Received: 1 November 2017 | Revised: 16 February 2018 | Accepted: 28 February 2018

DOI: 10.1002/ptr.6077

SHORT COMMUNICATION

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Combination of artemisinin-based natural compounds from *Artemisia annua* L. for the treatment of malaria: Pharmacodynamic and pharmacokinetic studies

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Funding information
Major National Science and Technology Program of China for Innovative Drug, Grant/Award Number: 2017ZX09101002-001-002; National Natural Science Foundation of China, Grant/Award Numbers: 81641002 and 81102752; Beijing Natural Science Foundation, Grant/Award Numbers: 7152020 and 2112010

Currently, the most effective antimalarial is artemisinin, which is extracted from the leaves of medicinal herb *Artemisia annua* L. (*A. annua*). Previous studies showed that the complex chemical matrix of *A. annua* could enhance both the bioavailability and efficacy of artemisinin. The present study aims to evaluate the efficacy and pharmacokinetic properties of a combination therapy based on artemisinin and 3 components from *A. annua* with high content (arteannuin B, arteannuic acid, and scopoletin). In vivo antimalarial activity was assessed following a 4-day treatment in murine malaria models (*Plasmodium yoelii* and *Plasmodium berghei*). Results showed that a much sharper reduction in parasitemia (~93%) was found in combination therapy compared with pure artemisinin (~31%), indicating pharmacodynamic synergism occurring between artemisinin and arteannuin B, arteannuic acid, and scopoletin. Multiple-dose pharmacokinetics further demonstrated that combination therapy results in increased area under the curve ($AUC_{0\rightarrow\infty}$), C_{max}, and $t_{1/2}$ by 3.78-, 3.47-, and 1.13-fold in healthy mice, respectively, and by 2.62-, 1.82-, and 1.22-fold in *P. yoelii*-infected mice, respectively. The calculated oral clearance of combination therapy in healthy and *P. yoelii*-infected mice was also reduced. These findings imply that specific components in *A. annua* might offer a possibility to develop new artemisinin-based natural combination therapy for malaria treatment.

KEYWORDS

ACTs, *Artemisia annua* L, artemisinin, nature component, pharmacokinetics

1 | INTRODUCTION

Malaria continues to be the most prevalent public health threat despite intensive international efforts to reduce its toll. In 2015, there were 212 million cases of malaria resulting in 429,000 deaths worldwide (WHO, 2016). In the 1970s, the principal antimalarial compound from extracts of medicinal herb *Artemisia annua* L. (*A. annua*) was unraveled and termed artemisinin by Chinese scientists. Currently, artemisinin and its derivatives have become essential components of treatment choice for uncomplicated and severe malaria in the form of artemisinin-based combination therapies (ACTs; Chrubasik & Jacobson, 2010; Saxena et al., 2016).

Investigators have shown that tea infusions of *A. annua* and oral consumptions of dried leaves of *A. annua* (pACT) might provide an effective and inexpensive treatment option for the impoverished patients in endemic regions (anamed, 2017; Weathers, Towler, Hassanali, Lutgen, & Engeu, 2014). The antimalarial activity of tea infusions and pACT could be explained by the properties of complex components in the whole plant, such as flavonoids, which may enhance the bioavailability of artemisinin and act in synergy with artemisinin. Whether other components in *A. annua*, especially those in relatively high content, contribute to the positive pharmacodynamic and pharmacokinetic interaction with artemisinin deserves further research.

The current study was planned to evaluate the efficacy of oral delivery of a combination therapy based on artemisinin, arteannuin B, arteannuic acid, and scopoletin (four components therapy, Figure 1). Thus, first, the in vivo antimalarial activity of pure artemisinin and the four components therapy in murine malaria models (*Plasmodium yoelii* and *Plasmodium berghei*) was assessed following a 4-day treatment. Then, the plasma pharmacokinetic profiles of artemisinin after multiple-dose regimens of pure artemisinin and four components therapy were compared in healthy mice and *P. yoelii*-infected mice.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Artemisinin, arteannuin B, arteannuic acid, and scopoletin (purities >98.4%) were isolated from *A. annua* extracts, and their structures were confirmed by high resolution mass spectrometry (MS) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Buspirone (purity >99%) was obtained from Sigma Co., Ltd (St. Louis, Mo, USA). Formic acid (analytical grade) was purchased from Beijing Yili Fine Chemicals Co., Ltd (Beijing, China). Methanol, acetonitrile, and methyl tert-butyl ether (Thermo Fisher Scientific Inc., MA, USA) were of HPLC grade. Sodium carboxymethyl cellulose was purchased from Beijing Fengli Jingqiu Commerce and Trade Co., Ltd (Beijing, China). Water used in the experiment was doubly distilled and deionized. Other chemicals were analytical reagent grade.

2.2 | Animal

ICR mice (male, 20 ± 2 g) were obtained from Beijing Vital River Experimental Animal Technical Co., Ltd (Beijing, China). All animal experiments were carried out under the guideline approved by the Ethics Committee of Capital Medical University.

2.3 | In vivo antimalarial efficacy

Stock solutions of chemicals

Artemisinin and the combination therapies were separately suspended in 0.5% sodium carboxymethyl cellulose by sufficient milling to get a stock solution of 10 mg artemisinin/ml.

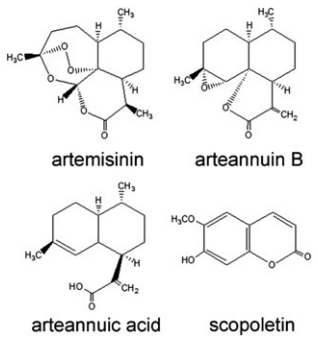

FIGURE 1 Structures of artemisinin, arteannuin B, arteannuic acid, and scopoletin

Experimental design

Infection of mice was established by intraperitoneal (*i.p.*) injection with 1×10^7 *P. yoelii*- or *P. berghei*-infected red blood cells. Then the infected mice were assigned into different treatment groups (n = 8), including normal saline control, artemisinin low dosage (25 mg/kg), artemisinin high dosage (100 mg/kg), arteannuin B–arteannuic acid–scopoletin combination (25 mg/kg for each component), and four components therapy (artemisinin–arteannuin B–arteannuic acid–scopoletin combination, 25 mg/kg for each component) groups. Three hours postinfection, the saline control or tested drugs were orally administrated once a day for 4 consecutive days.

Assessment of parasite burden after treatment

Twenty-four hours after the last dosing, blood samples were collected from tail vein of mice. Thin blood smear slides were air-dried, methanol-fixed, and stained in Giemsa for 30 min, followed by rinsing with distilled water and air-dried at room temperature and examined using a microscope (Eclipse 80i, Nikon Corporation, Tokyo, Japan). Once plasmodium is found in the blood by the microscopic examination, the infection state is confirmed. The inhibition ratio (%) for each group was calculated by the following equations:

$$\text{Inhibition ratio } (\%) = \frac{\text{infection ratio}_{\text{control group}} - \text{infection ratio}_{\text{treatment group}}}{\text{infection ratio}_{\text{control group}}} \times 100.$$

2.4 | Liquid chromatography and mass spectrometric condition

The concentration of artemisinin in plasma was determined using a validated method of liquid chromatography (LC)-MS/MS (Zhang, Gong, Qiu, Li, & Wang, 2016).

2.5 | Multiple-dose pharmacokinetics in mice

Infection of mice with *P. yoelii* was established by the same method described in in vivo antimalaria efficacy section, and the infection was validated through Giemsa staining. When the infection rate reached 20%, pharmacokinetic study was initiated. Once daily for 5 consecutive days, healthy or *P. yoelii*-infected mice were given an oral dose of pure artemisinin or four components therapy at an amount corresponding to 100 mg artemisinin/kg. After the fifth day of drug administration, blood samples were withdrawn from eyeballs of mice under anesthesia at predetermined times. Subsequently, the mice were sacrificed by cervical dislocation. Blood samples were centrifuged to separate the plasma and stored at −80 °C until analysis. The experiment was repeated in three independent experiments (n = 6). Plasma sample preparation and pharmacokinetic calculation were carried out as previously reported (Zhang et al., 2016).

2.6 | Statistical analysis

Statistical analyses were performed using SPSS Statistics software (version 17.0, IBM Analytics Inc., NY, USA). The data were analyzed by Student's t test for two groups and one-way analysis of variance for multiple groups; $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | *In vivo* antimalarial efficacy

The in vivo antimalarial activity was assessed in mice infected with *P. yoelii* and *P. berghei* after oral administration of pure artemisinin and the combination therapies. The representative Giemsa-staining images of different groups after a 4-day treatment are shown in Figure 2. The quantitative analysis demonstrated that treating with artemisinin low dosage (25 mg/kg) achieved ~31% inhibition ration (Figure 3). Arteannuin B, arteannuic acid, and scopoletin combination group has very low antiplasmodial effect themselves (~11%). However, artemisinin high dosage and the four components therapy groups achieved a much sharper reduction in parasitemia (~93%). More importantly, the four components therapy groups achieved the significant inhibition of parasitemia at a low dose of 25 mg/kg, compared with artemisinin high dosage group at a dose of 100 mg/kg. Therefore, in the case of combination of artemisinin with arteannuin B, arteannuic acid, and scopoletin, strong antimalarial efficacy was achieved at a quarter of the normal dose.

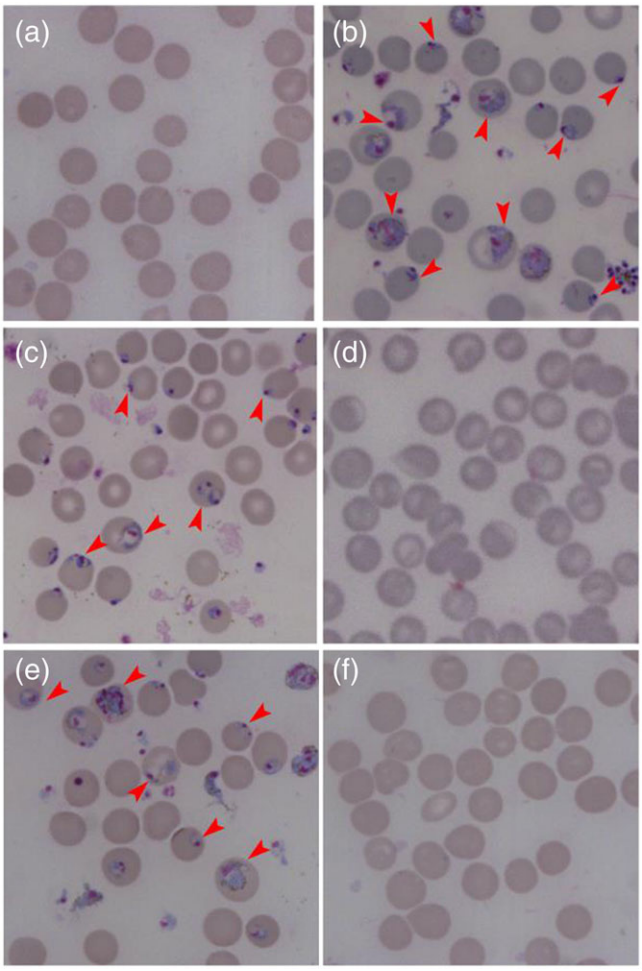

FIGURE 2 Representative Giemsa-staining images of different groups after a 4-day treatment, including uninfected control (a), normal saline (b), artemisinin low dosage (25 mg/kg, c), artemisinin high dosage (100 mg/kg, d), arteannuin B–arteannuic acid–scopoletin combination (25 mg/kg for each component, e), and four components therapy (25 mg/kg for each component, f). Red arrows indicate the *Plasmodium yoelii*-infected red blood cells [Colour figure can be viewed at wileyonlinelibrary.com]

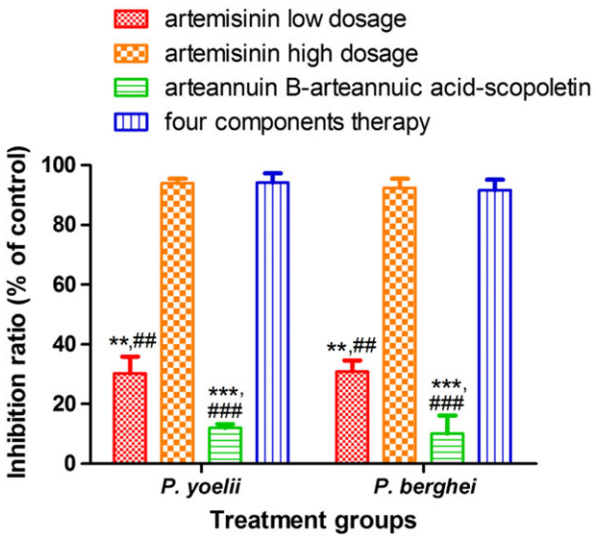

FIGURE 3 Inhibition ratio assay to qualitatively display the *in vivo* antimalarial efficacy of different treatment groups. ** and *** indicate $p < .01$ and .001 versus artemisinin high dosage group, respectively. ## and ### indicate $p < .01$ and .001 versus four components therapy group, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Bioanalytical method validation

Bioanalytical method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, and stability. No significant interference was observed from endogenous substances in blank plasma at the retention time of analytes or the internal standard. The respective retention times for artemisinin, arteannuin B, arteannuic acid, scopoletin, and buspirone were 6.82, 6.61, 7.46, 5.09, and 5.12 min. Calibration curves were linear from 2.00 to 1,000.00 ng/ml, with r values ≥.998. The intraday and interday accuracies of the analytes ranged from 93.57% to 105.71%, and the intraday and interday precisions were below 8.87%.

The mean recovery was ≥92.3% for artemisinin. The stability of analytes during the sample preparation at room temperature and storage conditions indicated that the analytes in plasma were stable for 24 hr at room temperature, 30 days in refrigerated conditions (−80 °C), and minimum of three freeze–thaw cycles at −20 °C. The data demonstrated that this developed method was accurate, reliable, and reproducible for the pharmacokinetic study of artemisinin.

3.3 | Multiple-dose pharmacokinetics in healthy and *P. yoelii*-infected mice

The mean plasma concentration-time curves of artemisinin after five consecutive daily oral administration of pure artemisinin or four components therapy in healthy and *P. yoelii*-infected mice were shown in Figure 4. It could be seen that the artemisinin level from the four components therapy group remained higher at all time points compared with those from pure artemisinin group in either healthy or *P. yoelii*-infected mice. The main pharmacokinetic parameters, estimated by

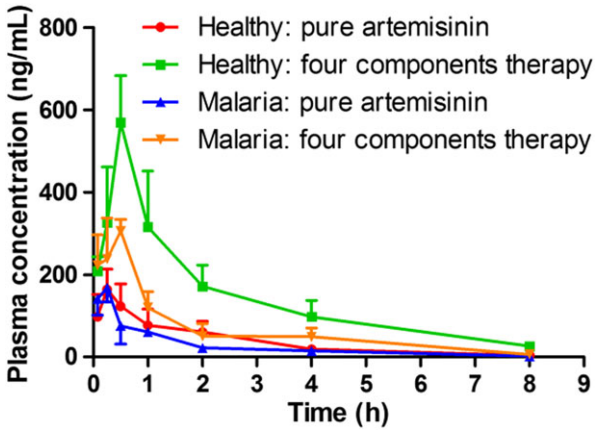

FIGURE 4 Plasma concentration-time curves of artemisinin following the multiple oral administration of pure artemisinin or four components therapy in healthy and *Plasmodium yoelii*-infected mice. The data are the average of three independent experiments and presented as the mean ± *SD* (*n* = 6) [Colour figure can be viewed at wileyonlinelibrary.com]

non-compartmental analysis, were summarized in Table 1. The area under the curve ($AUC_{0\rightarrow\infty}$), which determined the therapeutic effects, was 3.78- and 2.62-fold higher in four components therapy group when compared with those of pure artemisinin group in healthy and *P. yoelii*-infected mice, respectively, and the C_{max} showed 3.47- and 1.82-fold increase. Furthermore, oral coadministration of the four components achieved a longer $t_{1/2}$ and mean residence time ($MRT_{(0-\infty)}$) and lower clearance (CL) than pure artemisinin. The results indicated that arteannuin B, arteannuic acid, and scopoletin enhanced mean plasma artemisinin concentration and might contribute to the improved antimalarial efficacy.

In addition, the concentration-time profile obtained varied in two animal models. The mean plasma artemisinin concentration in *P. yoelii*-infected mice decreased greatly compared with that in healthy mice. For instance, the $AUC_{0-\infty}$ from *P. yoelii*-infected mice was 1.61- and 2.30-fold lower in pure artemisinin and four components therapy, respectively, compared with that from healthy mice. Additionally, the $t_{1/2}$ and $MRT_{(0-\infty)}$ obtained from *P. yoelii*-infected mice were relatively shorter, and the CL in *P. yoelii*-infected mice was increased at a proportion ranging from 63.64% (pure artemisinin) to 135.07% (four components therapy). The above findings thus suggested the significant influence of disease on artemisinin in vivo disposition.

4 | DISCUSSION

When used in tea infusion and oral consumption of *A. annua* dried leaf tablets, the bioavailability and efficacy of artemisinin was significantly enhanced due to the complex matrix of compounds (Suberu et al., 2013; Weathers, Towler, Hassanali, Lutgen, & Engeu 2014; Weathers & Towler, 2014). However, the validation of tea infusion and *A. annua* dried leaf tablets can be problematic because of the lack of sufficient information and standardization of the active compounds. We proposed that the antimalarial activity of artemisinin could be improved by fewer chemicals from *A. annua*. In a Beijing cultivar, the content of arteannuin B, arteannuic acid, and scopoletin could reach 0.4%, 0.2%, and 0.2%, respectively, which was quite close to that of artemisinin (0.3%). The four components were isolated with high extraction rate in our preliminary study. To evaluate the potential of using the combination of artemisinin, arteannuin B, arteannuic acid, and scopoletin (1:1:1:1, *w*/w) as a therapeutic agent, in vivo antiplasmodial assay (murine *P. yoelii* and *P. berghei*) and multiple-dose pharmacokinetic study were carried out.

Murine malaria model provides a good initial screen for identifying antiplasmodial activity of new drugs (Rocha et al., 2011; Upegui et al., 2015). Potency of the four components therapy was increased almost four times compared with pure artemisinin. Artemisinin combined with arteannuin B, arteannuic acid, and scopoletin showed pharmacodynamic synergistic effect, which exerted antiplasmodial effects greater than the sum of individual components. Arteannuic acid and arteannuin B had weak antimalarial activity on their own, whereas scopoletin is one of the major anti-inflammatory and anti-allergic constituents of *A. annua* (Cheng, Cheng, & Chang, 2012; Yao et al., 2012), which possesses the ability to activate lymphocytes and stimulate immunological functions, thereby enhancing the potency of artemisinin (Moon et al., 2007; Weathers et al., 2014).

Long treatment duration (3–7 days) is necessary to produce a clinical effect because of the short half-life of artemisinin. Therefore,

TABLE 1 Pharmacokinetic parameters of artemisinin after multiple oral administration of pure artemisinin or four components therapy in healthy and *Plasmodium yoelii*-infected mice

pharmacokinetic parameters	Unit	Healthy		Malaria	
		Artemisinin	Four components	Artemisinin	Four components
$AUC_{(0-t)}$	$hr{\cdot}ng{\cdot}ml^{-1}$	328 ± 40	1,205 ± 156	204 ± 18	524 ± 60
$AUC_{(0-\infty)}$	$hr{\cdot}ng{\cdot}ml^{-1}$	336 ± 42	1,271 ± 159	206 ± 18	540 ± 62
C_{max}	ng/ml	164 ± 50	569 ± 114	168 ± 35	306 ± 28
T_{max}	hr	0.25	0.50	0.25	0.50
$MRT_{(0-t)}$	hr	2.03 ± 0.34	2.35 ± 0.28	1.89 ± 0.20	2.27 ± 0.22
$MRT_{(0-\infty)}$	hr	2.23 ± 0.34	2.77 ± 0.29	1.94 ± 0.21	2.49 ± 0.24
$t_{1/2}$	hr	1.51 ± 0.18	1.70 ± 0.20	1.13 ± 0.18	1.38 ± 0.16
Ke	hr^{-1}	0.65 ± 0.06	0.20 ± 0.02	0.60 ± 0.07	0.81 ± 0.06
Vd	L/kg	646 ± 59	193 ± 22	795 ± 81	368 ± 34
CL	$L{\cdot}hr^{-1}{\cdot}kg^{-1}$	297 ± 31	78.7 ± 71	486 ± 50	185 ± 17

Note. The data are the average of three independent experiments and presented as the mean ± *SD* (*n* = 6).

pharmacokinetic study of multiple dose administration, which had an important practical significance, was performed in the current study. Compared with artemisinin alone, a remarkably increased AUC and C_{max} of artemisinin were obtained after multiple oral doses of the four components therapy, demonstrating a beneficial effect of the drug combination. There are many possible mechanisms for the enhanced bioavailability of the combination therapy. Preliminary studies showed that arteannuic acid, arteannuin B, and scopoletin may affect the binding of artemisinin with serum albumin in the blood circulation, which altered the distribution, metabolism, and excretion of artemisinin (Wang, Zhang, Li, Li, & Gong, 2014; Zhang et al., 2016). In addition, artemisinin undergoes extensive metabolism, which is mediated by cytochrome P450 metabolizing enzymes, especially CYP2B6 and CYP3A4 (Simonsson et al., 2003; Svensson & Ashton, 1999). Inhibition of metabolizing enzymes through combination therapy could be of great importance to improve the efficacy of artemisinin. As noted in recent publications, arteannuin B is a strong inhibitor of CYP3A4, and the AUC and therapeutic effect of artemisinin could be enhanced by the metabolism-dependent synergistic effect with arteannuin B (Cai, Zhang, Ji, & Xing, 2017). In the present study, a higher AUC, longer $t_{1/2}$, and lower CL in four components group suggested the inhibition of metabolism and excretion, which was in accordance with the previous report (Cai et al., 2017). The pharmacokinetic study further demonstrated that malaria infection leads to decreased AUC, C_{max}, and $t_{1/2}$ and increased CL as compared with that from healthy mice. For four component therapy, the effect of malaria infection was more significant, presumably by enhanced first-pass effect, auto-induction of cytochrome P450 metabolizing enzymes, and hepatic clearance caused by high drug concentration and pathological status (Navaratnam et al., 2000; Simonsson et al., 2003).

There are several indications that using arteannuin B, arteannuic acid, and scopoletin along with artemisinin is useful. Using a mixture of the three components has been linked to direct antiplasmodial activity, positive immunomodulatory effect, and P450 metabolizing enzymes inhibition, which benefit both the pharmacodynamic and pharmacokinetic effect of artemisinin. Indeed, there might be other mechanisms for the synergistic antiplasmodial action between artemisinin and arteannuin B, arteannuic acid, and scopoletin. More research about the synergistic mechanism and optimal ratio of the multicomponents combination therapies is necessary for the development of natural ACTs and new antimalarial strategies.

5 | CONCLUSION

In this study, we examined interactions between artemisinin and three components from *A. annua* for the development of an artemisinin-based natural ACTs. The results provided evidence for antiplasmodial synergism between artemisinin and arteannuin B, arteannuic acid, and scopoletin, with 2.62-fold increased exposure of artemisinin in the blood stream of *P. yoelii*-infected mice. Different mechanisms of action might contribute to the enhanced antiplasmodial activity of artemisinin in the four components therapy. Further work to clarify the synergistic mechanisms would be worthwhile, and this might lead to the development of new natural ACTs composed of artemisinin and other *A. annua* components, especially those having different synergistic mechanisms.

ACKNOWLEDGEMENTS

This study was supported by the Major National Science and Technology Program of China for Innovative Drug (2017ZX09101002-001-002), the National Natural Science Foundation of China (81641002 and 81102752), and the Beijing Natural Science Foundation (7152020 and 2112010).

CONFLICT OF INTEREST

We declare no conflict of interest.

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How to cite this article: Li J, Zhang C, Gong M, Wang M. Combination of artemisinin-based natural compounds from *Artemisia annua* L. for the treatment of malaria: Pharmacodynamic and pharmacokinetic studies. *Phytotherapy Research*. 2018;1–6. https://doi.org/10.1002/ptr.6077