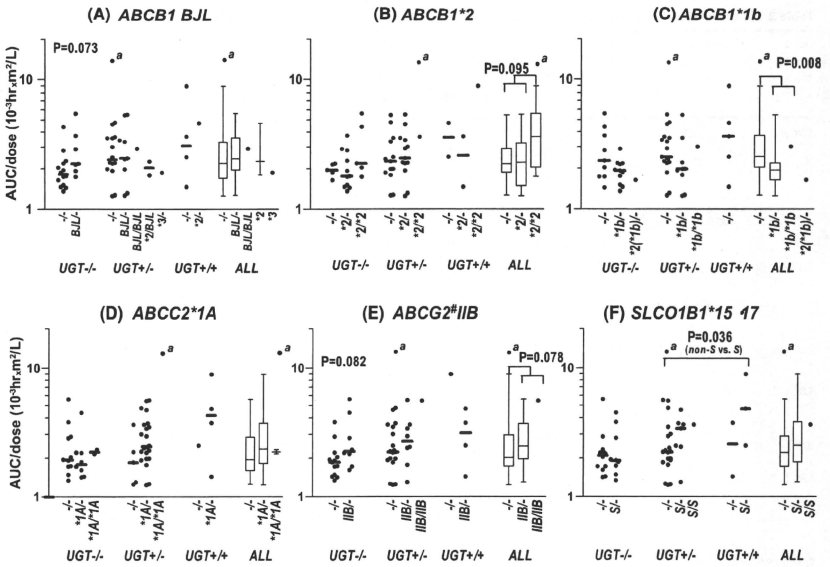

Fig. 1 Effects of transporter genotypes on SN-38 AUC/dose in irinotecan monotherapy ($N = 54$). *a* Excluded from statistical analysis. The *bars* represent the medians. *UGT+* = *UGT1A1*6* or **28*. **a** *BJL* contains −1789G>A, *2 (block 1) = 325G>A (E109K), **3* (block 1) = 304G>A (G102R); **b** *2 (block 2) contains 2677G>T (A893S); **c** **1b* (block 3) = IVS27-182G>T, *2 (block 3) = 3751G>A (V1251I); **d** **1A* contains −1774delG; **e** *IIB* contains 421C>A (Q141K) and IVS12 + 49G>T; **f** *S* = *SLCO1B1*15 · 17* containing 521T>C (V174A)

Effects of transporter genotypes on toxicities in irinotecan monotherapy

Since 80 and 100% of *UGT+/+* patients showed grade 3/4 neutropenia in the irinotecan monotherapy and combination therapy with cisplatin, respectively, neutropenia incidence was analyzed only in the *non-UGT+/+* population. Two patients were excluded from the analysis; one patient who showed an outlier SN-38 value (indicated as "*a*" in Fig. 1) and a second patient from the cisplatin-combination therapy group who discontinued irinotecan therapy.

In terms of incidence of grade 3/4 neutropenia in irinotecan monotherapy (Table 2), *ABCC2*1A*-dependent increases [0, 25.8 and 50.0% for −/−, **1A*/− and **1A*/**1A*, respectively; $P = 0.014$ (chi-square test for trend)] were observed in *UGT* (−/− and +/−) patients. Higher incidence with *ABCG2#IIB* was also found in *UGT* (−/− and +/−) patients [9.5% for −/− and 35.3% for *#IIB*/− and *#IIB*/#IIB*, respectively; $P = 0.049$ (Fisher's exact test)], and with *SLCO1B1*15 · 17(S)* in the *UGT+/−* patients [15.0, 28.6 and 100% for −/−, *S*/− and *S/S*, respectively; $P = 0.076$ (chi-square test for trend)].

Multiple regression analysis for the ANC nadir (logarithm-transformed values) was conducted. The final model [$R^2 = 0.466$, Intercept = 1.088 (log counts/μL), $N = 52$] revealed associations of *ABCC2*1A/*1A* (coefficient = −0.339 ± 0.088, $P = 0.0004$), *ABCG2#IIB* (−0.131 ± 0.067, $P = 0.057$) and *SLCO1B1*15 · 17* (−0.136 ± 0.066, $P = 0.046$) in addition to *UGT+/−* (−0.134 ± 0.073, $P = 0.074$) and *UGT+/+* (−0.238 ± 0.117, $P = 0.047$) and ANC at baseline (0.541 ± 0.226, $P = 0.021$), but association of *ABCB1*2/*2* was not significant (−0.158 ± 0.095, $P = 0.104$).

Although total incidence of grade 3 diarrhea was low (11%), an *ABCB1*2*-dependent increase was observed [0, 15.4 and 28.6% for −/−, *2/− and *2/*2, respectively; $P = 0.022$ (chi-square test for trend)]. Note that all patients who experienced grade 3 diarrhea had neither the *ABCC2*1C/G* nor *ABCG2#IIIC* genotypes.

Table 2 Effects of transporter genotypes on incidences of grade 3/4 neutropenia in Japanese patients treated with irinotecan monotherapy

Gene	Genotype	*UGT*−/−				*UGT*+/−				*UGT* (−/−, +/−)			
		No./total	%	*P* value		No./total	%	*P* value		No./total	%	*P* value	
				Exact[a]	Trend[b]			Exact[a]	Trend[b]			Exact[a]	Trend[b]
ABCB1	*BJL* (block 1)[c]												
	−/−	3/14	21.4	>0.1		4/15	26.7	>0.1	>0.1	7/29	24.1	>0.1	>0.1
	+/−	0/7	0.0			2/9	22.2			2/16	12.5		
	+/+					0/1	0.0			0/1	0.0		
	*2 group (block 2)												
	−/−	1/5	20.0	>0.1[d]	>0.1	5/14	35.7	>0.1[d]	>0.1	6/19	31.6	>0.1[d]	>0.1
	+/−	1/11	9.1			0/13	0.0			1/24	4.2		
	+/+	1/5	20.0			1/1	100			2/6	33.3		
	1b (block 3)[e]												
	−/−	2/9	22.2	>0.1		4/18	22.2	>0.1	>0.1	6/27	22.2	>0.1	>0.1
	+/−	0/11	0.0			2/9	22.2			2/20	10.0		
	+/+					0/1	0.0			0/1	0.0		
ABCC2	*1A												
	−/−	0/11	0.0	>0.1	0.031	0/5	0.0	>0.1		0/16	0.0	0.022	0.014
	+/−	2/8	25.0			6/23	26.1			8/31	25.8		
	+/+	1/2	50.0							1/2	50.0		
ABCG2	#*IIB*												
	−/−	0/13	0.0	0.042		3/19	15.8	>0.1	>0.1	3/32	9.4	0.049	0.057
	+/−	3/8	37.5			3/8	37.5			6/16	37.5		
	+/+					0/1	0.0			0/1	0.0		
SLCO1B1	*15 · 17												
	−/−	2/12	16.7	>0.1		3/20	15.0	>0.1	0.076	5/32	15.6	>0.1	>0.1
	+/−	1/9	11.1			2/7	28.6			3/16	18.8		
	+/+					1/1	100			1/1	100		

[a] Fisher's exact test for (−/−) versus (+/− and +/+)

[b] Chi-square test for trend

[c] Three patients bearing *2 (block 1) or *3 (block 1) were excluded

[d] Fisher's exact test for (−/− and +/−) versus (+/+)

[e] One patient bearing *2 (block 3) was excluded

Effects on toxicities in combination therapy with cisplatin

Since only four patients (6.0%) experienced grade 3 diarrhea from the cisplatin-combination therapy, association analysis for diarrhea was not done.

Grade 3/4 neutropenia incidence was higher with *ABCB1**2 [47.1, 63.3 and 85.7% for −/−, *2/− and *2/*2, respectively; $P = 0.073$ (chi-square test for trend)] in *UGT* (−/− and +/−) patients. In *UGT*−/− patients, a higher incidence was also observed with *ABCG2*#*IIB* [55.6, 83.3 and 100% for −/−, #*IIB*/− and #*IIB*/#*IIB*, respectively; $P = 0.075$ (chi-square test for trend)]. Conversely, the incidence was lower with *ABCG2*#*IIIC* [71.4% for −/−, and 25% for #*IIIC*/− and #*IIIC*/#*IIIC*, respectively; $P = 0.006$ (Fisher's exact test)] in *UGT* (−/− and +/−) patients. Notably, all patients homozygous for *ABCG2*#*IIB* ($N = 5$) or *SLCO1B1**15 · 17 ($N = 1$) experienced grade 3/4 neutropenia. The effect of *ABCC2**1A on neutropenia was not consistent among the *UGT* genotypes in contrast to the results from the monotherapy. Multiple regression analysis was not applied to the neutropenia parameters in the cisplatin-combination therapy because, as described in the next section, contributions of minor variations could not be ignored.

Minor genetic variations possibly related to grade 4 neutropenia

We have detected a number of rare non-synonymous variations of the transporter genes to which statistical analysis could not be applied. Since grade 4 neutropenia

Table 3 Minor genetic variations detected in non-*UGT*+/+ patients who experienced grade 4 neutropenia

ID	Gene	Genetic variation	
		Nucleotide change (amino acid substitution)	Haplotype[a]
b1	*ABCB1*	304G>C (G102R)	*Block 1 *3*
b2(B)[b]		1804G>A (D602N)	*Block 2 *12*
b3(B)[b]		1342G>A (E448K)	*Block 2 *14*
b4		3043A>G (T1015A)	*Block 2 *16*
b5		3751G>A (V1251I)	*Block 3 *2*
c1	*ABCC2*	1177C>T (R393W)	**7*
g1	*ABCG2*	376C>T (Q126X)	*Block 1 *4*
g2		1465T>C (F489L)	*Block 2 *2*
g3		1723C>T (R575X)	*Block 2 *5*
s1(S)[c]	*SLCO1B1*	1007C>G (P336R)	
s2		311T>A (M104K)	
u1	*UGT1A1*	−3279T>G, 1941C>G	*#60-#IB* (+/+)

[a] Defined in previous papers for *ABCB1* [26], *ABCC2* [27], *ABCG2* [28] and *UGT1A1* [35]

[b] Linked with *ABCB1*2* (*B*)

[c] Linked with *SLCO1B1*15 · 17* (*S*)

occurred in *non-UGT*+/+ patients at rates of 8.0% (4/50) in the irinotecan monotherapy and 20% (11/55) in the cisplatin-combination therapy, we investigated possible contributions of these minor transporter variations and another low-activity *UGT*-haplotype, *UGT1A1#60-#IB* [35], to severe neutropenia.

Among the rare variations detected, eleven heterozygous transporter genetic variations and one *UGT1A1#60-#IB* homozygote were found in *non-UGT*+/+ patients who experienced grade 4 neutropenia (Table 3). These variations include an amino acid substitution leading to reduced in vitro activity, *ABCG2* 1465T>C (F489L) [36], and the stop codons, *ABCG2* 376C>T (Q126X) and 1723C>T (R575X) [28].

Additive effects of transporter gene haplotypes on neutropenia

Since multiple transporters are involved in irinotecan PK/PD, severity of toxicity might depend on the number and combinations of the low-activity variants, each of which does not effectively affect PD. To examine this possibility, we surveyed relationships between ANC nadirs and combinations of haplotypes associated with grade 3/4 neutropenia ($P < 0.1$) and the minor variations associated with grade 4 neutropenia (listed in the previous section); the data for selected haplotypes/variations are depicted in Fig. 2. For the combination therapy with cisplatin (Fig. 2b), homozygous *SLCO1B1*15 · 17* was included, but *ABCC2*1A* was excluded since its effect in the cisplatin-combination therapy was not consistent among the *UGT* genotypes.

In the irinotecan monotherapy, ANC nadirs in most patients with either one or more of *ABCG2#IIB*, *SLCO1B1*15 · 17* and the minor variations were lower than the median ANC nadirs of both *UGT*−/− and *UGT*+/− patients without them (None) (Fig. 2a). In particular, the effects were more evident in patients bearing two or more of the selected haplotypes/variations (including the *UGT*+). Among the patients who experienced grade 3 or 4 neutropenia, 80% of patients had two or more candidate haplotypes/variations in the *UGT* (−/− and +/−) group (Fig. 2a).

In *UGT*+/− patients with the cisplatin-combination therapy, ANC nadirs of the patients with *ABCB1*2/*2*, *ABCG2#IIB/#IIB*, *SLCO1B1*15 · 17/*15 · 17* or any minor variations, and their combinations were lower than the median values of patients without these markers (None), except for one patient with *ABCB1*2/*2* and *SLCO1B1*15 · 17* (*B/B* + *S*/−) (Fig. 2b). Also, in *UGT*−/− and *UGT*+/− patients, the effects were more evident in the patients with two or more of the selected haplotypes/variations. Among the patients who experienced grade 4 neutropenia, 82% of patients had two or more candidate haplotypes/variations in the *UGT* (−/− and +/−) group (Fig. 2b).

It was noted that the additive effect of *g1* [*ABCG2* 376C>T (Q126X)] was not observed in the heterozygotes (*g1*/−), but was evident in the compound heterozygotes with another *ABCG2* genetic polymorphism, *#IIB*, (*G/g1*) (Fig. 2a, b).

Regarding the combined effects of the above transporter genotypes on SN-38 AUC values, higher levels were observed in patients with the candidate haplotypes/variations of two or more genes in the monotherapy, but this trend was not always evident in the cisplatin-combination therapy patients (data not shown).

Discussion

In this study, we showed possible additive effects of transporter and *UGT1A1* genotypes on irinotecan PK and PD. Since multiple transporters are involved in irinotecan PK, it is likely that a functional alteration of one of the responsible transporters can be compensated by other transporters; thus, changes in PK/PD parameters by transporter genotypes may not always be large. However, the overall elimination rate of irinotecan or its metabolites might be altered under the conditions of simultaneously reduced activities of multiple transporters, higher irinotecan doses, or reduced UGT activity.

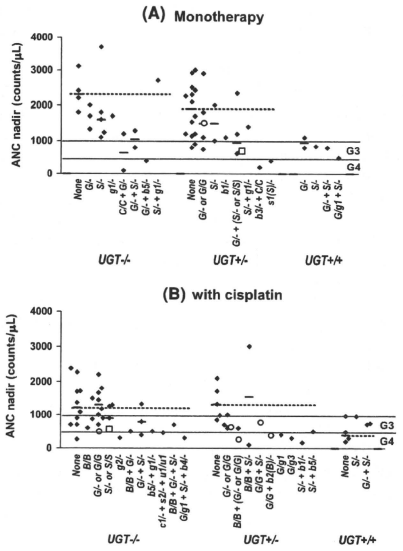

Fig. 2 Additive effects of transporter haplotypes/variations on ANC nadirs in irinotecan monotherapy (**a**) and combination therapy with cisplatin (**b**). *UGT+* = *UGT1A1*6* or **28*; *B* = *ABCB1*2*; *C* = *ABCC2*1A*; *G* = *ABCG2#IIB* (*open circle*, *#IIB/#IIB*); *S* = *SLCO1B1*15 · 17* (*open square*, **15 · 17/*15 · 17*); *b1–u1* = minor variations listed in Table 3. **a** *None* = *non-*(*C*, *G*, *S* or *minors*), **b** *None* = *non-*(*B*, *G*, *S* or *minors*). The *bar* in each genotype represents the median. The *dotted lines* in each *UGT* genotype show the median values of patients without any selected transporter polymorphisms/variations (*None*). The *lines* (G3 and G4) represent the border of grade 3 and 4 neutropenia

In the irinotecan monotherapy, the increasing effect of *ABCB1*2/*2* (block 2) on SN-38 AUC/dose was evident while contributions of *ABCB1 BJL* (block 1), *ABCB1*1b* (block 3), *ABCG2#IIB* and *SLCO1B1*15 · 17* were not significant in the multivariate analysis. For neutropenia, additive effects were suggested for *ABCC2*1A/*1A*, *ABCG2#IIB*, *SLCO1B1*15 · 17*, and possibly some minor genetic variations in addition to *UGT1A1*6* or **28* (Fig. 2a). The association of *ABCB1*2* (block 2) with grade 3 diarrhea was also observed.

In the combination therapy with cisplatin, an increase in the SN-38 AUC/dose by *ABCB1*2* and for a decrease by *ABCB1*1b* were observed, but the multivariate analysis did not show their significant contributions. Regarding neutropenia, additive effects of *ABCB1*2/*2*, *ABCG2#IIB/#IIB*, and possibly, *SLCO1B1*15 · 17/*15 · 17* and some minor variations were suggested (Fig. 2b).

Thus, in both regimens, the associations of *ABCB1*2* (block 2) with higher SN-38 AUC/dose levels and toxicities (diarrhea or neutropenia), and additive effects of *ABCG2#IIB* and *SLCO1B1*15 · 17* with *UGT1A1*6* or **28* on neutropenia were observed. The current study also suggests that combination genotypes with two or more genes could have a greater effect on neutrophil count reduction than a single gene, indicating a quantitative property of multiple genetic factors affecting phenotype. These findings could partly explain a large interindividual variation in irinotecan toxicities within each *UGT* genotype.

In this study, influences of the transporter genotypes on SN-38 AUC/dose did not always correlate to an influence on neutropenia as observed in the combination therapy with cisplatin and in the case of *ABCB1*2* (block 2) in the monotherapy. Although weak negative correlations were observed between the SN-38 AUC level and ANC nadir, the SN-38 AUC values of patients who exhibited grade 3/4 neutropenia (ANC nadir < 1,000 counts/μL) were fairly diverse, especially in the combination therapy with cisplatin (Fig. 3). It is likely that the extent of toxicities depends not only on systemic exposure levels of the active metabolite for which hepatic UGT activity is a large contributor, but also on the elimination from the target cells (neutrophil progenitor cells or enterocytes) where transporter function might be more critical.

Our previous study showed the association of *ABCB1* block 2 **2* [1236C>T, 2677G>T (A893S) and 3435C>T] with lower renal clearance of irinotecan and its metabolites [16]. The current data obtained in the irinotecan monotherapy also suggest higher AUC/dose for irinotecan, SN-38G, and SN-38 with *ABCB1*2/*2*. Since a high affinity of P-gp for irinotecan is known, lower elimination rate of irinotecan could also result in higher plasma levels of its metabolites. Other studies have also suggested associations of the haplotype 1236T–2677T (corresponding to our **2* group in this study) with a reduced excretion rate of P-gp substrates [37] and SN-38 [25], and associations of the haplotype 2677T–3435T (corresponding to our **2* group in this study) with paclitaxel-induced neutropenia [38].

For *ABCC2*, *ABCC2* −1774delG, a tagging SNP of **1A*, was reported to be associated with low promoter activity and cholestatic or mixed-type hepatitis [32]. Patients with *ABCC2*1A/*1A* together with *ABCB1*2/*2* or *ABCG2#IIB* showed higher values of SN-38 AUC (Fig. 1) and neutropenia in the monotherapy (Fig. 2a), but these trends were not evident in the *UGT−/−* patients treated with cisplatin-combination therapy (data not shown). Thus, the effects of *ABCC2* might be dependent on combinations with other genetic and non-genetic factors. Conflicting clinical outcomes of *ABCC2* 3972C>T, a marker of **1C/G*, were reported to cause higher AUC of irinotecan and its

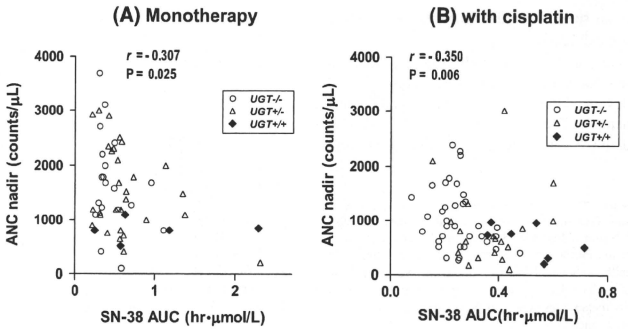

Fig. 3 Correlations between SN-38 AUC and ANC nadir in patients in irinotecan monotherapy (**a**) and combination therapy with cisplatin (**b**). *r* Spearman's rank correlation coefficient

metabolites in Caucasians treated with irinotecan monotherapy [18] and to lower the incidence of grade 3 diarrhea in Koreans treated with a combination therapy of irinotecan and cisplatin [24]. In the current study, no significant association of *ABCC2*1C/G* on PK/PD was observed in the monotherapy. Although a high incidence of grand 3/4 neutropenia was observed in patients with *ABCC2*1C/G* in the combination therapy with cisplatin, most patients also had *ABCG2#IIB* (data not shown); thus, the effect of *ABCC2*1C/G* remains obscure.

For *ABCG2*, the current study examined the association with the combinatorial haplotypes consisting of the three previously defined block haplotypes [28]. *ABCG2#IIB* contains the non-synonymous SNP 421C>A (Q141K), which was detected at higher frequencies in Asians and was reported to cause reduced expression of BCRP in vitro [36, 39–41]. In clinical studies, the association of 421C>A (Q141K) with higher plasma levels of diflomotecan was shown in Caucasians [42]. However, an association of this SNP with irinotecan PK/PD had not been shown [19, 24]. An association of 421C>A (Q141K) alone with irinotecan PK/PD was not significant in our hands (data not shown), but *#IIB* containing both 421C>A (Q141K) and IVS12 + 49G>T showed a moderate association with neutropenia. It is unclear whether the additional SNP IVS12 + 49G>T itself or another unknown linked SNP is causative for the reduced function. *ABCG2#IIIC* contains a non-synonymous SNP 34G>A (V12M) which has no influence on BCRP expression or activity in vitro [36, 39–41]. Our study showed no influence of *ABCG2#IIIC* on the SN-38 AUC/dose levels and neutropenia in the irinotecan monotherapy (data not shown), but did show a decreasing trend in grade 3/4 neutropenia in the combination therapy with cisplatin. In contrast, a report on Korean patients suggested the association of *ABCG2* 34G>A (V12M) with a higher incidence of grade 3 diarrhea in a combination therapy of irinotecan and cisplatin [24].

Among *SLCO1B1* polymorphisms, 521T>C (V174A), a tagging SNP of **15 · 17*, was demonstrated to reduce in vitro SN-38 influx [7], and clinical studies in Asians also showed its relevance to a higher SN-38 AUC and severe neutropenia in combination therapy of irinotecan with cisplatin [22–24]. Our results support these previous findings. Note that our **15 · 17* mainly consists of **17* [containing −11187G>A, 521T>C (V174A) and 388A>G (N130D)].

Taken together, the clinical data on transporter genotypes show variability among the studies. The reasons for these conflicting findings might be partly attributed to the ethnic differences in transporter genotypes and the regimens used. In addition, non-genetic factors, such as disease status and inflammation [43, 44], hepatic or renal function [45], and co-administered or pre-administered drugs, may also influence the clinical outcome.

The current study suggests combined effects of multiple haplotypes/variations on neutropenia. From clinical aspects of irinotecan therapy, the benefit of additional genotyping of transporters to predict severe toxicities should be clarified. Regarding grade 3 and 4 neutropenia, positive prediction values for two or more candidate genotypes including *UGT* (+) (Fig. 2) were 46 and 89% in the monotherapy and the cisplatin-combination therapy, respectively, which are low compared with *UGT+/+* (80 and 100%, respectively). Regarding grade 4 neutropenia, positive predictive values for these candidate genotypes were 15 and 41% in the monotherapy and the cisplatin-combination therapy, respectively, while for *UGT+/+*, they were 0 and 43%, respectively. Further studies using a

larger population size are needed to further elucidate the roles of these candidate markers.

In conclusion, the current study suggests there are additive effects for several transporter genotypes on the SN-38 AUC level and the reduction of neutrophil counts in irinotecan therapy. The clinical benefits of additional genotyping of these candidate markers should be further delineated.

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Simple and sensitive HPLC method for determination of amrubicin and amrubicinol in human plasma: application to a clinical pharmacokinetic study

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ABSTRACT: A simple and sensitive high-performance liquid chromatographic (HPLC) method was developed for determination of amrubicin and its metabolite amrubicinol in human plasma. After protein precipitation with methanol without evaporation procedure, large volume samples were injected and separated by two monolithic columns with a guard column. The mobile phase consisted of tetrahydrofuran–dioxane–water (containing 2.3 mM acetic acid and 4 mM sodium 1-octanesulfonate; 2:6:15, v/v/v). Wavelengths of fluorescence detection were set at 480 nm for excitation and 550 nm for detection. Under these conditions, linearity was confirmed in the 2.5–5000 ng/mL concentration range of both compounds. The intra- and inter-day precision and intra- and inter-day accuracy for both compounds were less than 10%. The method was successfully applied to a clinical pharmacokinetic study of amrubicin and amrubicinol in cancer patients. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: amrubicin; amrubicinol; cancer; protein precipitation; monolithic column

Introduction

Amrubicin, a completely synthetic 9-aminoanthracycline, is an active anticancer agent. Both amrubicin and amrubicinol, the C-13 hydroxy active metabolite of amrubicin, are inhibitors of the DNA topoisomerase II mediated cleavable complex. The antitumor activity of amrubicinol is 10–100 times greater than that of the parent compound *in vitro* (Yamaoka *et al.*, 1998). In phase I/II trials conducted in Japan the recommended dose of amrubicin was determined to be 45 mg/m^2 for three consecutive days every 3 or 4 weeks. In phase II trials of amrubicin monotherapy the response rate of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) was found to be 75.8% (Yana *et al.*, 2007) and 27.9% (Sawa *et al.*, 2006), respectively. Amrubicin was approved in Japan for the treatment of SCLC and NSCLC in December 2002. The major problem with amrubicin is hematological toxicity. An incidence of grade 3 or 4 toxicity of 76.8% has been found for neutropenia, 54.7% for leucopenia, 26.0% for anemia, 22.1% for thrombocytopenia and 35% for the more serious toxicity, febrile neutropenia (Kato *et al.*, 2006). The severity of these toxicities varies from individual to individual. Neutropenia has been reported to be associated with the area under the curve of the plasma amrubicinol concentration, which is one of the major pharmacokinetic (PK) parameters (Matsunaga *et al.*, 2006). A prospective PK and pharmacodynamic (PD) study was planned in our institution, the National Cancer Center Hospital (Tokyo, Japan), to evaluate the PK and PD parameters of amrubicin and amrubicinol and to develop an individualized dosing strategy for amrubicin.

Development of a simple and sensitive HPLC method for determination of amrubicin and amrubicinol in human plasma was required to conduct the PK/PD study. Four methods, including two HPLC methods (Noguchi *et al.*, 1998; Matsunaga *et al.*, 2006), an HPLC-MS-MS method (Yanaihara *et al.*, 2007) and a UPLC-MS-MS method (Li *et al.*, 2008), have already been reported. The two HPLC methods (Noguchi *et al.*, 1998; Matsunaga *et al.*, 2006) involve preparation by liquid–liquid extraction and solid-phase extraction, respectively, and they lack sensitivity because of low recovery and loss during processing. The HPLC-MS-MS method (Yanaihara *et al.*, 2007), on the other hand, involves preparation by solid-phase extraction, but the lower limits of quantification (LOQ) of amrubicin and amrubicinol is 20 ng/mL, which is higher than the plasma concentration of amrubicin 24 h after an intravenous bolus and higher than the concentration of amrubicinol, stated in the application for the approval of amrubicin. However,

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Abbreviations used: NSCLC, non-small cell lung cancer; PD, pharmacodynamic; PK, pharmacokinetic; SCLC, small cell lung cancer.

although an UPLC-MS-MS method by protein precipitation (Li *et al.*, 2008) improved the LOQ and preparation procedure, it is not general measurement equipment in hospitals because the equipment is too expensive.

A monolithic column, which has applied for bioanalysis with in the past 10 years, has a lower back-pressure than packed beds (Nguyen *et al.*, 2006). Many applications of analysis using monolithic column have been investigated in μ-HPLC and capillary electrochromatography. Otherwise, few methods refer to the use of a monolithic column by HPLC. The advantage of using monolith columns is that sensitivity can be improved by a long column and large volume samples can be injected.

There are many methods of sample preparation, including protein precipitation, liquid–liquid extraction and solid-phase extraction. Protein-precipitation is the simplest method in terms of procedure and technique. Moreover, recovery with protein precipitation is higher than that with liquid–liquid extraction or solid-phase extraction because amrubicin and amrubicinol have both hydrophilic sites and hydrophobic sites.

The aim of this study was to use monolithic columns to develop a simple and sensitive HPLC method for determination of amrubicin and amrubicinol in human plasma.

Experimental

Chemicals

Amrubicin and amrubicinol were provided by Dainippon Sumitomo Pharmaceuticals Co. Ltd (Osaka, Japan). The chemical structure of amrubcin and amrubicinol are shown in Fig. 1. All other chemicals and reagents used were of analytical reagent grade or HPLC grade and were purchased from Wako (Osaka, Japan).

Chromatographic Instrumentation and Conditions

The chromatographic system consisted of an LC-20AD pump, a SIL-20AC auto sampler, an RF-10AXL fluorescence detector and C-R8A Chromatopac integrator with a CTO-10A oven (Shimadzu, Kyoto, Japan). Two connected Onyx Monolithic C_{18} (100 × 4.6 mm) columns were used with an Onyx Monolithic C_{18} Guard Cartridge (10 × 4.6 mm; Phenomenex, Torrance, CA, USA). Effluent was monitored with a fluorescence detector set at an excitation wavelength of 480 nm and a detection wavelength of 550 nm. The mobile phase consisted of tetrahydrofuran–dioxane–water (containing 2.3 mM acetic acid and 4 mM sodium 1-octanesulfonate; 2:6:15, v/v/v) pumped at flow rate of 0.9 mL/min at a column temperature of 35°C.

Preparation of Standards and Plasma Samples

Stock solutions of amrubicin and amrubicinol were stored in plastic microtubes to avoid adsorption to glassware, and stored at −80°C. Working solutions were obtained by diluting the stock solutions with acetonitrile. Blank plasma samples for use in validating the method were obtained from healthy volunteers. The patient plasma samples were obtained from the National Cancer Center Hospital. Both the blank plasma samples and patient plasma samples were stored at −80°C until analyzed. Heparin sodium salt was added to patient blood samples to prevent coagulation. Then the blood samples were centrifuged at 5000 rpm for 10 min.

Extraction Procedure

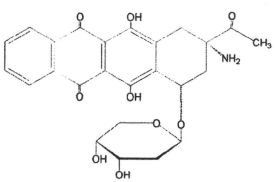
Plasma (100 μL) in a 1.5 mL screw-capped tube was diluted with 20 μL of 16 mM citric acid–16 mM Na_2HPO_4–0.9% NaCl solution, and after adding 480 μL of methanol the tube was shaken for 30 min. The mixture was then centrifuged for 10 min at 12,000 rpm. The supernatant was filtered through an UltraFree-MC filter (Millipore, Tokyo, Japan), and 250 μL of the solution was transferred into auto sampler vials and vortex-mixed with a 500 μL of 16 mM citric acid–16 mM Na_2HPO_4–0.9% NaCl solution. A 450 μL volume of the solution was injected into the HPLC system for analysis.

Validation

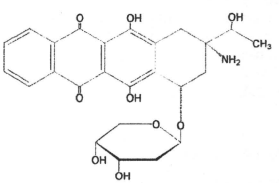
Specificity. The specificity of the method was evaluated by comparing different blank plasma samples and plasma samples spiked with amrubicin and amrubicinol. The blank plasma samples were collected from nine volunteers.

Accuracy, precision and recovery. Accuracy and precision were determined by replicate analysis ($n = 6$) of plasma samples spiked with three concentrations of amrubicin and amrubicinol: 10, 100 and 1000 ng/mL. Accuracy was evaluated as relative error (RE), and precision was evaluated as coefficient of variation (CV). Recovery was assessed by comparing the results of analyses of extracted plasma samples and unextracted standards containing the same concentrations.

Calibration curve. The LOQ was determined from the peak and the standard deviation of the noise level (SN). The LOQ was defined as the concentration of amrubicin and amrubicinol resulting in a peak height of 10 times SN. The calibration curve was generated by linear regression of the peak areas (y) of amrubicin and amrubicinol against the corresponding concentrations (x) of amrubicin and amrubicinol in plasma.



amrubicin



amrubicinol

Figure 1. Chemical structures of amrubicin and amrubicinol.

Analysis of Patient Samples

For the analysis of plasma concentration of amrubicin and amrubicinol, plasma samples were obtained from lung cancer patients treated with 40 mg/m^2 of amrubicin. All patients were enrolled in the prospective PK/PD study, which was aimed to evaluate the correlation between PK and PD of amrubicin and amrubicinol. Written informed consent was obtained from all patients. This study was approved by the Ethical Review Board of National Cancer Center Hospital and is ongoing. The plasma samples were obtained from blood samples collected immediately before injection, and immediately after the injection, and 5, 15 and 30 min, and 1, 2, 4, 8 and 24 h after the end of injection. Each sample was determined in triplicate.

Results

Specificity

No endogenous interference was observed at the retention times of amrubicin and amrubicinol. The retention time of amrubicin and amrubicinol was approximately 8.5 and 10.2 min, respectively. Representative chromatograms of the blank plasma sample, the plasma sample spiked and the patient plasma sample are shown in Fig. 2. The capacity factors (k') of amrubicin and amrubicinol were 1 and 1.4, respectively.

Accuracy, Precision and Recovery

The results for intra- and inter-day accuracy, precision and recovery are shown in Table 1. Intra-day accuracy ranged between −4.1 and 0.8% for amrubicin and between −9.8 and −2.1% for amrubicinol. Inter-day accuracy was between −3.1 and 3.0% for amrubicin and between −4.0 and 2.3% for amrubicinol. Intra-day precision was 1.4–8.8% for amrubicin and 1.3–4.2% for amrubicinol. Inter-day precision was 2.7–8.8% for amrubicin and 5.3–5.5% for amrubicinol. Recovery was greater than 95% at all concentrations (10, 100 and 1000 ng/mL) of amrubicin and amrubicinol.

Lower Limit of Quantitation

The LOQ was 2.5 ng/mL for both amrubicin and amrubicinol. At that level the coefficient of variation (CV) was 8.3% for amrubicin and 3.2% for amrubicinol ($n = 6$).

Linearity

Under the chromatographic conditions described, linearity and the appropriate correlation coefficient were achieved for amrubicin within the concentration range from 2.5 to 5000 ng/mL. The linear regression equation for amrubicin was $y = 526.3x + 6156.3$, and the correlation coefficient (r) was 0.999. Similar results were obtained for amrubicinol with the concentration range from 2.5 to 5000 ng/mL. The linear regression equation for amrubicinol was $y = 662.9x + 2947.7$, and the correlation coefficient (r) was 0.996.

Analysis of Patient Plasma Samples

The amrubicin and amrubicinol in the patient plasma samples were separated well under the optimal chromatographic conditions. Figure 2(C) shows a chromatogram of amrubicin and amrubicinol in a plasma sample from a patient who was treated at dose of 40 mg/m^2 of amrubicin. Figure 3 shows the concentration–time profiles for amrubicin and amrubicinol after an intravenous bolus. The amrubicin concentrations ranged between 4.3 and 2504 ng/mL, and the amrubicinol concentrations ranged between 3.0 and 18.5 ng/mL. These concentrations were similar to the concentrations stated in the application for approval of amrubicin.

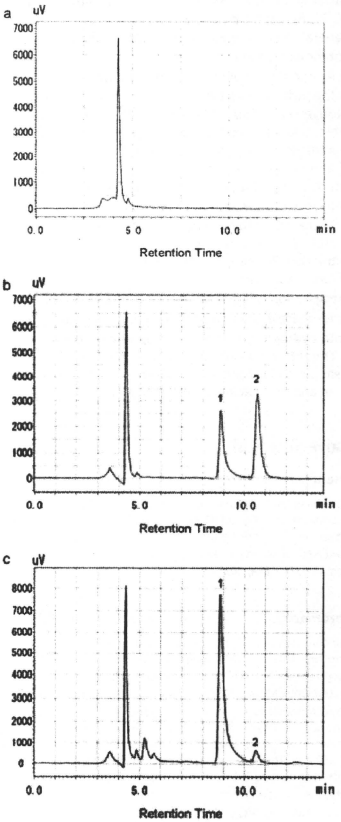


Figure 2. Representative HPLC chromatogram. (a) Blank plasma sample; (b) plasma sample spiked with 100 ng/mL amrubicin and amrubicinol; (c) patient plasma sample obtained 2 h after anintravenous bolus dose of 40 mg/m^2 of amrubicin, Peaks:1 = amrubicin; 2 = amrubicinol. For chromatographic condition see Experimental section.

Discussion

We developed a simple and sensitive method of determination for amrubicin and amrubicinol by HPLC. In our method protein

Table 1. Intra-day and inter-day accuracy, precision and recovery of the HPLC method for amrubicin and amrubicinol

	Nominal concentration (ng/mL)	*n*	Experimental concentration (mean ± SD, ng/mL)	Precision (% CV)	Mean recovery (%)	Accuracy (%RE)
Intra-day						
Amrubicin	10	6	9.9 ± 0.6	5.9	98.9	−1.1
	100	6	95.9 ± 1.3	1.4	100.0	−4.1
	1000	6	1007.6 ± 88.2	8.8	100.7	0.8
Amrubicinol	10	6	9.0 ± 0.4	4.2	98.5	−9.8
	100	6	97.8 ± 1.3	1.3	97.8	−2.1
	1000	6	954.9 ± 14.6	1.5	95.5	−4.5
Inter-day						
Amrubicin	10	6	9.7 ± 0.3	2.7	98.0	−3.1
	100	6	102 ± 8.6	8.4	100.0	2.0
	1000	6	1029.8 ± 90.9	8.8	100.7	3.0
Amrubicinol	10	6	10.0 ± 0.5	5.3	99.3	0.1
	100	6	96.0 ± 5.5	5.7	99.3	−4.0
	1000	6	1023.2 ± 56.4	5.5	100.4	2.3

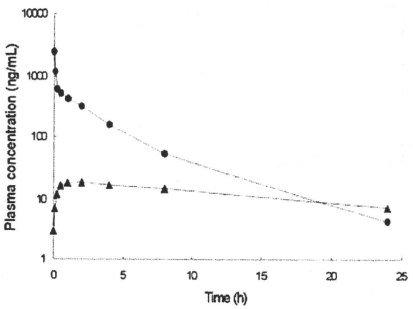


Figure 3. Plasma concentrations vs time curves of amrubicin (circles) and its metabolite amrubicinol (triangles) in a patient treated with 40 mg/m^2 of amrubicin.

precipitation is used to prepare the samples, and monolithic columns are used to make determination.

During the past decade monolithic columns have emerged as an alternative to traditional packed-bed columns. Monolithic columns are structurally very different from packed-bed columns. The most interesting characteristic of monoliths is their high external porosity resulting from the structure of the network of through-macropores. Another interesting characteristic is the structure of the stationary phase skeleton, which consists of a network of small, thin threads of porous silica. These structural characteristics allow the combination of the low hydraulic resistance of the column to the stream of mobile phase and an enhancement of the column of the rate of the mass transfer of the sample molecules through the column. In this way, the monolithic column improves back-pressure. Yunsheng *et al.* (2003) investigated the utility of monolithic column for direct HPLC-MS-MS analysis. Although access to the matrix in biological samples was prevented in analysis by packed-bed columns, the monolith column with high porosity could remove matrix macromolecules.

In this study, we evaluated packed-bed columns, such as Sumipax ODS A-212 (5 μm, 6 mm × 15 cm), Synergi Hydro-RP (4 μm, 4.6 mm × 15 cm), Luna C_{18} (4 μm, 4.6 mm × 15 cm) and Luna C_{18} (3 μm, 4.6 mm × 15 cm). The LOQ using Sumipax ODS A-212 was 5 ng/mL for both amrubicin and amrubicinol. Using the other three columns, we could not obtain sufficient result (data not shown). We considered the reason why small particles prevented the access of macromolecules in biological samples.

We connected two monolithic columns in tandem like a long column, which made it possible to determine low concentrations of amrubicin and amrubicinol without high pressure. As a result, the sensitivity of our method is equal or superior to that of other methods, including methods that use HPLC-MS-MS or UPLC-MS-MS, and its sensitivity is adequate for performance of the analyses in the PK study.

The sample preparation procedure in this method is based on protein precipitation, because it is simple. Moreover, recovery by protein precipitation is higher than by liquid–liquid extraction or solid-phase extraction, because amrubicin and amurbicinol have both hydrophilic sites and hydrophobic sites. Methanol was selected for protein precipitation, because methanol makes shaper peaks and enables better separation than other organic solvent (data not shown). We added an appropriate amount of buffer, which prevents broad peaks, to the samples after protein precipitation.

The fluorescence detector was set at an excitation wavelength of 480 nm and a detection wavelength of 550 nm. In the excitation wavelength, the highest energy should be obtained in the 480 nm region at the level of excitation lamps, according to the proposal for determination of anthracyclines (Sepaniak and Yeung, 1980). We set the excitation wavelength at 480 nm. Since the most sensitive detection wavelength for amrubicin and amrubicinol was 550 nm, we used it as the detection wavelength in our method. The mobile phase was a modification of a previous report (Noguchi *et al.*, 1998). 1-Octanesulfonate improved separation compared with other ion-pair agents including 1-heptanesulfonate, which used Noguchi's method (data not

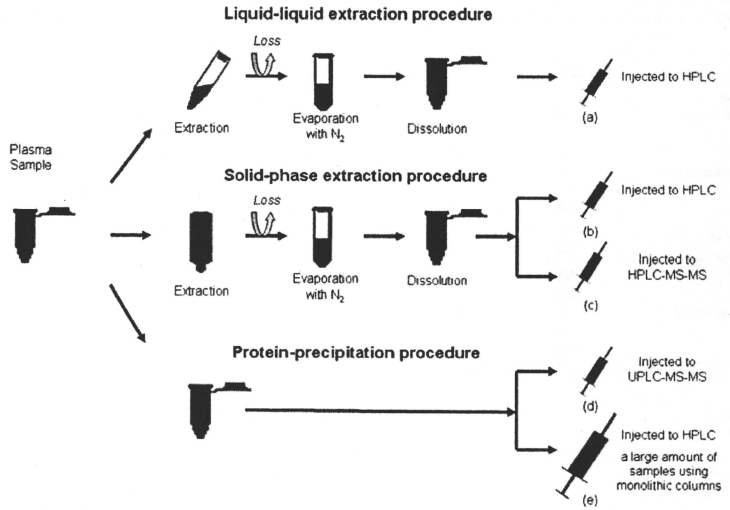


Figure 4. Four methods previously described methods for determination of amrubicin and amrubicinol in plasma; (a) HPLC method (Noguchi *et al.*, 1998) with sample preparation by liquid–liquid extraction; (b) HPLC method (Matsunaga *et al.*, 2006) with sample preparation by solid-phase extraction; (c) HPLC-MS-MS method (Yanaihara *et al.*, 2007) with sample preparation by solid-phase extraction; (d) UPLC-MS-MS method (Li *et al.*, 2008) with sample preparation by protein precipitation; (e) HPLC method with sample preparation by protein precipitation in this paper.

shown). We added 1-octanesulfonate to mobile phase as an ion-pair agent.

Previous reports have described four methods for determination of amrubicin and amrubicinol. The UPLC-MS-MS method (Li *et al.*, 2008) is the most sensitive of the four; however, UPLC-MS-MS is not widely available in hospitals. The other methods involve problems in relation to application to PK studies, such as low recovery or loss during processing (Fig. 4). A more simple and sensitive method that can be performed with equipment that is generally available was needed for analysis in hospitals.

We validated our method under Guidance for Industry of the Food and Drug Administration in Bioanalytical Method Validation, with regard to specificity, accuracy, precision, recovery and calibration curve for concentrations ranging from 2.5 to 5000 ng/mL, which were thought to be clinically relevant range for amrubicin and amrubicinol concentrations in plasma. Both the inter-day and intra-day accuracy and precision of the method were adequate. Our method provides good sensitivity, and was able to detect all points in our PK study.

Conclusion

A simple and sensitive HPLC method was developed for determination of amrubicin and amrubicinol in human plasma. In our method, we selected a monolithic column for determination and protein precipitation for preparation, and it was validated sufficiently. This method can be used clinically because the required equipment and technique are simple. The PK/PD study of amrubicin is ongoing, and a therapeutic drug monitoring study by this HPLC method is in the planning stage.

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Phase I study of TLR9 agonist PF-3512676 in combination with carboplatin and paclitaxel in patients with advanced non-small-cell lung cancer

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This phase I, open-label study investigated the Toll-like receptor 9 agonist, PF-3512676, in combination with carboplatin and paclitaxel in Japanese patients with advanced, non-small-cell lung cancer (NSCLC). Patients (n = 12) with treatment-naive stage IIIB or IV NSCLC received single-agent PF-3512676 subcutaneously once during the first 7 days (monotherapy phase) in three escalating dose levels (0.1, 0.2, and 0.4 mg/kg) followed by a combination phase during which patients received 0.1 or 0.2 mg/kg PF-3512676 subcutaneously on days 8 and 15 of each 3-week cycle of carboplatin (area under the curve, 6 mg × min/mL) and paclitaxel (200 mg/m^2). Safety and pharmacokinetics of PF-3512676 were assessed during monotherapy and combination therapy phases. PF-3512676 was tolerable as monotherapy or in combination with chemotherapy in patients with NSCLC. Most common treatment-related, non-hematologic adverse events (AEs) throughout the study were injection-site reactions (n = 12, 100%) and flu-like symptoms (n = 11, 91.7%) that were each grade 1 or 2 in all but one patient. All patients experienced neutropenia and leukopenia (≥grade 3 in 11 [91.7%] and seven [58.3%] patients, respectively). One patient in dose level 2 had a dose-limiting toxicity: grade 3 rash and grade 3 increase in γ-glutamyltransferase during combination therapy. Mean PF-3512676 half-life ranged from 4.8 to 21.6 h (longer with higher doses). Four (33%) patients had objective responses (one complete response, three partial responses), and seven (58%) patients achieved stable disease. PF-3512676 as monotherapy and in combination with chemotherapy had an acceptable safety profile in Japanese patients with treatment-naive NSCLC. (*Cancer Sci* 2010; 101: 188–195)

Worldwide, lung cancer accounts for 1.3 million deaths per year, and cancers of the lung, trachea, and bronchus are the leading cause of cancer-related death in Japanese men. Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of lung cancers,[1] and the vast majority (~70%) of cases of NSCLC are locally advanced or metastatic at diagnosis.[2] The current standard first-line treatment for patients with stage IIIB or IV NSCLC and good performance status is doublet chemotherapy with a platinum agent (e.g. carboplatin or cisplatin) in combination with paclitaxel, docetaxel, gemcitabine, or vinorelbine.[3,4] This treatment is associated with objective response rates of approximately 20% to 40% and median survival of 8 to 10 months, which is not considered satisfactory to patients.[4,5] Therefore, development of more effective treatment regimens is warranted for the unmet medical needs of patients with advanced NSCLC.

Toll-like receptors (TLRs) are a family of specialized immune receptors that induce protective immune responses upon detection of highly conserved pathogen-expressed molecules. To date, 10 different TLRs have been identified in humans.[6,7] Each TLR binds one or more distinct pathogen-expressed molecules and can function as an immune system 'alarm signal,' leading to initiation of appropriate host immune defenses.[6,8] In humans, TLR9 is expressed primarily by plasmacytoid dendritic cells (pDCs) and B cells. It recognizes unmethylated cytosine-phosphate-guanine (CpG) dinucleotide sequences commonly found in bacterial and viral DNA.[9] TLR9 can also be stimulated using synthetic oligodeoxynucleotides (ODNs) containing one or more unmethylated CpG dinucleotide motifs. This stimulation leads to activation of type 1 helper T cell (T_H1)-like innate immunity, including upregulated production of interleukin (IL)-6, IL-12p40, interferon-alpha (IFN-α), and IFN-inducible chemokines such as interferon-γ-inducible protein 10 (IP-10).[10] Innate immune activation with a TLR9 agonist may enhance tumor antigen presentation and promote an antitumor immune response.

PF-3512676 (formerly known as CpG 7909) is a TLR9 agonist that has been tested in clinical trials for the treatment of patients with several types of cancer.[11] This synthetic CpG ODN can induce potent innate and adaptive immune T_H1 responses, and to a lesser extent, T_H2 immune responses in murine models.[9–12] Preclinical evidence supporting the use of PF-3512676 in lung cancer was provided by studies with a murine Lewis lung cancer model in which mice treated with PF-3512676 in combination with paclitaxel had significantly prolonged survival compared to mice treated with either drug given alone ($P < 0.0001$).[13] This preclinical evidence, combined with the promising clinical activity of PF-3512676 in other types of advanced cancer, supported investigation in patients with NSCLC. In non-clinical studies in mice investigating efficacy of PF-3512676 plus paclitaxel in the metastatic Renca renal cell carcinoma (RCC) models, survival following treatment with PF-3512676 was longer with regional divided dosing and weekly administration compared with temporal divided dosing using twice-weekly administration. Furthermore, in early clinical studies, elevations of IP-10 observed after dosing with PF-3512676 returned to baseline levels after about 1 week. Therefore, PF-3512676 was administered weekly with rotation of administration sites in clinical studies. Chemotherapy and PF-3512676 were not co-administered because chemotherapy was intended to cause decomposition of tumor cells and release of tumor antigens. PF-3512676 was administered after chemotherapy so that pDCs activated through the TLR9 pathway might present these antigens, thus increasing the number of antigen-specific, cytotoxic T cells.

The safety of PF-3512676 has been studied in more than 800 subjects, including more than 400 cancer patients. The extensive human clinical experience demonstrates that PF-3512676 is safe and well tolerated. In phase I studies in Western patients, 0.0025 to 0.81 mg/kg PF-3512676 weekly subcutaneous doses have

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been evaluated. To date, no organ dysfunction meeting the protocol-defined dose-limiting toxicity (DLT) criteria has been reported in any of these studies, and the maximum tolerated dose has not been defined.

In phase I or II studies of single-agent PF-3512676, the minimum dose level at which objective response was reported was 0.16 mg/kg weekly in patients with cutaneous T-cell lymphoma or RCC and 6 mg (approximately 0.10 mg/kg) weekly in patients with advanced melanoma.[14–16] A subsequent, randomized phase II study in Western patients with chemotherapy-naive NSCLC investigated PF-3512676 (0.2 mg/kg) in combination with standard taxane/platinum doublet chemotherapy (n = 74) and chemotherapy alone (n = 37).[17] The PF-3512676 dose of 0.2 mg/kg was selected to be above the minimum dose associated with antitumor activity in phase I and II studies of single-agent PF-3512676 and below the dose level (0.24 mg/kg) that, in the same single-agent studies, had been well tolerated for up to 6 months by the majority of patients. In the randomized phase II NSCLC study, the response rate in the PF-3512676 plus chemotherapy arm was higher than that in the chemotherapy-alone arm (30% *vs* 19% confirmed response rate, respectively). In addition, there was a trend toward improved median overall survival with addition of PF-3512676 to chemotherapy (12.3 months compared with 6.8 months for chemotherapy alone, P = 0.188). One-year survival was 50% and 33% for PF-3512676 plus chemotherapy and chemotherapy alone, respectively. Common adverse events (AEs) considered related to treatment with PF-3512676 and not to chemotherapy were injection-site reactions and flu-like symptoms. Other, less-common AEs considered related to treatment with PF-3512676 were febrile neutropenia, anemia, and thrombocytopenia. Overall, a 0.2 mg/kg dose of PF-3512676 in combination with taxane/platinum doublet chemotherapy appeared to have promising antitumor activity as well as a favorable safety profile and was recommended for further study in patients with advanced NSCLC.[17]

The present phase I study was conducted to investigate the safety and pharmacokinetics of PF-3512676 both as monotherapy and in combination with carboplatin and paclitaxel as first-line therapy for Japanese patients with advanced NSCLC.

Patients and Methods

Patients. Patients aged 20 to 75 years with histopathologically or cytologically diagnosed, previously untreated stage IIIB or IV NSCLC were eligible. To enroll in the study, patients were required to have a life expectancy ≥3 months, an Eastern Cooperative Oncology Group performance status (ECOG PS) of ≤1, and at least one measurable lesion of ≥20 mm according to Response Evaluation Criteria in Solid Tumors (RECIST).[18] Patients were also required to have adequate renal, liver, and bone marrow function (serum creatinine <1.5 × upper limit of normal [ULN], total bilirubin <1.5 × ULN, aspartate aminotransferase and alanine aminotransferase <2.5 × ULN, absolute neutrophil count ≥2000/mm^3, platelets ≥100 000/mm^3, and hemoglobin ≥10 g/dL).

Patients were excluded if they had brain or central nervous system metastases that were symptomatic or requiring treatment; any other malignancies within the past 5 years (except non-melanoma skin cancer or adequately treated *in situ* cervical cancer, gastric cancer, or colorectal cancer); autoimmune or antibody-mediated diseases; possible hypersensitivity to ODNs or castor oil; or hepatitis B or C infection. In addition, patients were excluded if they had participated in any other clinical trials; had received other investigational drugs within the previous 3 months; were pregnant or lactating; had uncontrolled infections or hypertension; had certain cardiac abnormalities; or required chronic treatment with therapeutic doses of systemic corticosteroids.

This study was conducted according to the Declaration of Helsinki and its amendments, Japanese Good Clinical Practice guidelines, and in agreement with the Institutional Review Board at the National Cancer Center Hospital (Tokyo, Japan). All patients provided written informed consent prior to study procedures.

Study design and treatments. This was an open-label phase I study in patients with advanced NSCLC. Patients received single-agent PF-3512676 subcutaneously on day 1, followed by 7 days of observation. If safety was confirmed, the patient immediately proceeded to the combination therapy phase. During combination therapy, carboplatin (area under the curve [AUC] 6 mg × min/mL) and paclitaxel (200 mg/m^2) were administered by intravenous (i.v.) infusion on day 1 and PF-3512676 was administered subcutaneously on days 8 and 15 of a 3-week cycle. Treatments were administered for a maximum of six cycles. Dexamethasone (20 mg) and chlorpheniramine maleate (10 mg) were administered by i.v. infusion 1 h before and ranitidine (50 mg) by i.v. infusion at least 30 min before each administration of paclitaxel.

During the monotherapy phase, patients in dose levels 1 and 2 were to be administered 0.1 mg/kg and 0.2 mg/kg PF-3512676, respectively. These doses were to be maintained during the combination therapy phase. Patients in dose level 3 were to receive 0.4 mg/kg PF-3512676 in the monotherapy phase and 0.2 mg/kg during the combination therapy phase. The three treatment arms with a maximum dose of 0.4 mg/kg PF-3512676 during the monotherapy phase were designed to establish one of the primary endpoints of this study: the pharmacokinetic (PK) profile of PF-3512676 in Japanese patients. Another study objective was to determine whether the same dose (0.2 mg/kg) of PF-3512676 that was used in combination with chemotherapy in the phase II and III studies of this agent in Western patients with NSCLC would also be recommended in Japanese patients. Therefore, PF-3512676 in dose level 3 was reduced from 0.4 mg/kg to 0.2 mg/kg when patients moved from the monotherapy to the combination phase. Patients with no DLT in the monotherapy phase could move immediately into the combination phase. For patients in level 3 only, any DLT observed during the monotherapy phase would have led to extension of the duration of this phase of the study by 1 week; if severity of toxicity decreased to ≤grade 1, patients would then continue into the combination therapy phase. A DLT was defined as any of the following: ≥grade 3 febrile neutropenia accompanied by infection; ≥grade 3 non-hematologic toxicity; ≥grade 3 injection site reaction; ≥grade 3 thrombocytopenia requiring transfusion; grade 4 flu-like symptoms; grade 4 neutropenia lasting 7 days; or grade 4 thrombocytopenia. DLT evaluation took place during monotherapy and the first cycle of combination therapy. PF-3512676 activates the immune system, and commonly associated AEs include flu-like symptoms and mild neutropenia believed to be the result of transient migration of neutrophils into peripheral tissues. This is distinct from bone-marrow suppression and may not necessarily be an indication of an increased risk of infection. Therefore, in this study, ≥grade 3 neutropenia was not considered a DLT unless it was accompanied by infection. If, following a DLT, continuation of study was judged to be possible with dose reduction of chemotherapeutic agents, and if study protocol dose-reduction criteria were satisfied, treatment could be continued. The dose of carboplatin could also be reduced to AUC 4.5 mg × min/mL and/or paclitaxel to 150 mg/m^2 if, in the absence of a DLT, patients had specific, predesignated hematologic or non-hematologic adverse events. These dose modifications were based on those reported for the Four-Arm Comparative Study.[19] The planned sample size for dose levels 1 and 3 was three patients each. If one DLT was observed in dose level 1 or 3, three additional patients were to be enrolled. The planned number of patients in dose level 2 in

this study was predefined to be six patients. If >1 DLT was observed in dose levels 1 or 2, the study would not have progressed to the next level. Dose level 2 in this study was the same dose used in preceding clinical studies in Western patients.

Primary endpoints were evaluation of safety and PK of PF-3512676 during the monotherapy and combination therapy phases. Secondary endpoints included evaluation of patient immune function and objective tumor response according to RECIST.

Pretreatment assessment and follow-up studies. History, physical examination (including temperature, blood pressure, heart rate, and weight) ECOG PS, and routine laboratory studies were performed at baseline, before each treatment cycle, and at end of the study. Routine laboratory studies included serum electrolytes, renal and liver function tests, complete blood count and differential white blood cell counts, coagulation studies, and urinalysis. Physical examination and complete blood count were also performed on days 2, 3, and 4 of the monotherapy phase and on days 1, 8, 9, 10, 11, and 15 of the first cycle of combination therapy. After patients completed one cycle of monotherapy and one cycle of combination therapy, these tests were performed on days 1, 8, and 15 of all other cycles of combination therapy. An electrocardiogram was performed at baseline as well as at 3 and 24 h after administration of PF-3512676 monotherapy. Severity of AEs and other symptoms were evaluated according to Common Terminology Criteria for AEs (CTCAE) version 3.0. Relevant radiologic studies to assess measurable and evaluable disease were repeated after every other cycle, and responses were scored according to RECIST.

Pharmacokinetics. To compare the PK of PF-3512676 in the monotherapy phase with its PK during the combination therapy phase, blood samples were collected predose and at 1, 2, 3, 5, 7, 10, 24, 48, 72, and 96 h postdose in the monotherapy phase as well as predose and at 1, 2, 3, 5, 7, 10, 24, 48, 72, and 96 h postdose on day 8 of the first cycle of the combination therapy phase. For each sample, 4 mL of whole blood was collected in a tube containing EDTA-2K dipotassium salt. Collected samples were centrifuged at 1000*g* for 10 min, and resultant plasma was stored in aliquots at or below −70°C until analysis. Concentrations of PF-3512676 were determined by Pharmaceutical Product Development (Richmond, VA, USA) using a validated hybridization assay with capture and detection probes complementary to either the 3′ or 5′ portions of the molecule. Pharmacokinetic parameters were calculated and summarized using descriptive statistics.

Pharmacodynamics. To evaluate patient immune function, blood samples were collected to measure the serum concentrations of IP-10, IL-6, IFN-α, IL-12p40, monocyte chemotactic protein-1 (MCP-1), and C-reactive protein (CRP). Serum samples were collected predose and at 1, 3, 7, 24, 48, 72, 96, and 168 h postdose of PF-3512676 in the monotherapy phase. During the combination therapy phase, samples were collected on day 8 of the first cycle of combination therapy predose and at 1, 3, 7, 24, 48, 72, 96, and 168 h postdose. For each sample, ≥3 mL of whole blood was collected, stored at room temperature for 30 min, and then centrifuged at 1000*g* for 10 min. Resultant serum was stored in aliquots at or below −70°C until analysis. Serum levels of IFN-α, IL-12p40, and MCP-1 were determined by the Human Custom Three-Plex Beads Kit (Invitrogen/Biosource, Carlsbad, CA, USA). Multianalyte profiling was performed on the BioPlex® Suspension Array System, and acquired fluorescence data were analyzed by the BioPlex Manager software versions 4.1 (BioRad Laboratories, Hercules, CA, USA). The levels of CRP, IP-10, and IL-6 were determined by ELISA (enzyme-linked immunosorbent assay). C-reactive protein was quantified with the C-reactive Protein (hsCRP) EIA kit (ALPCO Diagnostics, Salem, NH, USA). Interleukin-6 and IP-10 were detected using the Quantikine® HS Human IL-6 Immunoassay kit and Quantikine® Human CXCL10/IP-10 Immunoassay kit (R&D systems, Minneapolis, MN, USA), respectively. The levels of IFN-α, MCP-1, IL12-p40, and CRP were determined at Mitsubishi Chemical Medicine (Tokyo, Japan). The levels of IP-10 and IL-6 were determined at Quest Pharmaceutical Services (Newark, DE, USA).

Results

Patient characteristics. From June 2006 to March 2007, a total of 12 patients were enrolled, and all patients were treated with PF-3512676 monotherapy and at least one cycle of combination therapy. There were seven male and five female patients in this study, and median age was 60 (range, 41–69) years (Table 1). Most patients had stage IV disease (8/12, 67%) and adenocarcinoma (9/12, 75%). Forty-two total cycles of combination therapy were administered, and the median number of combination therapy cycles per patient was four (range, 1–6).

Safety. A list of any-grade AEs with incidence of 30% or more in either the monotherapy phase or the entire study (both monotherapy and combination therapy phases) is presented in Table 2. Many treatment-related AEs observed during the combination therapy phase were likely to be at least in part related to PF-3512676, as they also developed in patients during the monotherapy phase. Treatment-related AEs that occurred in >30% of patients during monotherapy included injection-site reactions (n = 12, 100%), flu-like symptoms (n = 11, 91.7%), lymphocytopenia (n = 6, 50.0%), leukopenia (n = 4, 33.3%), and anemia (n = 4, 33.3%). Neutropenia was also observed (n = 3, 25.0%). Through the entire study period the most common treatment-related AEs of any grade were injection-site reactions, neutropenia, and leukopenia (n = 12, 100% for each); anemia, flu-like symptoms, and lymphocytopenia (n = 11, 91.7% each) were also very common.

Only injection-site reactions and flu-like symptoms occurred with similar frequency in both monotherapy and combination therapy phases, suggesting these AEs were most closely related to treatment with PF-3512676. Certain AEs such as thrombocytopenia, monocytopenia, and malaise that were observed during the combination therapy phase were not observed at all during monotherapy phase, suggesting they were most closely related to chemotherapy.

Seven patients discontinued study therapy; one patient in dose level 1 discontinued as the result of progressive disease, while the remaining six patients (85.7%) discontinued as a result of

Table 1. Characteristics of patients

Enrolled patients, *n*	12
Age (years), median (range)	60 (41–69)
Gender, *n* (%)	
Men	7 (58)
Women	5 (42)
Baseline ECOG performance status	
0	7
1	5
Histologic classification of NSCLC, *n* (%)	
Adenocarcinoma	9 (75)
Squamous cell carcinoma	2 (17)
Other	1 (8)
Clinical stage, *n* (%)	
IIIB	4 (33)
IV	8 (67)

ECOG, Eastern Cooperative Oncology Group; NSCLC, non-small-cell lung cancer.

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Table 2. Treatment-related adverse events occurring in >30% of patients in either the PF-3512676 monotherapy phase or entire study (both monotherapy and combination therapy phases)

Level† (evaluable patients, *n*)	Patients, *n* (%)															
	Entire study (monotherapy phase + combination therapy phase)								Monotherapy phase							
	Level 1 (*n* = 3)		Level 2 (*n* = 6)		Level 3 (*n* = 3)		All levels (*n* = 12)		Level 1 (*n* = 3)		Level 2 (*n* = 6)		Level 3 (*n* = 3)		All levels (*n* = 12)	
	All grades	≥Grade 3	All grades	≥Grade 3	All grades	≥Grade 3	All grades	≥Grade 3	All grades	≥Grade 3	All grades	≥Grade 3	All grades	≥Grade 3	All grades	≥Grade 3
Adverse events, hematologic																
Leukopenia	3	2	6	3	3	2	12 (100)	7 (58.3)	2	0	1	0	1	0	4 (33.3)	0
Neutropenia	3	3	6	5	3	3	12 (100)	11 (91.7)	1	0	1	0	1	0	3 (25.0)	0
Lymphocytopenia	2	2	6	1	3	1	11 (91.7)	4 (33.3)	2	0	3	0	1	1	6 (50.0)	1 (8.3)
Anemia	3	1	5	1	3	1	11 (91.7)	3 (25.0)	1	0	2	0	1	0	4 (33.3)	0
Thrombocytopenia	2	0	2	2	3	0	7 (58.3)	2 (16.7)	0	0	0	0	0	0	0	0
Monocytopenia	1	0	1	0	3	0	5 (41.7)	0	0	0	0	0	0	0	0	0
Adverse events, non-hematologic																
Injection-site reactions	3	0	6	0	3	1	12 (100)	1 (8.3)	3	0	6	0	3	0	12 (100)	0
Flu-like symptoms	2	0	6	0	3	1	11 (91.7)	1 (8.3)	2	0	6	0	3	0	11 (91.7)	0
Anorexia	1	1	4	0	2	1	7 (58.3)	2 (16.7)	0	0	2	0	0	0	2 (16.7)	0
Malaise	2	0	3	0	2	0	7 (58.3)	0	0	0	0	0	0	0	0	0
ALT increased	1	0	3	0	2	0	6 (50.0)	0	0	0	0	0	0	0	0	0
Constipation	0	0	3	0	2	0	5 (41.7)	0	0	0	0	0	1	0	1 (8.3)	0
Diarrhea	1	0	4	0	0	0	5 (41.7)	0	0	0	2	0	0	0	2 (16.7)	0
AST increased	0	0	2	0	2	0	4 (33.3)	0	0	0	0	0	0	0	0	0
Nausea	1	0	3	0	0	0	4 (33.3)	0	0	0	1	0	0	0	1 (8.3)	0

†Level 1: (Mono) PF-3512676 0.1 mg/kg → (Combo) PF-3512676 0.1 mg/kg + carboplatin AUC 6 + paclitaxel 200 mg/m^2; Level 2: (Mono) PF-3512676 0.2 mg/kg → (Combo) PF-3512676 0.2 mg/kg + carboplatin AUC 6 + paclitaxel 200 mg/m^2; Level 3: (Mono) PF-3512676 0.4 mg/kg → (Combo) PF-3512676 0.2 mg/kg + carboplatin AUC 6 + paclitaxel 200 mg/m^2.
ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; Combo, combination therapy; Mono, monotherapy.

AEs or laboratory abnormalities (one patient in dose level 1, three patients in dose level 2, and two patients in dose level 3). All of the discontinuations resulting from AEs or laboratory abnormalities occurred during combination therapy, and the AEs that led to discontinuation varied. The patient in dose level 1 discontinued as a result of grade 2 nausea and grade 2 vomiting that were related to both PF-3512676 and chemotherapy. One patient in dose level 2 discontinued after having multiple hematologic AEs that were related to both PF-3512676 and chemotherapy: grade 4 anemia, and grade 2 neutropenia and leukopenia. Another patient in dose level 2 discontinued after having PF-3512676-related, grade 2 flu-like symptoms (this event was considered unrelated to chemotherapy). The third discontinuation in dose level 2 was the result of grade 3 increase in γ-glutamyltransferase that was considered related to PF-3512676 and chemotherapy and a grade 3 rash considered related to chemotherapy, but not to PF-3512676. One discontinuation in dose level 3 was the result of grade 2 peripheral neuropathy that was considered to be related to paclitaxel. The other was a patient who developed PF-3512676-related grade 3 anorexia and flu-like symptoms (these events were considered unrelated to chemotherapy).

Although all patients reported treatment-related AEs of ≥grade 3, no serious AEs were reported. No DLTs occurred during the monotherapy phase. One patient in level 2 experienced a DLT in the combination therapy phase. This patient developed grade 3 rash and grade 3 increase in γ-glutamyltransferase on days 9 and 10 of the first cycle of combination therapy, respectively. Both events decreased to grade 2 by day 13 of the same cycle and to grade 1 after completion of the DLT observation period. The patient discontinued study therapy as a result of these AEs. No further DLTs were observed. Therefore, the study progressed to the highest planned dose level.

Efficacy. Of 12 patients treated with PF-3512676 and chemotherapy, one patient (8%) achieved a complete response (CR) and three patients (25%) had partial responses (PRs). All objective responses were among patients treated in dose levels 1 and 2. In addition, seven patients (58%) had stable disease (SD).

Pharmacokinetics. The plasma concentration profiles of PF-3512676 were similar in the monotherapy and combination therapy phases (Fig. 1), and overall pharmacokinetic parameters of PF-3512676 were not different with addition of chemotherapy (Table 3). Median time to highest plasma concentration ranged from 2–3 h and mean peak plasma concentration (C_{max}) of PF-3512676 appeared to be dose dependent. Furthermore, mean half-life ($t_{1/2}$) of PF-3512676 varied with dose, ranging from 4.8 to 21.6 h during the monotherapy phase and from 7.9 to 9.5 h in combination therapy phase, with longer $t_{1/2}$ for higher doses of PF-3512676. Based on these PK data, accumulation of PF-3512676 was not observed in this study.

Pharmacodynamics. IFN-α, IL-12p40, IL-6, IP-10, CRP, and MCP-1 were evaluated following treatment with PF-3512676

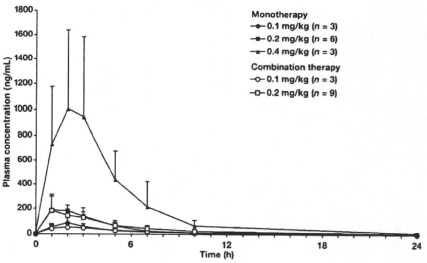

Fig. 1. Pharmacokinetics (PK) of PF-3512676 were similar during monotherapy and combination therapy phases. To compare the PK of PF-3512676 in the monotherapy phase with the PK of the combination therapy phase, blood samples were collected predose and at 1, 2, 3, 5, 7, 10, 24, 48, 72, and 96 h postdose in the monotherapy phase as well as predose and at 1, 2, 3, 5, 7, 10, 24, 48, 72, and 96 h postdose on day 8 in the first cycle of the combination therapy phase. A custom-designed hybridization enzyme-linked immunosorbent assay was used. Mean plasma concentration ± SD of each time point for each group is shown.

during both monotherapy and combination therapy phases for all dose levels. For each assayed cytokine or protein, detected levels began to escalate at approximately 3 h postdose, but time to peak concentration varied from approximately 24 to 96 h (Fig. 2). Levels returned to predose concentrations by ~168 h postdose. Pharmacodynamic profiles of the cytokines and proteins during the combination therapy phase were similar to their corresponding profiles in the monotherapy phase, although there was a trend toward lower peak cytokine and protein levels in the combination therapy phase. However, it must be noted that there was considerable variation in individual predose and maximum concentrations. Cytokine and protein profiles of patients who achieved objective responses were not different from those of patients without evidence of antitumor activity.

Discussion

This phase I study was conducted to examine the safety and PK of PF-3512676 as a single agent and in combination with carboplatin/paclitaxel therapy in Japanese patients with previously untreated NSCLC. Treatment with carboplatin and paclitaxel is a standard approach for patients with advanced NSCLC in Japan.[19] American Society of Clinical Oncology guidelines for treatment of previously untreated stage IV NSCLC recommend combination chemotherapy, but suggest stopping this

Table 3. Pharmacokinetics of PF-3512676

Dose level	*n*	Mean C_{max}, ng/mL (SD)	Mean $AUC_{(0-\infty)}$, ng × h/mL (SD)	Median t_{max}, hours (range)	Mean $t_{1/2}$, hours (SD)
Monotherapy					
0.1 mg/kg	3	90 (36)	376 (73)	2 (2–3)	4.8 (3.4)
0.2 mg/kg	6	217 (90)	856 (127)	2 (1–3)	12.8 (14.0)
0.4 mg/kg	3	1010 (633)	5270 (2450)	2 (2–2)†	21.6 (16.4)
Combination therapy					
0.1 mg/kg	3	55 (19)	379 (55)	3 (2–3)	7.9 (6.2)
0.2 mg/kg	9	226 (124)	1340 (775)	2 (1–3)	9.5 (6.9)

†All patients had reached maximum concentration of PF-3512676 by 2 h postdose. AUC, area under the curve; C_{max}, peak plasma concentration; SD, standard deviation; $t_{½}$, half-life; t_{max}, time to maximum plasma concentration.

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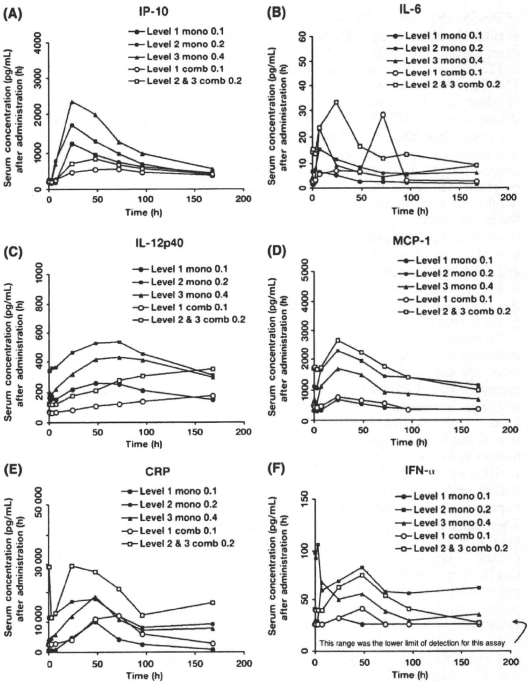

Fig. 2. Pharmacodynamics of cytokines, monocyte chemotactic protein-1 (MCP-1), and C-reactive protein (CRP) were similar during monotherapy and combination therapy phases of the study. The levels of (A) interferon-γ-inducible protein 10 (IP-10), (B) interleukin (IL)-6, (C) IL-12p40, (D) MCP-1, (E) CRP, and (F) interferon-alpha (IFN-α) were evaluated in patient sera at predose (0 h) and at 1, 3, 7, 24, 48, 72, 96, 168 h postdose of PF-3512676 during the monotherapy and combination therapy phases. Sample collection for the combination therapy phase occurred during the first cycle of treatment, and predose was on day 8 before treatment. In each case, the level of the assayed protein began to escalate after 3 h. The time at which highest expression was achieved varied but generally returned to baseline by 168 h postdose. comb, combination therapy; h, hour; mono, monotherapy.

treatment if patients do not respond after three or four cycles.[4] Furthermore, in a recent phase III trial in Japanese patients, the median number of cycles of first-line platinum-based chemotherapy was three.[20] In this phase I study, patients received a median of four cycles of chemotherapy combined with PF-3512676. Therefore, SC delivery of PF-3512676 was considered tolerable either as monotherapy or in combination therapy at the highest doses tested in this study (0.4 mg/kg and 0.2 mg/kg, respectively).

Through the entire study period, the most common treatment-related AEs of any grade were injection-site reactions, neutropenia, leukopenia, anemia, flu-like symptoms, and lymphocytopenia. Injection-site reactions and flu-like symptoms were likely related to treatment with PF-3512676 alone, as they occurred with similar frequency in both the monotherapy and the combination therapy phases. There was no clear dose relationship for these AEs during PF-3512676 monotherapy. Other AEs (eg, leukopenia, neutropenia, lymphocytopenia, anemia, and anorexia) observed during both phases of the study were probably related to treatment with both PF-3512676 and chemotherapy, because they occurred more frequently during the combination therapy phase than the monotherapy phase. There was no indication of cumulative toxicity. These safety results are similar to those from a previous phase II study in Western patients.[17] In that study, the most common AEs related to PF-3512676 and not to chemotherapy were mild to moderate injection-site reactions and flu-like symptoms. Other less common AEs considered related to treatment with PF-3512676 were neutropenia, anemia, and thrombocytopenia.

Across this study, the most frequently occurring AEs of ≥grade 3 were hematologic (e.g. neutropenia, leukopenia, or lymphocytopenia). Hematologic AEs were observed at all dose levels and were qualitatively similar to those reported with carboplatin and paclitaxel doublet chemotherapy.[21] When evaluating safety in studies of doublet chemotherapy, it is important to note that the incidence of ≥grade 3 neutropenia after doublet chemotherapy may be higher in Japanese patients[19] than in Western patients.[5,20,22] Although the small number of patients included in this study precludes a definitive comparison, 11 patients (91.7%) in the present study had ≥grade 3 neutropenia, and this is similar to the frequency reported (84%) in Japanese patients with NSCLC receiving doublet chemotherapy alone.[19]

Because the administration and observation periods were brief in this phase I study, patient blood samples were not analyzed for immunopathological changes that could potentially be indicative of autoimmune events. However, no symptoms suggestive of autoimmune disease were observed. Some patients in other PF-3512676 clinical trials developed positive tests for anti-DNA antibodies. The potential significance of these serologic results is not yet clear.

The PK profiles of PF-3512676 observed during the monotherapy and combination therapy phases were similar. The effect

of PF-3512676 on the PK of carboplatin and paclitaxel was not evaluated in this study. Median time required to achieve maximum plasma concentration (2–3 h) was consistent across all PF-3512676 doses with or without the addition of chemotherapy. The C_{max} increased with the dose administered and was highest in dose level 3 monotherapy in which patients received 0.4 mg/kg PF-3512676. The time required to clear drug from the body appeared to be dose dependent; shortest $t_{1/2}$ (4.8 h) was found in the 0.1 mg/kg dose level monotherapy phase, and longest $t_{1/2}$ (21.6 h) was observed in the 0.4 mg/kg monotherapy phase. However, these data may be confounded by the small number of patients per group and resultant high SD as well as the assay sensitivity level at the lowest dose level. Therefore, it is unclear whether clearance is truly dose dependent. Linearity was also not clearly defined because of the small sample size and the large variation in PK parameters.

The objective response rate (33%) in this study was similar to the rate of confirmed responses (30%) found in the previous phase II study.[17]

Treatment with PF-3512676 alone or in combination with chemotherapy, regardless of dose, modulated several cytokines and other proteins. Immunomodulation was transient, and all increases had dissipated by ~168 h postdose. The most robust responses observed were increases in the levels of IP-10 and IL-6, and this was consistent with the T_H1-like pattern of activation of the innate immune system previously observed in normal human volunteers subcutaneously injected with PF-3512676.[10] IP-10 is a potent chemokine for activated T lymphocytes and regulates cell proliferation, apoptosis, and adhesion molecule expression.[23] Its elevation is indicative of TLR9 activation. There appeared to be a trend toward reduced stimulation of cytokine and chemokine production in the combination therapy phases compared with monotherapy. Although the relevance of this finding is unclear, it should be noted that in this study design, patients who received monotherapy were treatment-naive, while patients who received combination therapy had already received monotherapy with PF-3512676. Increasing the single-agent dose to 0.4 mg/kg seemed to result in a similar pattern of cytokine and chemokine production to that observed with lower doses. Cytokine and chemokine profiles from patients who achieved CR or PR were similar to those from patients without evidence of antitumor activity. However, the small sample size in this study may have confounded these results, and further investigation in future, larger studies would be required for confirmation.

In addition to the present study, PF-3512676 has been investigated in two phase III clinical studies in which combination with platinum-based doublet chemotherapy was compared with platinum-based doublet chemotherapy alone in Western patients with previously untreated advanced NSCLC.[24,25] In those studies, addition of PF-3512676 to doublet chemotherapy did not produce an improvement in overall survival and was associated with increased toxicity. After completion of the study described in this manuscript and based on results from these phase III studies, all clinical trials of PF-3512676 in combination with cytotoxic chemotherapy agents for treatment of NSCLC were discontinued. However, clinical trials in other settings and in combination with targeted or immunotherapeutic agents are ongoing or planned.

In conclusion, PF-3512676 as a single agent and in combination with carboplatin and paclitaxel had an acceptable safety profile in Japanese patients with treatment-naive NSCLC, and PF-3512676 showed evidence of immune activation in the study. It is, therefore, still considered to have potential utility as an anticancer agent.

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