-17, Santa Cruz Biotechnology, Dallas, Tx, USA), anti-p62 (Q-19, Santa Cruz Biotechnology), and anti-Pol II (N-20, Santa Cruz Biotechnology) rabbit polyclonal antibodies were used. Goat anti-rabbit IgG polyclonal antibody (AP132, Chemicon International Inc., Billerica, MA, USA) was used as a secondary antibody.

2.3. siRNAs

Three siRNAs were used for siRNA transfection. The siRNA sequences that were synthesized by Invitrogen are shown below.

Non-target siRNA:
- sense strand: 5'-AUUCUAUCACACUCGUGACuu-3'
- antisense strand: 5'-GUCACGCUAGUGAUAGAAUUu-3'

hMED15-191:
- sense strand: 5'-AAACAUGGCUCUCUAUCUCCUGCU-3'
- antisense strand: 5'-CAGCAAGGAUAUGGAGAGCGAUA-3'

hMED15-304:
- sense strand: 5'-UUCAAUGGAUAUGGAGAGCGAUA-3'
- antisense strand: 5'-AAGCUUCCGUCAGUGAUCCUGAA-3'

hMED15-1558:
- sense strand: 5'-UUCAAUGGACGCUCCUCAGCUUGCU-3'
- antisense strand: 5'-UGGACACAGGAAGCAGCUGUGCGAA-3'

2.4. Luciferase reporter assay

HeLa S3 and MCF-7 cells were cultured at 37°C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 7 mg/mL of penicillin-streptomycin, 5% calf serum, and 29.2 mM glutamine.

2.5. Cell culture

HeLa S3 cells were seeded in 24-well plates at a density of 4 x 10⁵ cells/well. After 1 day, siRNA oligos (final 12nM) were transfected into cells using Lipofectamine 2000 (Life Technologies Japan, Tokyo, Japan). Cells were cultured for 2.5 days, and, after washing with PBS, were cotransfected with 100ng of pE1b-TATA-luciferase reporter plasmid, 0.5ng of pRL-TK (with Renilla luciferase used as an internal control), and 0.5 ng of VP16-pM or 10 ng of SREBP1a-pM. After 1 day, the cells were lysed and their transcription activities were quantitated using a Picagene Dual SeaPansy Luminescence kit (Wako Pure Chemical Industries, Osaka, Japan).

2.6. Immunofluorescence

To examine the subcellular colocalization of hMed15, hTFIIEβ, and hCDK7, HeLa S3 cells were seeded on coverslips and were transfected with HA/Flag-Med15-pIRESneoII using Lipofectamine 2000. The cells were then double-immunostained with anti-hTFIIEβ or hCDK7 rabbit polyclonal antibody, and anti-HA mouse
monoclonal antibody (12CA5). Axela Fluor 488 Goat anti-rabbit IgG and Axela Fluor 555 Goat anti-mouse IgG (Life Technologies Japan, Tokyo, Japan) were used as secondary antibodies. For the immunofluorescence analysis, confocal laser microscope, LSM 700 (Zeiss, Jena, Germany), was used to examine the intracellular localization of the red and green fluorescence.

2.7. Chromatin immunoprecipitation (ChIP) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

MCF-7 cells grown in 10 cm dishes to 80–90% confluence were fixed in 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by addition of 125 mM glycine, and cells were incubated for 5 min at room temperature before being washed with PBS. Fixed cells were collected in a 1.5 mL tube by scraping, and then 300 μL of lysis buffer (50 mM Tris•HCl [pH 8.1], 10 mM EDTA, and 1% SDS) was added to each tube. Cells were completely disrupted by chromatin shearing using a Bioruptor sonication device (UCD-250, Cosmo Bio, Tokyo, Japan) at 200W for 25–30 cycles (30 sec on, 30 sec off). Cell lysates were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was diluted 10-fold with dilution buffer (16.7 mM Tris•HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, and 1.1% Triton X-100), then incubated overnight at 4°C with 2 μg of the indicated antibodies. Forty microliters of protein G Dynabeads were suspended in Dynabeads blocking buffer (10 mM Tris•HCl [pH 7.5], 1 mM EDTA, 1 mg/mL BSA, and 0.4 mg/mL salmon sperm DNA) and incubated for 3h at 4°C. The beads were then washed with 1 mL of low-salt buffer (20 mM Tris•HCl [pH 8.1], 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, and 0.1% SDS), 1 mL of high-salt buffer (20 mM Tris•HCl [pH 8.1], 500 mM NaCl, 2 mM EDTA, 1% TritonX-100, and 0.1% SDS), 1 mL of LiCl buffer (10 mM Tris•HCl [pH8.1], 250 mM LiCl, 1 mM EDTA, 1% NP-40, and 1% sodium deoxycholate), and 1 mL of TEN buffer (16 mM Tris•HCl [pH 7.5], 1 mM EDTA, and 0.5% NP-40). After washing, immunocomplexes were eluted by incubation in 100 μL of elution buffer (1% SDS and 100 mM NaHCO₃) for 1 h at room temperature, and the eluate was collected. Cross-links were reversed by overnight incubation at 65°C. Next, the eluate was treated with RNaseA for 1 h at 37°C, followed by treatment with Proteinase K for 2 h at 37°C. For qPCR, DNA templates were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA), and the purified DNA was quantified using SYBR® Premix Ex Taq™ II (TaKaRa, Otsu, Japan) on an Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA). Total RNA (350 ng) purified from siRNA-transfected cells using the Nucleo Spin RNA II kit (TaKaRa) was subjected to reverse transcription (RT) using PrimeScript™ RT Master Mix (TaKaRa). Synthesized cDNA was quantitated using SYBR® Premix Ex Taq™ II (TaKaRa) on an Mx3000P QPCR system (Agilent Technologies).

2.8. Primer sets for qPCR

The following primer sets were used in qPCR analyses of the p21WAF1 gene:

**Forward Primers**

-2283 5'-AGCAGGCTGTGGCTCTGATT-3'
-20 5'-TATATCAGGGCCGCGCTG-3'
+4001 5'-AGTCACTACGCCCTGGAGTCAA-3'

**Reverse Primers**

-2283 5'-CAAAATAGCCACCAGCCTCTTCT-3'
-20 5'-GGCTCCACAAGGTGACTTC-3'
+4001 5'-GGAGAGTGAGTTTGCCCATGA-3'

3. Results and Discussion

3.1. Knockdown of hMED15 reduces cell growth rate

Yeast MED15 (yMED15) was initially called Gal11 in *Saccharomyces cerevisiae* (5). yMED15 involves in galactose metabolism in tight collaboration with yeast transcriptional activator GAL4 and its deficiency causes slow growth (12). Since MED15 was determined to be one of the Mediator subunit conserved among eukaryotes, we could easily imagine that hMED15 plays essential roles in human. Therefore, to study the hMED15 functions using human living cells, three siRNAs (hMED15-191, hMED15-304, and hMED15-1558) were designed to knockdown its expression (Figure 1A). Treatment of HeLa cells with each siRNA clearly knocked down hMED15 expression at the protein level (Figure 1B). Among three, siRNA

![Figure 1. Effects of siRNA-mediated knockdown of hMED15 on HeLa S3 cell growth.](image-url)

A. hMED15 mRNA (ORF, 2364 nt)

B. Cell Count (3x10⁴ cells)

C. y tubulin

Figure 1. Effects of siRNA-mediated knockdown of hMED15 on HeLa S3 cell growth. (A) The positions of sequences targeted by three different hMED15 siRNAs (hMED15-191, hMED15-304, and hMED15-1558) are indicated on the hMED15 mRNA. (B) The effects of siRNA-mediated knockdown on hMED15 protein expression. Western blot analyses of hMED15 and y-tubulin (used as a control) levels after siRNA treatment. NC: Non-targeting control siRNA. (C) The effects of siRNA-mediated knockdown on cell growth. HeLa S3 cells were treated with siRNA and cell growth was monitored for 4 days.
hMED15-191 showed the best knockdown efficiency and thus was used for further studies.

Since yMED15 deletion causes slow growth, the effects of hMED15 knockdown on HeLa cell growth were tested (Figure 1C). As expected, the growth rate was reduced to one fifth of the non-target siRNA treated cells. It will be easily imagined that deletion of hMED15 may cause reduction of the galactose metabolism because yMED15 (GAL4) has been demonstrated to be involved that. And this may cause the reduction of energy production in cells and, ultimately, may reduce the growth rate.

3.2. Knockdown of hMED15 reduced Mediator-dependent transcription activation

In yeast, yMED15 assists the transcriptional activation activity of GAL4. We investigated the effects of hMED15 on the activity of two different transcription activators (Gal4-VP16 and Gal4-SREBP1a) in HeLa cells (Figure 2). After the knockdown of hMED15 using hMED15-191 siRNA, transcription was drastically reduced (lane 2 versus lane 4 in Figures 2A and 2B). Expressing increasing amounts of hMED15 restored transcription (lane 4 versus lanes 6 and 8 in Figures 2A and 2B). It is worth noting that we used Gal4-VP16, a transcription factor with no direct interaction with hMED15, and Gal4-SREBP1a, a transcription factor that does interact. These results suggest that hMED15 promotes transcriptional activation.

3.3. hMED15 colocalizes with TFIIE and TFIIH in the nucleus

Because our results suggest hMED15 functions in transcriptional activation, we next tested whether hMED15 colocalizes with the general transcription factors TFIIE and TFIIH (Figure 3). HeLa S3 cells were immunostained with antibodies against hMED15 and hTFIIEβ (Figure 3A). As Figure 3 shows, hMED15 was localized in the nucleoplasm (Figures 3A, left panels), while hTFIIEβ was both in the nucleoplasm and on the nuclear envelope (Figure 3A, second panel). The similar localization was observed for hCDK7 subunit of TFIIH (Figure 3B, second panel). These results suggest that hMED15 (and the Mediator complex that contains it) promotes transcriptional activation in the nucleoplasm together with the general transcription factors TFIIE and TFIIH.

3.4. hMED15 colocalizes with TFIIE and TFIIH at the p53 binding site and the promoter region of the p21 gene

Figure 2. Effects of siRNA-mediated knockdown of hMED15 on transcriptional activation. (A) The effects of siRNA-mediated knockdown on transcriptional activation by Gal4-VP16. Lanes 3–8: siRNA hMED15-191 was introduced in HeLa S3 cells and to knockdown hMED15. Lanes 5–8: hMED15 wild-type was overexpressed in increasing amounts. (B) The effects of siRNA-mediated knockdown on transcriptional activation by Gal4-SREBP1a. Lanes 3–8: siRNA hMED15-191 was used for knockdown. Lanes 5–8: hMED15 wild-type was overexpressed in increasing amounts.

Figure 3. Localization of hMED15, hTFIIE, and hTFIIH in HeLa S3 cells. (A) Immunostaining of cellular hMED15 and the hTFIIEβ subunit of hTFIIH. hMED15 was stained in red and hTFIIEβ was stained in green, using Alexa-Fluor 594- or Alexa-Fluor 488-conjugated secondary antibodies, respectively. Those stained cell pictures were merged. Nuclei were stained with DAPI (shown in dark blue). (B) Immunostaining of cellular hMED15 and the hCDK7 subunit of hTFIIH. hMED15 was stained in red and hCDK7 was stained in green, using Alexa-Fluor 594- or Alexa-Fluor 488-conjugated secondary antibodies, respectively. Those stained cell pictures were merged. Nuclei were stained with DAPI (shown in dark blue).
To study the functional association of hMED15 with two general transcription factors, TFIIE and TFIIH, we used the human cyclin-dependent kinase inhibitor p21, which is dependent on p53 for transcriptional activation (Figure 4). When MCF-7 cells are treated with the MDM2 inhibitor Nutlin-3, p53 binds to the p53-binding site (p53BS1) and stimulates p21 transcription. Figure 4A illustrates the p21 gene locus structure and the positions of the primer sets used for qPCR. The Nutlin-3 treatment recruited hMED15, hTFIIEα, and the hp62 subunit of TFIIH to p53BS1 (position 1) and the promoter region (position 2). Pol II was recruited only to the promoter region (position 2). Knockdown of hMED15 abolished the binding of hMED15, hTFIIEα, and hp62 to those sites, whereas Pol II remained bound, although the amount was slightly reduced. When hMED15 was knocked down during Nutlin-3 treatment, the binding of Pol II to the promoter increased, and a smaller amount of hTFIIEα bound there. These results demonstrate that, during transcriptional activation of p21, hMED15 colocalizes with TFIIE and TFIIH at the upstream p53-binding site and the promoter region. Given MED15’s established role in yeast transcription activation, as well as its effects on both the p21 gene and the genome overall, we believe this study provides compelling evidence that hMED15 plays a key role in transcription activation.

Recently, we extensively studied the human Mediator head module subunit hMED17 (Kikuchi and Ohkuma, in preparation). This hMED17 also binds to the general transcription factors and functions positively in transcriptional activation. We assume that the Mediator tail subunit hMED15 and hMED17 function collaboratively in response to the transcriptional activator. These two subunits may evoke drastic conformational change of the whole Mediator complex upon transcriptional activator binding. We will study this mutual association in the near future.

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References


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**Original Article**

**R-eriodictyol and S-eriodictyol exhibited comparable effect against H₂O₂-induced oxidative stress in EA.hy926 cells**

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**Summary**

Eriodictyol is a flavanone well-known for its antioxidative activity. Due to a chiral carbon atom in position C-2, eriodictyol always exist in racemic form. In order to study the antioxidant activity under H₂O₂-induced oxidative stress of each enantiomer, enantiomers of eriodictyol were resolved by high-performance liquid chromatography (HPLC), using a Chiral Amylose-C column as chiral stationary phase. Online coupling HPLC-circular dichroism (CD) method was used for the determination of elution order and the absolute configurations of the two eluates. The protective effects of racemic and enantiomeric eriodictyol against H₂O₂-induced cytotoxicity with EA.hy926 cells were tested. The results showed that the two enantiomers of eriodictyol and the corresponding racemate were equipotent, suggesting that the configuration of the C-2 chiral center does not influence the cytoprotective activity against H₂O₂-induced oxidative stress in EA.hy926 cells.

**Keywords:** Eriodictyol, enantiomer, cytoprotective activity, oxidative stress

**1. Introduction**

Eriodictyol, 5,7,3',4'-tetrahydroxyflavanone (Figure 1), is a chiral flavanone presented in citrus fruits and herbal products. This flavanone is well known for its beneficial health-related properties, such as antioxidant (1), antiinflammatory (2), and antimicrobial (3) activity. Recent studies have shown that eriodictyol can provide cytoprotective effect in UV-irradiated keratinocytes (4), induces long-term protection in ARPE-19 cells (5), protect neuron-like PC12 cells against H₂O₂-induced injury (6), prevents early retinal and plasma abnormalities in streptozotocin induced diabetic rats (7, 8).

Eriodictyol is a chiral molecule with an asymmetric center at position C-2 and can occur in two enantiomeric form, R-eriodictyol and S-eriodictyol (Figure 1). It has long been established that stereochemistry is one of the important determinant of the biological, pharmacological, and toxicological properties of many nutrients (e.g. D-glucose, L-ascorbic acid) (9) and drugs (e.g. thalidomide) (10). Thioctic acid, a naturally occurring antioxidant, played protective role in central nervous system injury related to oxidative stress, and only (+)-thioctic acid showed pronounced effect both in H₂O₂ induced cell injury or in vivo experiment, while (‒)thioctic acid was inactive (11). Naringenin, which is structurally similar with eriodictyol, showed stereospecific anti-inflammatory potential in vitro (12). This suggested that enantiomers of flavanone might have different behaviors in pharmacological action and metabolic process (13). It is necessary to consider the stereochemistry of flavanone when studying the biological activities. However, most of published results on the biological activities of flavanone in vitro are available for the racemate, little is known about the influence of the stereochemical configuration of flavanone on their biological activity due to the lack of readily available pure flavanone enantiomers.

**Figure 1. Structures of RS-, R- and S-eriodictyol.**
For the separation of enantiomers of eriodictyol, a couple of methods have been previously reported, such as capillary electrophoresis (14), micellar electrokinetic chromatography (15), and chiral HPLC (16).

In our previous papers, we also described the resolution of enantiomers of some flavonones by chromatography on a chiral phase, and we reported the absolute configuration determination by CD spectra (17). However, no bioactivity study was carried out using pure enantiomers of eriodictyol. As a continuation of our research on small molecule antioxidants, this study was designed to investigate the effects of the stereochemical configuration of eriodictyol on its cytoprotective effects against oxidative stress. We have first prepared the pure enantiomeric forms of eriodictyol by chiral phase HPLC, assigned the absolute configuration by the online coupling HPLC-CD method. Furthermore, the cytoprotective abilities of R- and S-eriodictyol were tested against H2O2-induced EA.hy926 cell injury.

2. Materials and Methods

2.1. Chemicals and reagents

Racemic eriodictyol was isolated from Draccephalum rupestre by the procedure of ethanol extraction, polyamide column separation and recrystallization (18). The purity was proved to be above 98% by HPLC analysis. The structure identification was carried out by 1H and 13C nuclear magnetic resonance (NMR). R- and S-eriodictyol were purified by chiral HPLC from racemic eriodictyol, the purities were confirmed to be above 96% by chiral HPLC-UV/CD analysis. HPLC-grade n-hexane and 2-propanol were from Spectrum Chemical MFG Corp. (Gardena, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Solarbio Science & Technology Co. Ltd. (Beijing, China). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) and 4',6-diamidino-2-phenylindole (DAPI) was from Sigma-Aldrich (Saint Louis, MO, USA). Annexin V-FITC apoptosis detection kit was purchased from Bestbio (Shanghai, China).

2.2. Chromatographic system and conditions

The HPLC-UV was performed on Agilent 1260 HPLC system, equipped with quaternary pump, diode array detector and an autosampler (Agilent, Palo Alto, LA, USA). The HPLC-CD was performed on a JASCO LC-Net II/ADC HPLC system, equipped with PU-2089 plus pump, CD-2095 plus CD detector and a 7125 Rheodyne injector with 20 μL sample loop (Jasco, Tokyo, Japan). The column (250 mm × 4.6 mm) was amyllose tris-3, 5-dimethylphenyl carbamate (Chiral Amylose-C) coated on 5 μm silica gel. The column was obtained from YMC Co. (Kyoto, Japan). Experiments were performed at ambient temperature. All solvents were degassed in an ultrasonic bath prior to use. The flow rate was 0.5 mL/min. Once a new chromatographic condition was adopted, the column was equilibrated for at least 1 h before injection. Sample of eriodictyol was diluted in methanol to a concentration of 0.1 mg/mL for HPLC-UV and 0.5 mg/mL for HPLC-CD. The prepared HPLC sample solutions were filtered through a nonsterile 0.45 μm PTEE syringe filter. UV and CD detection were performed at 284 nm. The CD spectra of the enantiomers were obtained by stopped-flow scanning at each chromatographic peak by CD detector from the wavelength range of 220-420 nm. Column void volume (t0) was measured by injection of tri-tert-butylbenzene as a non-retained marker. The retention factor (k) was calculated as k1 = (t1 − t0)/t0 and k2 = (t2 − t0)/t0, where t1 and t2 are the retention times for the first and second eluting enantiomers, respectively. The separation factor (α) was calculated as α = k1/k2. The resolution factor was evaluated according to Rr = 2(t2−t1)(w1 + w2), i.e. the peak separation divided by the mean value of the baseline widths. Retention times (t) were mean values of two replicate determinations.

2.3. Cell culture and treatment

Human endothelial-like immortalized cells (EA.hy926) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in a humidified incubator containing 5% CO2. 2H2O2-induced EA.hy926 cell injury.

2.4. Measurement of cell viability

Cell viability was monitored by two kinds of method. The first method is MTT assay (19). In brief, 1 × 10^4 cells per well were seeded in a 96-well plate and incubated overnight. Cells were pretreated with several concentrations of compound for 2 h before exposure to H2O2 for 24 h. After addition of 20 μL 2.0 mg/mL MTT solution, the cells were incubated at 37°C for 4 h, the plate was centrifuged and the medium was removed. For each well, 100 μL DMSO was added and crystals were dissolved by shaking the plate at room temperature. Absorbance was measured at 570 nm by a microplate reader (Biorad, Model 680, Hercules, CA, USA). Triple wells were used for each sample and the experiments were repeated at least three times to get means and standard deviations.

The second method is the real time cellular analysis (RTCA) by using the xCELLigence system (ACEA Biosciences, San Diego, CA, USA), which monitors cell growth in response to treatment in real-time (20). Cells grow on top of electrodes so that the impedance varies based on the number of cells attached and the quality of cell-electrode interaction. Electrode impedance, which is displayed as Cell Index (CI), can be used to monitor cell viability, number, morphology, and cell adhesion (21). Cells (10,000/well) were seeded overnight and then treated with each chemical alone or in combination, and cell growth was monitored.
2.5. DAPI nuclear staining

EA.hy926 cells (2 × 10^5 cells/well) in 12-well plates were exposed to compounds for 24 h, then cells were fixed in ice-cooled acetone-methanol (1:1) mixture for 5 min, after rinsing with PBS, cells were stained with DAPI (2 mg/mL) for 15 min at room temperature. Cells were viewed and photographed under fluorescence microscopy (Olympus IX71, Olympus Co., Tokyo, Japan). Apoptotic cells were recognized based on characteristic observations including the presence of condensed, fragmented and degraded nuclei.

2.6. Apoptosis assays

Apoptotic rates were analyzed by flow cytometry using an annexin V-FITC/PI kit (Bestbio, Shanghai, China) according to the manufacturer’s instruction. Briefly, cells were treated with compounds for 24 h, and then 1 × 10^6 cells were harvested, washed twice with ice-cold PBS, and evaluated for apoptosis by double staining with annexin V-FITC and propidium iodide in binding buffer using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.7. Measurement of intracellular Reactive oxygen species (ROS)

ROS levels were determined using DCFH-DA as fluorescent probes (22). The cells were treated with H2O2 for 24 h after being pretreated with or without compounds for 2 h, washed cells with PBS, then incubated cells in fresh medium containing 10 μg/mL DCFH-DA at 37°C for 30 min. Subsequently, the cells were trypsinized and diluted with PBS to approximate 1 × 10^6 cells per mL, analyzed with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

2.8. Statistical analysis

One way analysis of variance (ANOVA) and post hoc multiple comparison Bonferroni test were used to determine the significant difference between two groups. Results are presented as the mean ± SD. p < 0.05 was considered to be significant.

3. Results and Discussion

3.1. Chiral separation of eriodictyol

The effect of mobile-phase on the separation process was examined by modifying the ratio of n-hexane and 2-propanol. The chromatographic parameters, capacity factor (k), separation factor (α), and resolution factor (Rs) for the resolved eriodictyol are given in Table 1. The table showed that eriodictyol could be resolved with good separation factors (α) and resolution factors (Rs) on Chiral Amylose-C CSP column by optimizing the mobile phase composition. Typical enantiomeric separations of eriodictyol on Chiral Amylose-C column and mobile phase composition are shown in Figure 2. The optimized mobile phase consisted of 30% n-hexane and 70% 2-propanol, and the flow rate was 0.5 mL/min. The good resolution obtained for eriodictyol allowed us to separately collect the individual enantiomers used for the biological assays. R- and S-eriodictyol (3.0 mg each) were purified using the above-mentioned optimized HPLC condition as shown in Figure 2G.

The elution order was easily determined by the online HPLC-CD method. It has been previously reported that a negative CD signal at 280-290 nm of flavanone is related to the S-configuration at C-2, whereas a positive CD signal establish an R-configuration (14). As evidenced

<table>
<thead>
<tr>
<th>Eluent</th>
<th>k1</th>
<th>k2</th>
<th>Rs</th>
<th>α</th>
</tr>
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<tr>
<td>2-Propanol</td>
<td>0.37</td>
<td>0.47</td>
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<td>n-Hexane-2-propanol 5:95</td>
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<tr>
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<td>0.64</td>
<td>1.49</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Figure 2. Typical HPLC chromatograms of enantiomeric resolution of eriodictyol on Chiral Amylose-C CSP. Mobile phase: (A) 2-propanol; (B) n-hexane-2-propanol, 5:95 (v/v); (C) n-hexane-2-propanol, 10:90 (v/v); (D) n-hexane-2-propanol, 15:85 (v/v); (E) n-hexane-2-propanol, 20:80 (v/v); (F) n-hexane-2-propanol, 25:75 (v/v); (G) n-hexane-2-propanol, 30:70 (v/v), at 0.5 mL/min in all cases.

Table 1. Chromatographic results for enantiomeric resolution of eriodictyol on Chiral Amylose-C CSP.
by the positive and negative CD signals at 284 nm, the \( R \)-enantiomer eluted as the first peak (Figure 3). Beside to obtain CD signal at a chosen \( \lambda \), the complete CD spectrum of the eluting peak could also be obtained in a stop-flow mode. Based on the obtained CD spectra, the absolute configuration of the eluates could also be determined.

3.2. Effects of racemic and enantiomeric eriodictyol on \( H_2O_2 \)-induced cytotoxicity in EA.hy926 cells

To evaluate the efficacy of racemic and enantiomeric eriodictyol on \( H_2O_2 \)-induced cytotoxicity, EA.hy926 cell line was used. Firstly, cells were treated with 200 \( \mu M \) \( H_2O_2 \) in the presence or absence of racemic and enantiomeric eriodictyol (5, 10, 15, 20 \( \mu M \)), and the cell viability was assessed by performing MTT assay. All the compounds tested were clearly able to block the cytotoxic effects of \( H_2O_2 \) on EA.hy926 cells, and also both enantiomers and the corresponding racemate were almost equipotent (Figure 4A).

Figure 3. HPLC-CD chromatograms and CD spectra of the eluted peaks of eriodictyol on Chiral Amylose-C. Mobile phase: \( n \)-hexane-2-propanol, 25:75 (v/v).

Figure 4. Protective effects of \( RS \)-, \( R \)- and \( S \)-eriodictyol on \( H_2O_2 \)-induced EA.hy926 cell injury. (A) EA.hy926 cells were treated with 200 \( \mu M \) \( H_2O_2 \) alone or co-treated with indicated concentrations of \( RS \)-, \( R \)- and \( S \)-eriodictyol for 24 h, and cell viability was determined by MTT assay. Data are presented as mean \( \pm SD \) of three independent experiments. \( * p < 0.05 \) versus untreated cells and \( ** p < 0.01 \) versus \( H_2O_2 \)-treated cells. (B) EA.hy926 cells were treated with 200 \( \mu M \) \( H_2O_2 \) alone or co-treated with indicated concentrations of \( RS \)-, \( R \)- and \( S \)-eriodictyol, and cell viability was determined by the xCELLigence live cell analysis system.

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The xCELLigence live cell analysis system can be used as a rapid monitoring tool for cellular viability and be applied in toxicity testing of xenobiotics using in vitro cell cultures. For the assay of the protective effects of racemic and enantiomeric eriodictyol against H₂O₂-induced cytotoxicity, the xCELLigence live cell analysis system was used as the second testing method. After seeding the EA.hy926 cells to E-plates, the proliferation, attachment and spreading of the cells was monitored every 15 min by the xCELLigence system. Approximately 24 h after seeding, the cells were treated with DMSO, H₂O₂, racemic/enantiomeric eriodictyol, or in combination, and cell growth was monitored for a period of up to 48 h. H₂O₂ (200 μM) could elicit significant cytotoxicity in EA.hy926 cells, which displayed as sharp CI decreasing, whereas treatment with racemic/enantiomeric eriodictyol (10 and 20 μM) alone did not affect cell growth. However, cotreatment significantly improved cell survival as judged by slight CI decreasing compared with H₂O₂ treatment alone (Figure 4B). Consistent with the results tested by MTT assay, both enantiomers and the corresponding racemate of eriodictyol were equipotent.

3.3. Effects of enantiomeric eriodictyol on DNA condensation and H₂O₂-induced apoptosis in EA.hy926 cells

DAPI staining revealed that nuclear DNA condensation and nuclear fragmentation occurred after treatment with 200 μM H₂O₂ for 24 h. Pretreatment with both R- and S-eriodictyol inhibited these apoptotic features (Figure 5). Annexin V cell surface staining followed by flow cytometry analysis also showed similar results. After exposure to 200 μM H₂O₂ for 24 h, the apoptotic rate of cells increased from 8.95 ± 0.2% to 33.59 ± 2.0%. Pretreatment with both racemic and enantiomeric...
eriodictyol (20 μM) reduced the rate of apoptosis. These results indicated that R- and S-eriodictyol have anti-apoptotic effects against H2O2-induced apoptosis in EA.hy926 cells, and the anti-apoptotic effects were almost the same for the two enantiomers (Figure 6).

3.4. Effects of enantiomeric eriodictyol on intracellular ROS production

To determine the effects of compounds on ROS induction, DCFH-DA and flow cytometry were used to detect intracellular peroxide levels. As shown in Figure 7, when EA.hy926 cells were exposed to 200 μM H2O2 for 24 h, the intracellular ROS levels increased significantly compared with untreated cells. Treatment with both R- and S-eriodictyol attenuated the increase of ROS induced by H2O2 in a dose-dependent manner, and the inhibiting intracellular ROS effects were almost the same for the two enantiomers.

4. Conclusion

Oxidative stress is an imbalance between the production of ROS and antioxidant defense mechanisms, potentially leading to cellular damage. Oxidative stress has a key role in the development of cardiovascular and/or cerebrovascular diseases. This phenomenon is mainly mediated by an enhanced ROS production by the vascular endothelium with its consequent dysfunction. Eriodictyol was chosen as an antioxidant as increasing evidences indicates the protection activity of the compound in many kinds of cellular disorders characterized by ROS overproduction (1, 7). Although there are a certain number of investigations detailing the antioxidant activity of the racemic eriodictyol (1, 4, 5, 7), no studies have properly assessed the differences in activity between the enantiomers of eriodictyol. This work reports for the first time the comparison of effects of R- and S-eriodictyol against H2O2-induced oxidative stress in EA.hy926 cells. The results showed that eriodictyol could be resolved well on Chiral Amylose-C column. The two enantiomers of eriodictyol appeared to be almost equally effective in inhibiting H2O2-induced cell viability reduction and cell apoptosis, and also equipotent in deceasing intracellular ROS levels.

Acknowledgments

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References

5. Johnson J, Maher P, Hanneken A. The flavonoid,


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HDAC1/3 dual selective inhibitors - New therapeutic agents for the potential treatment of cancer

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Summary Histone deacetylases (HDACs) have attracted a great deal of interest as anticancer drug targets, and many HDAC inhibitors (HDACIs) have displayed clinical efficacy in treating specific tumors. However, all of these agents have significant toxicity, including fatigue, nausea, vomiting, thrombocytopenia, and neutropenia. Thus, increased effort is being directed toward developing selective HDACIs that are tolerated better and cause fewer adverse reactions. This article focuses mainly on the N-hydroxycinnamamide-based HDAC 1/3 dual inhibitors, and this article outlines the anticancer potential of these inhibitors. Since selective HDAC1/3 inhibitors may cause fewer adverse reactions than selective pan-HDACIs and selective Class I inhibitors in clinical settings, further study of their mechanism of anticancer activity and optimization of their structure is warranted.

Keywords: Epigenetic, HDACs, selective HDACIs, HDAC1/3 selective inhibitors, anti-cancer agent

Histone deacetylases (HDACs) are a class of zinc-dependent metalloproteinases that catalyze the removal of acetyl groups from lysine residues on histones and non-histone proteins. This action results in a “closed” chromatin configuration, thereby regulating the expression of genes, which include tumor suppressor genes (1,2). HDAC inhibitors (HDACIs) have attracted a great deal of interest as anticancer drug agents. Over the past 10 years, over 490 clinical trials of more than 20 HDACI candidates as anticancer agents have been conducted. Three HAACIs, vorinostat (SAHA, Zolinza®), romidepsin (FK-228, Istodax®), and belinostat (PXD101, Beleodaq®) have been approved for the treatment of hematologic tumors. In clinical use as anti-cancer agents (such as vorinostat, panobinostat, belinostat, and abexinostat), many HDACIs inhibit a broad spectrum of HDACs, including Class I, II, and IV isoforms. Although these HDACIs have promising efficacy in treating specific tumors, they all exhibit significant toxicity, including fatigue, nausea, vomiting, thrombocytopenia, and neutropenia (3). Thus, increased effort is being directed toward developing HDACIs that selectively inhibit certain classes or a single isoform. This should result in agents that are tolerated better and cause fewer adverse reactions. Several selective Class I, class IIa, and HDAC6 inhibitors (Figure 1) have been reported, but only a few selective Class I inhibitors are used clinically (4-7). Selective HDAC6 inhibitors are expected to be beneficial since they may cause fewer adverse reactions. However, the literature indicates that such small molecules have not played a prominent role in cancer therapy, with the exception of their combination with other chemotherapeutics (8). Selective Class I HDACIs such as MS-275 and MGCD0103 (HDAC1, 2, and 3-selective) are the most studied selective HDACIs in clinical use or in development. However, these therapeutic have been found to have similar toxicity profiles and overall tolerability in comparison to pan-HDACIs (9). A reasonable explanation for this is that selective Class I HDACIs in clinical use may not be selective enough to offer a superior therapeutic benefit over pan-inhibitors. Given this fact, several improved selective inhibitors have been described (Figure 2). These inhibitors have better potency and selectivity for HDAC1 and 2 versus HDAC3 (10,11). Medicinal chemists have worked to develop more selective HDACIs, such as inhibitors targeting individual isoforms.

Recently, a series of N-hydroxycinnamamide-based HDAC 1/3 dual inhibitors were described by the current
authors’ laboratory. This work represents the first report of such selective inhibitors with oral activity. The representative compound 11r had low nanomolar IC₅₀ values in response to HDAC1 (11.8 nM) and HDAC3 (3.9 nM) and micromolar or submicromolar IC₅₀ values in response to other HDACs such as HDAC2, HDAC4, HDAC6, HDA8, and HDAC11 (Figure 3). Both in vitro and in vivo studies demonstrated that these HDAC1/3 dual inhibitors could help treat cancer. In vitro, some of the selective inhibitors block the proliferation of cancer cell lines, including those of solid and hematologic tumor cells, better than pan-HDAC inhibitor vorinostat (Table 1). Western blot analysis of procaspase 3 and flow cytometry analysis revealed that the potent HDAC1/3 dual selective inhibitors significantly induce cancer cell apoptosis in a time-dependent and dose-dependent manner.
manner. An In vivo study in a subcutaneous U937 xenograft model revealed that the most potent and selective compound was 11r, which inhibited tumor growth 55.1% (Table 2). Moreover, mice treated with 11r had no significant weight loss and no signs of liver or spleen toxicity (I2). More detailed study of their mechanism of anticancer activity and optimization of their structure to improve transcellular permeability and isoform selectivity are underway in this laboratory.

Selective HDAC1/3 inhibitors are selective for HDAC1 and 3 versus HDAC2, so this type of selective inhibitor may offer a better therapeutic approach over pan-HDACIs. In addition to their activity against cancer, HDACIs have been studied in the treatment of neurodegenerative disorders, including Huntington’s disease and Friedreich’s ataxia. Thus far, a phase I clinical study of selective Class I HDACi RG2833 for the treatment of Friedreich’s ataxia has begun, and phase II clinical trials of selective SIRT 1 inhibitors to treat Huntington’s disease (HD) have been conducted. Moreover, HDACIs, and particularly hydroxamate-based inhibitors, have surprisingly been found to show synergistic activity with antifungal agents against fungal species at concentrations that are not toxic to the host. One example is HDACi MGCD290, which was discovered by MethyGene. This inhibitor has been found to have activity against fungal pathogens (including azole-resistant yeasts) especially when used in combination withazole antifungals (I3). Human HDACs are related to yeast transcriptional regulators and have similar sequences to yeast Rpd3, Hda1, and Sir2, so an interesting question is whether selective HDACIs have the potential to exhibit such antifungal activity. The anticancer activity of selective HDAC1/3 inhibitors has been verified, but their potential use in other ways, such as treatment of neurodegenerative disorders and fungal infection, has yet to be explored. Thus, HDACIs need to be studied a great deal more.

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References


Table 1. In vitro antiproliferative activity of representative compound 11r and positive control SAHA (I2)

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937</td>
<td>K562</td>
</tr>
<tr>
<td>11r</td>
<td>0.16</td>
</tr>
<tr>
<td>SAHA</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Table 2. In vivo antitumor activity of representative compound 11r and positive control SAHA* (I2)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tumor growth inhibition (TGI)</th>
<th>Relative increment ratio (T/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11r</td>
<td>55.1%</td>
<td>37%</td>
</tr>
<tr>
<td>SAHA</td>
<td>32.1%</td>
<td>47%</td>
</tr>
</tbody>
</table>

* An in vivo study was conducted using a subcutaneous U937 xenograft model. Treatment groups were given compound 11r (100 mg/kg/d) or SAHA (100 mg/kg/d) orally for 16 days.

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Drug development for controlling Ebola epidemic – A race against time

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Summary The Ebola outbreak in West Africa this year is causing global panic. The high mortality of this disease is largely due to lack of effective preventive vaccines or therapeutic drugs. Realizing the gravity and urgency in controlling the epidemic, governments and drug companies across the world have taken many strong measures to speed up the process of drug development. Several representative candidate drugs that demonstrate potent anti-Ebola activity in preclinical studies have been pushed forward to higher research stages to obtain an earlier official license. It is expected that proven preventive or therapeutic regimens could be established in the near future.

Keywords: Africa, vaccine, ZMapp, TKM-Ebola, jk-05

The Ebola outbreak identified in Guinea in March this year is currently getting worse across the world. According to the statistics of World Health Organization (WHO), a total of 9,216 confirmed, probable, and suspected cases of Ebola virus disease (EVD) and 4,555 deaths have been reported in seven affected countries including Guinea, Liberia, Nigeria, Senegal, Sierra Leone, Spain, and the United States of America up to the end of 14 October, 2014 (1). It was predicted by WHO that the number of patients and deaths might continue increasing from hundreds to thousands per week in the coming months if control measures are not significantly improved (2). Facing such a grim situation, an alarming fact is that there are no proven therapies or vaccines against this deadly disease. However, it is a comfort that this epidemic on the verge of being out of control has eventually caused comprehensive attention in the international society. Drug development for controlling the epidemic enters the speedway in the world.

ZMapp, an experimental drug developed by Mapp Biopharmaceutical (USA), is a combination of three humanized monoclonal antibodies that are produced in genetically modified tobacco plants (Table 1) (3). In preclinical studies, it provided a survival benefit in nonhuman primates that were experimentally infected with the virus (4,5). In addition, a recent study demonstrated that this drug was capable of rescuing rhesus macaques that had developed advanced EVD (3). Although it has never been tested in humans, the drug has been given to 7 patients with EVD on the basis of 'compassionate use', thus far. In these cases, 5 have survived and 2 have died. In the near future, the US government will provide funding, expertise, and technical support to Mapp Biopharmaceutical to accelerate the development of ZMapp. According to the signed contract of both parties, the government will provide the drug maker with an initial funding of US 24.9 million dollars over 18 months to support work toward acquiring US Food and Drug Administration (FDA) approval of the drug (6).

Another drug that has been recently approved by FDA for testing in Ebola patients is TKM-Ebola, a cocktail of small interfering RNA produced by Tekmira Pharmaceuticals, Canada (Table 1) (7). In a preclinical study, macaques were administered TKM-Ebola after Zaire Ebola virus challenge and the survival of animals was examined. Results demonstrated that 2 of 3 macaques were protected from lethal Ebola virus infection when given 4 postexposure treatments, whereas all 4 macaques were protected when given seven postexposure treatments (8). This study suggested that TKM-Ebola might be effective for people infected with Ebola virus. Unfortunately, during the phase I trial of this drug, it was observed that TKM-Ebola induced cytokine release in participants and thus had been put on clinical hold by FDA earlier this summer. However, in response to the Ebola outbreak in West Africa this year, the FDA consequently
modified its clinical hold to allow the drug company to propose studies in patients infected with the virus.

jk-05, a small molecule compound developed by Academy of Military Sciences (AMC) of China, has broad-spectrum antiviral activities and the phase I trial on its clinical safety has been completed (Table 1) (9). This drug is capable of inhibiting the RNA polymerase of Ebola virus selectively, resulting in a suppressing effect on virus replication. Studies demonstrated that jk-05 possesses antiviral activity against Ebola virus both in vitro and in vivo (9). On August 29, 2014, jk-05 was approved by the General Logistics Department of Chinese People’s Liberation Army for further development of this drug.

In September this year, a meeting consisting of Ebola scientists, industry executives, clinical-trials experts, ethicists, and regulatory officials was convened by WHO to identify and prioritize the most promising products for use in clinical trials against EVD. Besides ZMapp, TKM-Ebola, and jk-05, another four agents including two drugs (favipiravir and BCX4430) and two vaccines (cAd3-ZEBOV and VSV-EBOV) were considered as potential medications against EVD by the delegates (Table 1). Favipiravir, developed by Toyama Chemical of Japan, is an experimental anti-viral drug currently undergoing phase III clinical trials for influenza and was approved in Japan for stockpiling against influenza pandemics in 2014 (10). This drug was also demonstrated to be effective in a mouse model of EVD in preclinical studies. Favipiravir gave 100% protection against aerosol Ebola virus E718 infection in mice when administered at 1 h post-challenge and continuing twice daily for 14 days (11). In addition, initiation of favipiravir administration at day 6 post infection induced rapid Zaire Ebola virus clearance, reduced biochemical parameters of disease severity, and prevented a lethal outcome in 100% of the animals (12). These promising findings suggest favipiravir is a candidate for treatment of Ebola hemorrhagic fever. BCX4430, developed by BioCryst Pharmaceuticals (USA), shows broad-spectrum antiviral effectiveness against a range of RNA virus families including Ebola and Marburg viruses (13). BCX4430 has been demonstrated to protect against Ebola virus in rodent models (13). The development of this drug for use in humans is being fast-tracked. cAd3-ZEBOV and VSV-EBOV are experimental vaccines developed by GlaxoSmithKline (GSK, UK) and National Microbiology Laboratory (NML, Canada), respectively. cAd3-ZEBOV is derived from a chimpanzee adenovirus, Chimp Adenovirus type 3 (ChAd3), genetically engineered to express glycoproteins from the Zaire and Sudan species of Ebola virus to provoke an immune response against them (14). VSV-EBOV is based on the vesicular stomatitis virus, which has been genetically engineered to express Ebola glycoproteins so as to provoke an immune response against real Ebola virus (15). Phase I trials of these two vaccines were commenced in September and October 2014 (16). If this phase is completed successfully, the vaccines would be fast tracked for use in this Ebola outbreak.

Outbreaks of Ebola epidemics have occurred several times in parts of Africa since EVD was first identified in Zaire (now the Democratic Republic of Congo) in 1976. Not until this outbreak spread out of Africa did the international community realize the gravity and urgency against this epidemic. In the past several months, various aid including medical resources, armed forces, and donations from USA, European countries, China, etc. have arrived in African nations hardest hit by Ebola and played an important role in helping control the epidemic. The research and development of anti-Ebola drugs has correspondingly been speeded up in medically advanced countries. It is never too late to turn. Cooperation and collaboration across the world may be the best ‘drug’ in controlling epidemic diseases in this global village.

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**Table 1. Potential anti-Ebola drugs or vaccines in development**

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References

6. McCarthy M. FDA allows second experimental drug to be tested in Ebola patients. BMJ. 2014; 349:g5103.
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