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Genetic testing for *UGT1A1*28* and *6 in Japanese patients who receive irinotecan chemotherapy

Polymorphisms of the *UDP-glucuronosyltransferase* (*UGT*) *1A1* gene, such as *UGT1A1*28* and *UGT1A1*6*, can cause severe neutropenia and diarrhea in patients who receive irinotecan [1, 2]. Homozygosity for *UGT1A1*28* is associated with less efficient glucuronidation of SN-38, the active metabolite of irinotecan, resulting in increased plasma SN-38 concentrations. Four pharmacogenetic trials have demonstrated an association between *UGT1A1*28* genotype and irinotecan-induced hematologic toxicity, diarrhea, or both [3]. In response to these findings, the United States Food and Drug Administration has approved genetic testing for *UGT1A1*28* and recommends that the initial dose of irinotecan is reduced by at least one level in patients who are homozygous for *UGT1A1*28*, albeit the effectiveness of such testing remains to be confirmed prospectively. *UGT1A1*6* is also associated with severe irinotecan-related toxicity [4]. Given that the area under the time versus concentration curve ratio (SN-38 glucuronide/SN-38) seen in patients homozygous for *UGT1A1*28* and *6 are almost equal [4], the impact of these variants on glucuronidation capacity of UGT1A1 for SN-38 is almost the same. The distribution of genotypes associated with these polymorphisms varies considerably among ethnic groups. *UGT1A1*28* is found in Japanese and whites, but the allele frequency in Japanese is lower than that in whites [2, 4]. *UGT1A1*6* is found in Japanese, but not in whites [4]. Homozygosity for *UGT1A1*28* or *UGT1A1*6* and heterozygosity for both *UGT1A1*6* and *UGT1A1*28* are associated with severe irinotecan-related neutropenia in Japanese patients [1, 4]. The Ministry of Health, Labour and Welfare in Japan has therefore recently approved genetic testing for *UGT1A1*28* and *6.

The value of genetic testing for *UGT1A1* depends on genotype frequency and the association of genetic variants with irinotecan-induced toxicity. The higher the frequency of toxicity-related polymorphisms, the greater is the number of patients who would benefit from genetic testing. Large prospective studies are needed to accurately estimate the distribution of *UGT1A1* polymorphisms in a given population. We have carried out the largest prospective study to date, examining the distributions of *UGT1A1*28* and *UGT1A1*6* genotypes in 300 Japanese patients (male/female, 172 of 128) with various solid tumors (200 colorectal, 43 gastric, 15 ovarian, 14 breast, 10 lung, and 18 others).

All patients gave written informed consent, and the study protocol was approved by the Institutional Review Board of Saitama Medical University. Genotyping was carried out as described elsewhere [5].

*UGT1A1*28* and *UGT1A1*6* were in Hardy–Weinberg equilibrium ($P > 0.05$). Only 2 of 300 patients were *UGT1A1*28* homozygotes (0.7%) (Table 1). The frequency of homozygosity for *UGT1A1*28* was much lower than that in other prospective studies in Japan (2.3%, 4 of 176) [4]. The frequency of *UGT1A1*6* homozygosity was 5.7% (Table 1), higher than that reported previously (2.8%) [4]. Eleven patients were both heterozygous for *UGT1A1*6* and *UGT1A1*28* (3.7%). The combined frequency of patients with two 'risk alleles' (i.e. *28/*28, *6/*6, and *6/*28) was 10.1% (95% confidence interval, 6.8% to 14.0%). Such patients might be at increased risk for irinotecan-related neutropenia. Given the genotype frequencies of *UGT1A1*28* and *UGT1A1*6*, genetic testing for *UGT1A1* might not be essential for identifying homozygotes for *UGT1A1*28*, but useful for identifying homozygotes for *UGT1A1*6* as well as heterozygotes for *UGT1A1*6* and *UGT1A1*28*, thereby avoiding severe irinotecan-induced toxicity in Japanese patients. The present results and considerations are likely to have application across East Asia. Prospective evaluations of genetic testing for *UGT1A1* polymorphisms, encompassing both medical aspects and cost effectiveness, appear to be warranted, especially in East Asian countries including Japan.

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Table 1. Genotype frequencies of *UGT1A1*28* and *UGT1A1*6* in Japanese

Genotype	*n* (%)	95% confidence interval (%)
*UGT1A1*1/*1*[a]	135 (45.0)	39.3–50.8
*UGT1A1*1/*28*	47 (15.7)	11.7–20.3
*UGT1A1*1/*6*	88 (29.3)	24.1–34.8
*UGT1A1*28/*28*	2 (0.7)	0.1–2.4
*UGT1A1*6/*6*	17 (5.7)	3.3–8.9
*UGT1A1*6/*28*	11 (3.7)	1.8–6.5

[a]*UGT1A1* allele without *28 or *6 was defined as *1.

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Re: *UGT1A1*28* Genotype and Irinotecan-Induced Neutropenia: Dose Matters

We read with interest the paper from Hoskins et al. (1) on the meta-analysis of the studies that assessed the association of irinotecan dose with the risk of irinotecan-related toxic effects for patients with the *UGT1A1*28/*28* genotype. They indicated that the risk of hematologic toxicity was strongly associated with *UGT1A1*28* genotype at higher irinotecan doses (>150 mg/m²), not at lower doses (≤150 mg/m²).

We congratulate the authors for their excellent work. We would like to present support for their work from the ongoing study to establish the appropriate dose-adaptation strategy for irinotecan among Japanese patients who are heterozygous for both *UGT1A1*28* and *UGT1A1*6* or homozygous for each variant. *UGT1A1*6*, which contributes to the hepatic metabolism of SN-38 (the active metabolite of irinotecan), is more prevalent than *UGT1A1*28* in Asian populations (2,3). In the Japanese population, the metabolic ratio of the area under the curve (AUC) for SN-38/AUC for SN-38 glucuronide was statistically significantly higher in patients who were heterozygous for both *UGT1A1*28* and *UGT1A1*6* or homozygous for *UGT1A1*6* than in those with other genotypes (P = .004, Mann-Whitney U test) (4).

In the study, the dose of irinotecan was escalated from 25 to 150 mg/m² because the Japanese package insert information limits the dose for irinotecan to 150 mg/m² in each biweekly regimen when patients do not experience dose-limiting toxicity, which was defined as grade 4 neutropenia or grade 3 or 4 nonhematologic toxic effects according to the *Common Toxicity Criteria*, version 3.0, of the National Cancer Institute. Genotyping and pharmacokinetic analyses were performed simultaneously as previously reported (4). All patients gave written informed consent approved by the Institutional Review Board of Saitama Medical University.

In plots of metabolic ratios at the administered dose of irinotecan for individual patients, gradual changes in the metabolic ratio of patients were observed according to the dose escalation, with one exception that of patient A (Fig. 1). Patients A, B, and C were homozygous for *UGT1A1*6*, and patient D was heterozygous for both *UGT1A1*28* and *UGT1A1*6*. Patient A with a primary cancer whose site was unknown had no dose-limiting toxicity but refused to continue irinotecan treatment. During dose escalation, patient B with ovarian cancer experienced grade 4 neutropenia at an irinotecan dose of 100 mg/m², and patient C with lung cancer experienced grade 3 diarrhea with grade 3 neutropenia at a dose of 75 mg/m². Patient D with colon cancer experienced grade 4 neutropenia at an irinotecan dose of 150 mg/m².

Although the dosage of irinotecan in our study was lower than that in Hoskins

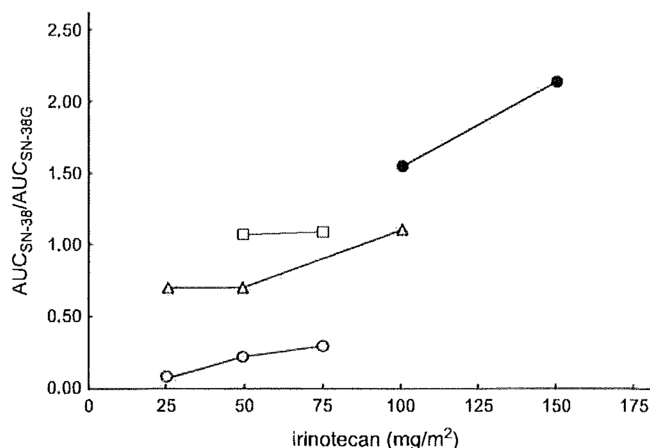

Fig. 1. The metabolic ratio of the area under the curve (AUC) for SN-38/AUC for SN-38 glucuronide (SN-38G) vs the dose of irinotecan. Patients A, B, and C were homozygous for *UGT1A1*6*. Patient D was heterozygous for both *UGT1A1*28* and *UGT1A1*6*. **Squares** = patient A; **triangles** = patient B; **open circles** = patient C; **solid circles** = patient D.

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et al. (1), the intrapatient variability in metabolic ratio according to the administered irinotecan dose might explain, in part, the association between irinotecan dose and the risk of irinotecan-related hematologic toxic effects in patients who were expected to have low *UGT1A1* activity. In addition, the interpatient heterogeneity of metabolic ratio among patients with a mutated *UGT1A1* genotype may likely be due to factors other than *UGT1A1* genotype, either genetic or nongenetic, as discussed by Hoskins et al. (1). Further studies on the pharmacokinetics, pharmacodynamics, and pharmacogenetics of irinotecan are needed to clarify these issues.

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Notes

The authors take full responsibility for the design of the study, the collection of the data, the analysis and interpretation of the data, the decision to submit the manuscript for publication, and the writing of the manuscript.

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Response

We thank Dr Ichikawa and colleagues for their kind comments. Their preliminary irinotecan pharmacokinetic data from Japanese cancer patients with low-activity *UGT1A1* genotypes who participated in an irinotecan dose escalation study provide some additional support for the findings of our study (1). The ratios of the areas under the concentration time curves of SN-38 to SN-38G for three of the four patients appear to increase with increasing dose of irinotecan. This observation, although based on few patients, indicates that the extent of SN-38 glucuronidation decreased with increasing irinotecan dose. We look forward to seeing the results on completion of their study.

On the basis of the findings of a few initial studies, the US Food and Drug Administration (FDA) made the recommendation that patients with the *UGT1A1*28/*28* genotype should receive a lower starting dose of irinotecan, and the package insert of the drug was amended accordingly (1,2) in the interest of patient safety. Our findings indicate that, for *UGT1A1*28/*28* patients, *UGT1A1*28* genotype–based dosing of irinotecan is likely to improve patient safety if they are being treated with high doses of irinotecan (>250 mg/m^2) but not if they are being treated with low doses (<150 mg/m^2). Therefore, genotype-based dosing may not be necessary for all patients (1). Our findings also indicate that the current recommendations for irinotecan dosing may be too broad and so the irinotecan package label should be fine tuned to acknowledge the effect of irinotecan dose on the association between *UGT1A1*28* genotype and hematologic toxicity. Many media outlets reported our findings with bold titles that implied an FDA error. Rather, we view FDA pharmacogenetic label updates as an iterative process in which refinement of the prescribing information is performed at intervals dictated by the robustness of the new data.

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ORIGINAL REPORT

Phase III Study, V-15-32, of Gefitinib Versus Docetaxel in Previously Treated Japanese Patients With Non–Small-Cell Lung Cancer

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A B S T R A C T

Purpose

This phase III study (V-15-32) compared gefitinib (250 mg/d) with docetaxel (60 mg/m^2) in patients (N = 489) with advanced/metastatic non–small-cell lung cancer (NSCLC) who had failed one or two chemotherapy regimens.

Methods

The primary objective was to compare overall survival to demonstrate noninferiority for gefitinib relative to docetaxel. An unadjusted Cox regression model was used for the primary analysis.

Results

Noninferiority in overall survival was not achieved (hazard ratio [HR], 1.12; 95.24% CI, 0.89 to 1.40) according to the predefined criterion (upper CI limit for HR ≤ 1.25); however, no significant difference in overall survival (*P* = .330) was apparent between treatments. Poststudy, 36% of gefitinib-treated patients received subsequent docetaxel, and 53% of docetaxel-treated patients received subsequent gefitinib. Gefitinib significantly improved objective response rate and quality of life versus docetaxel; progression-free survival, disease control rates, and symptom improvement were similar for the two treatments. Grades 3 to 4 adverse events occurred in 40.6% (gefitinib) and 81.6% (docetaxel) of patients. Incidence of interstitial lung disease was 5.7% (gefitinib) and 2.9% (docetaxel). Four deaths occurred due to adverse events in the gefitinib arm (three deaths as a result of interstitial lung disease, judged to be treatment related; one as a result of pneumonia, not treatment related), and none occurred in the docetaxel arm.

Conclusion

Noninferiority in overall survival between gefitinib and docetaxel was not demonstrated according to predefined criteria; however, there was no statistically significant difference in overall survival. Secondary end points showed similar or superior efficacy for gefitinib compared with docetaxel. Gefitinib remains an effective treatment option for previously treated Japanese patients with NSCLC.

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INTRODUCTION

In Japan, patients with advanced non–small-cell lung cancer (NSCLC) who fail first-line platinum-based therapy often receive second-line docetaxel.[1,2] However, docetaxel has been associated with significant levels of toxicity, especially grades 3 to 4 neutropenia (40% to 67% and 63% to 73% for docetaxel 75 mg/m^2 and 60 mg/m^2, respectively).[1-4] In North America and in European countries, docetaxel,[3,4] pemetrexed,[2] and erlotinib[5] are approved second-line treatments for NSCLC.[3,6]

In phase II trials (IDEAL 1 and 2), the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (Iressa; AstraZeneca, London, United Kingdom) 250 mg/d showed response rates of 12% to 18% and median survival of 7.0 to 7.6 months in patients who had pretreated advanced NSCLC.[7,8] A subset of Japanese patients in IDEAL 1 demonstrated a higher response rate (27.5%) and longer median survival (13.8 months) compared with the overall population.[9] A phase III study (Iressa Survival Evaluation in Lung Cancer) in patients who had previously treated refractory NSCLC

showed that gefitinib was associated with a nonsignificant trend toward improved overall survival versus placebo.[10] Preplanned subgroup analyses demonstrated a statistically significant increase in survival for gefitinib compared with placebo in patients of Asian origin (hazard ratio [HR], 0.66; 95% CI, 0.48 to 0.91; $P = .010$; median survival, 9.5 *v* 5.5 months) and in never-smokers (HR, 0.67; 95% CI, 0.49 to 0.92; $P = .012$; median survival, 8.9 *v* 6.1 months).[10,11]

Reported here is the first phase III study to compare the effects of targeted therapy (gefitinib) with chemotherapy (docetaxel) on overall survival in Japanese patients with advanced/metastatic (stages IIIB to IV) or recurrent NSCLC who failed one or two chemotherapy regimens.

METHODS

Study Design

This multicenter, randomized, open-label, postmarketing clinical study (V-15-32) compared gefitinib with docetaxel in Japanese patients who had pretreated, locally advanced/metastatic (stages IIIB to IV) or recurrent NSCLC. Patients were randomly assigned by using stratification factors of sex (female *v* male), performance status (PS; 0 to 1 *v* 2), histology (adenocarcinoma *v* others), and study site.

The primary end point was overall survival, and the study aimed to show noninferiority of gefitinib versus docetaxel. Secondary end points were progression-free survival (PFS), time to treatment failure, objective response rate (ORR), disease control rate (DCR), quality of life (QoL), disease-related symptoms, safety, and tolerability.

A late protocol amendment included exploratory end points, such as EGFR gene copy number, protein expression, and mutation status of tumor tissue.

Patients

Patients age 20 years or older were eligible if they had the following: histologically or cytologically confirmed NSCLC (stages IIIB to IV) not amenable to curative surgery or radiotherapy, or postoperative recurrent NSCLC; failure of prior treatment with one or two chemotherapy regimens ($\geq$ 1 platinum-based regimen); life expectancy of 3 months or greater; WHO PS 0 to 2; and measurable disease by Response Evaluation Criteria in Solid Tumors (RECIST). To improve recruitment, the protocol was amended approximately 6 months after study initiation to allow patients without measurable lesions to participate. This was not expected to greatly impact the primary end point.

Treatment

Gefitinib 250 mg/d was administered orally; docetaxel was administered every 3 weeks as a 1-hour intravenous infusion of 60 mg/m^2 (ie, the approved dose in Japan). Patients received treatment until disease progression, intolerable toxicity, or discontinuation for another reason. Poststudy treatment was at physician and patient discretion; a switch to other study treatment was prohibited unless requested by the patient.

Assessments

Overall survival was assessed from date of random assignment to date of death as a result of any cause, or data were censored at the last date the patient was known to be alive. Tumor response by RECIST was performed at baseline, every 4 weeks for the first 24 weeks, and every 8 weeks thereafter. Complete response (CR) or partial response (PR) was confirmed on the basis of two consecutive examinations that were at least 28 days apart. Investigator assessment of best overall tumor response was used for the primary analysis; sensitivity analyses were performed with independent response evaluation committee assessment. PFS was defined as the time from random assignment to the earliest occurrence of disease progression or death from any cause; patients who had not progressed or died at data cutoff were censored at last tumor assessment. QoL was assessed with the FACT-L questionnaire at baseline and every 4 weeks during study treatment until week 12. The FACT-L total score and trial outcome index (TOI; sum of FACT-L physical well-being + functional well-being + additional concerns subscales) were calculated. Disease-related symptoms were assessed weekly with the FACT-L lung cancer subscale (LCS). Improvement was defined as an increase from baseline of at least six points for FACT-L or TOI, or an increase of at least two points for LCS, on two visits that were at least 28 days apart. Adverse events (AEs) were monitored and graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC; version 2.0). Routine laboratory assessments were performed. EGFR gene copy number was determined by fluorescent in situ hybridization (FISH).[12] EGFR mutations were assessed by direct sequencing of exon 18 to 21 of chromosome 7. EGFR protein expression was measured by immunohistochemistry with the DAKO EGFR pharmaDxTM kit (DAKO, Glostrup, Denmark).[10]

Statistical Analysis

The primary overall survival analysis was conducted in the intent-to-treat (ITT) population by estimating the HR and two-sided 95.24% CI for gefitinib versus docetaxel, derived from a Cox regression model without covariates (significance level adjusted because of interim analysis). Noninferiority was to be concluded if the upper CI limit was $\leq$ 1.25. Superiority was concluded if the upper CI limit was less than 1. A total of 296 death events were required for 90% power to demonstrate noninferiority, with the assumption that gefitinib had better overall survival than docetaxel (median survival, 14 *v* 12 months[4]), and the study plan was to recruit 484 patients.

Robustness of the primary conclusion was assessed by supportive analyses in the per-protocol population and by using a Cox regression model with covariate adjustment for sex (male *v* female), PS (0 or 1 *v* 2), tumor type (adenocarcinoma *v* other), smoking history (ever *v* never), number of prior chemotherapy regimens (1 *v* 2), age at random assignment (< 65 years *v* $\geq$ 65 years), time from diagnosis to random assignment (< 6 *v* 6 to 12 *v* > 12 months), and best response to prior chemotherapy (CR/PR *v* stable disease [SD] *v* progressive disease not assessable/unknown).

Preplanned subgroup analyses were performed on the basis of these covariates. Subgroups were first assessed for evidence of randomized treatment effect by subgroup interactions, to ensure that outcomes between subgroups were likely to be different; then, the subgroups for which evidence existed were examined further.

For PFS, the HR and its 95% CI for gefitinib versus docetaxel were calculated for the population that was assessable for response (defined as patients with $\geq$ 1 measurable lesion at baseline by RECIST) by using a Cox regression model without covariates. Supportive analyses were performed in the ITT population by using a model adjusted for covariates. Overall survival and PFS were summarized with Kaplan-Meier methods.

The ORR (proportion of CR + PR) and the DCR (proportion of CR + PR + SD $\geq$ 12 weeks) were estimated in the assessable-for-response population and were compared between treatments by generating an odds ratio and a 95% CI from a logistic regression model that included covariates.

The exploratory analysis of biomarker subgroups was performed with similar methods to the overall and clinical subgroup analyses when possible.

RESULTS

Patients

From September 2003 to January 2006, 490 patients were randomly assigned from 50 institutes. In the ITT population, 245 patients were randomly assigned to gefitinib, and 244 patients were randomly assigned to docetaxel; one patient was excluded because of a Good Clinical Practice violation (Fig 1). Treatment groups were generally well balanced for baseline demographics (Table 1), except for some small imbalances in smoking history (7% fewer never-smokers and 10% more ex-smokers in the gefitinib arm). The overall population was representative of an advanced, pretreated NSCLC population in a clinical trial setting in Japan. The median (range) duration of treatment for gefitinib was 58.5 (4 to 742) days and, for docetaxel, was 3 (1 to 12) cycles.

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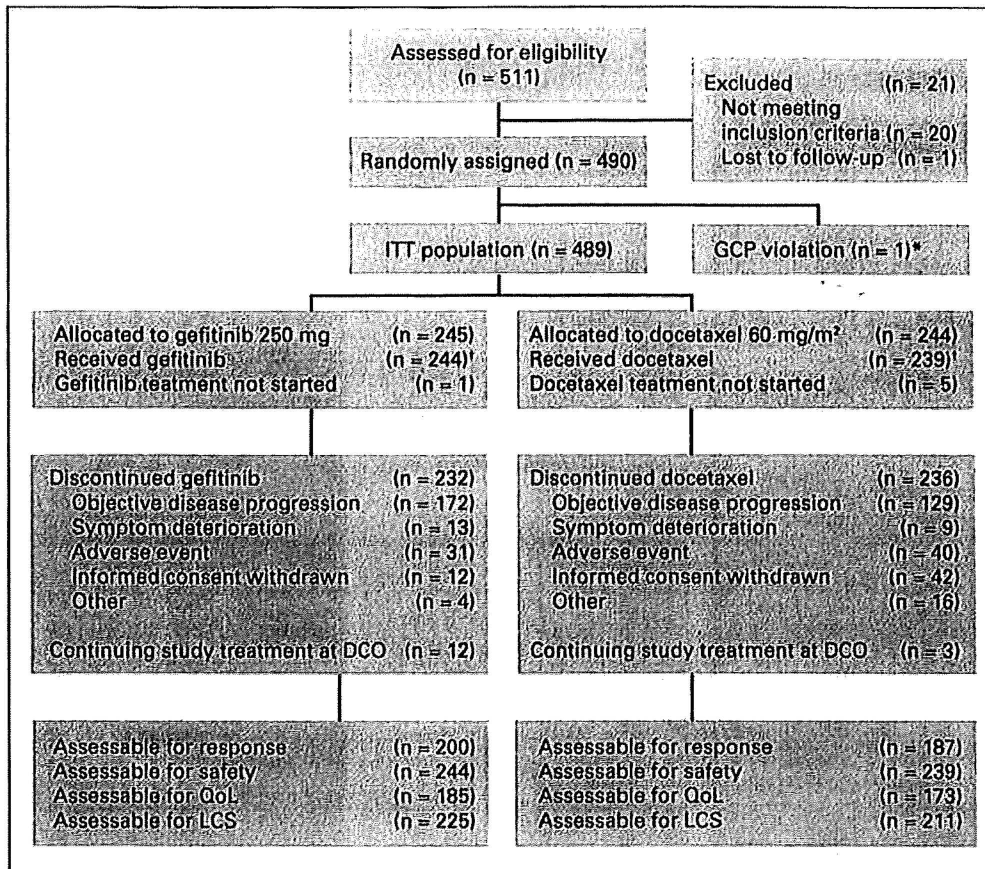

Fig 1. Study flow. (*) Allocated to the docetaxel group. (†) The safety analysis, conducted according to treatment received, was performed on this population. ITT, intent to treat; GCP, Good Clinical Practice; DCO, data cutoff date for overall survival (October 31, 2006); QoL, quality of life; LCS, Lung Cancer Subscale.

Poststudy, 36% of gefitinib-treated patients received subsequent docetaxel, and 40% received no other therapy except for gefitinib; 53% of docetaxel-treated patients received subsequent gefitinib, and 26% received no other therapy except for docetaxel.

Survival

At data cutoff for overall survival (October 31, 2006), overall mortality was 62.6%, and median follow-up was 21 months. Noninferiority in overall survival was not achieved (HR, 1.12; 95.24% CI, 0.89 to 1.40) according to the predefined criterion (upper CI limit for HR ≤ 1.25). However, no statistically significant difference in overall survival was apparent (P = .330; Fig 2A).

A supportive Cox analysis, which took into account imbalances in known prognostic factors, showed an HR of 1.01 (95% CI, 0.80 to 1.27; P = .914), which suggested that a demography imbalance that favored docetaxel may have had some impact on the primary, unadjusted, overall survival result.

The median survival and the 1-year survival rates were 11.5 months and 47.8%, respectively, for gefitinib and were 14.0 months and 53.7%, respectively, for docetaxel.

PFS

There was no significant difference between treatments in PFS in the unadjusted analysis (HR, 0.90; 95% CI, 0.72 to 1.12; P = .335); median PFS was 2.0 months with both treatments (Fig 2B). Similar PFS results were obtained from supportive Cox regression analysis adjusted for covariates (HR, 0.81; 95% CI, 0.65 to 1.02; P = .077).

Tumor Response

For ORR, gefitinib was statistically superior to docetaxel (22.5% *v* 12.8%; odds ratio, 2.14; 95% CI, 1.21 to 3.78; P = .009; Table 2). Gefitinib was similar to docetaxel in terms of DCR (34.0% *v* 33.2%; odds ratio, 1.08; 95% CI, 0.69 to 1.68; P = .735). The primary ORR results that were based on investigator judgment were generally consistent with those obtained from independent response evaluation committee assessment.

Symptom Improvement and QoL

Gefitinib showed statistically significant benefits compared with docetaxel in QoL improvement rates (FACT-L: 23.4% *v* 13.9%; P = .023; TOI: 20.5% *v* 8.7%; P = .002; Table 2), but there were no significant differences between treatments in LCS improvement rates (22.7% *v* 20.4%; P = .562).

Subgroup Analyses

Survival outcomes were generally consistent across subgroups, with the exception of best response to prior chemotherapy (treatment by subgroup interaction test P = .017). For patients with best response to prior chemotherapy of progressive disease, overall survival was numerically longer on gefitinib than on docetaxel, whereas patients with a best response of SD had significantly longer survival on docetaxel than on gefitinib (HR, 1.58; 95% CI, 1.09 to 2.27; P = .015; Fig 3A). However, the result was not supported by the PFS (Fig 3B) or ORR results in this subgroup, which favored gefitinib.

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Table 1. Baseline Patient Characteristics in Intent-to-Treat Population

Characteristic	Gefitinib (n = 245) No.	Gefitinib (n = 245) %	Docetaxel (n = 244) No.	Docetaxel (n = 244) %
Age, years				
≤ 64	138	56.3	135	55.3
≥ 65	107	43.7	109	44.7
Sex				
Male	151	61.6	151	61.9
Female	94	38.4	93	38.1
WHO performance status				
0	85	34.7	93	38.1
1	149	60.8	141	57.8
2	11	4.5	10	4.1
Smoking status				
Ever	174	71.0	157	64.3
Never	71	29.0	87	35.7
Histology				
Adenocarcinoma	192	78.4	188	77.0
Squamous cell carcinoma	37	15.1	41	16.8
Other	16	6.5	15	6.2
Time from diagnosis to random assignment, months				
< 6	70	28.6	60	24.6
6-12	99	40.4	96	39.3
> 12	76	31.0	87	35.7
Disease stage at diagnosis				
IIIB	47	19.2	50	20.5
IV	159	64.9	150	61.5
Recurrent	39	15.9	44	18.0
Number of prior chemotherapy regimens				
1	212	86.5	201	82.4
2	33	13.5	42	17.2
Best response to previous chemotherapy				
CR/PR	113	46.1	106	43.4
SD	91	37.1	101	41.4
PD/NA/unknown	41	16.7	37	15.2
Target lesions at baseline				
Yes	201	82.0	187	76.6
No	44	18.0	57	23.4

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA, not assessable.

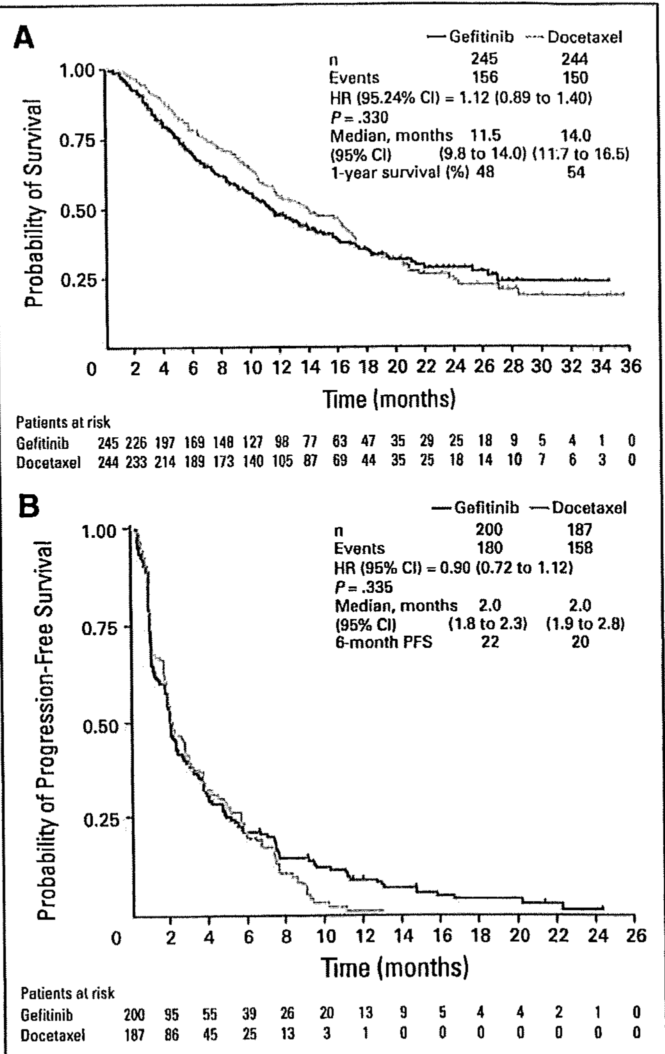

Fig 2. (A) Overall survival in the intent-to-treat population; (B) Progression-free survival (PFS) in the assessable-for-response population. HR, hazard ratio.

Safety

Gefitinib was associated with fewer dose interruptions or delays than docetaxel (26% *v* 52%, respectively). There were no clinically relevant differences in the frequencies of serious AEs or discontinuations of study treatment as a result of AEs between treatment groups (Table 3). Fewer NCI-CTC grades 3 to 4 AEs occurred with gefitinib compared with docetaxel (40.6% *v* 81.6%). There were four deaths as a result of AEs in the gefitinib arm (three as a result of interstitial lung disease that was considered by the investigator to be treatment related; one as a result of pneumonia that was not considered treatment-related), and none in the docetaxel arm.

The most common AEs with gefitinib were rash/acne (76.2%) and diarrhea (51.6%), and the most common AEs with docetaxel were neutropenia (79.5%) and alopecia (59.4%; Table 4). There was a higher incidence of grades 3 to 4 neutropenia with docetaxel (73.6%) compared with gefitinib (8.2%). Interstitial lung disease events occurred in 5.7% (n = 14) and 2.9% (n = 7) of patients who received gefitinib and docetaxel, respectively (Table 3).

Biomarkers

Of the 74 EGFR biomarker samples provided, 53 to 60 were assessable (depending on biomarker). Because of the late protocol amendment, these samples were from long-term survivors who were recruited early or from patients who were recruited later in the study. Compared with the overall study population, this subgroup was over-representative of some stratification factors on both treatment arms: good PS, females, never-smokers, greater than 12 months from diagnosis to random assignment, and best response to prior chemotherapy of CR/PR. There were insufficient events to allow meaningful evaluation of overall survival in relation to biomarker status, and the PFS and ORR data should be interpreted with caution.

Thirty-one (54.4%) of 57 patients had EGFR mutation–positive tumors, and 42 (70.0%) of 60 had EGFR FISH–positive tumors. There

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Table 2. Response Rates and Improvement Rates

Rate	Gefitinib: Total No. of Assessable Patients	Gefitinib: %	Docetaxel: Total No. of Assessable Patients	Docetaxel: %	Analysis: OR	Analysis: 95% CI	Analysis: P
Response*	200		187				
Overall		22.5		12.8	2.14	1.21 to 3.78	.009
Disease control		34.0		33.2	1.08	0.69 to 1.68	.735
Improvement							
FACT-L	185	23.4	173	13.9	1.89	1.09 to 3.28	.023
TOI	185	20.5	173	8.7	2.72	1.44 to 5.16	.002
LCS	225	22.7	211	20.4	1.15	0.72 to 1.81	.562

Abbreviations: OR, odds ratio; FACT-L, Functional Assessment of Cancer Therapy—Lung (Japanese version 4-A, which includes two additional Japan-specific questions in the subscale on social/family well-being); TOI, trial outcome index; LCS, lung cancer subscale.
*Overall response rate consists of complete response plus partial response rates. Disease control rate consists of the complete response plus partial response rates plus those with stable disease for at least 12 weeks.

was a high degree of overlap between EGFR mutation and clinical characteristics (eg, high frequency in females, in those with adenocarcinoma, and in never-smokers). EGFR mutation–positive patients appeared to have better PFS than EGFR mutation–negative patients on both treatments (gefitinib-positive *v* gefitinib-negative HR, 0.33; 95% CI, 0.11 to 0.97; 17 events; docetaxel HR, 0.15; 95% CI, 0.04 to 0.57; 15 events). In addition, EGFR FISH–positive patients appeared to have better PFS than EGFR FISH–negative patients on both treatments (gefitinib-positive *v* gefitinib-negative HR, 0.75; 95% CI, 0.28 to 1.98; 18 events; docetaxel HR, 0.45; 95% CI, 0.14 to 1.41; 16 events). There were no clear PFS differences between gefitinib and docetaxel in any biomarker subgroups, although the number of events was small and the CIs for the HRs were wide. PFS could not be assessed for EGFR protein expression because of the small number of events in the expression-negative group. For EGFR mutation–positive patients, the ORR was 67% (six of 9 patients) with gefitinib administration and 46% (five of 11 patients) with docetaxel administration. For EGFR FISH–positive patients, the ORR was 46% (five of 11) with gefitinib administration and 33% (six of 18) with docetaxel administration. For EGFR expression–positive patients, the ORR was 36% (five of 14) with gefitinib administration and 31% (four of 13) with docetaxel administration. There were no responses among EGFR mutation–negative, or EGFR FISH–negative, patients, and there was one response (13%) of eight EGFR expression–negative patients who received docetaxel.

DISCUSSION

V-15-32 is the first phase III study to compare gefitinib versus docetaxel in previously treated Japanese patients who have advanced NSCLC. Both gefitinib and docetaxel demonstrated efficacy and tolerability, and findings were consistent with previous experience for both agents in Japan.

Although noninferiority in overall survival for gefitinib versus docetaxel was not proven, there was no statistically significant difference between the two treatments. The original statistical assumption was that gefitinib would have 20% longer survival than docetaxel; hence, the relatively small sample size for a noninferiority study. However, since the study was initiated, data from postmarketing experience in Japan (the SIGN study[13]) and substantial switching to the alternative study treatment on progression in V-15-32 indicated that it would be more likely that gefitinib and docetaxel had similar overall survival. With the assumption of equal survival, the chance (power) of showing noninferiority with this study size is reduced to 48%. The median survival with gefitinib 250 mg/d in our study was consistent with previous experience in Japan (11.5 *v* 13.8 months for Japanese subset of IDEAL 1).[9] Docetaxel demonstrated a longer median survival in V-15-32 (14.0 months) compared with previous Japanese studies (7.8 to 9.4 months).[1,4,14]

In line with increasingly available therapy for NSCLC since the trial was designed and with standard practice in Japan, a large proportion of patients received additional anticancer therapy after discontinuation of the randomly assigned study treatment. Crossover was greater than initially expected, and differences in the number and types of patients who received these poststudy treatments complicated interpretation of survival results. A greater proportion of patients who received docetaxel received poststudy therapy compared with those who received gefitinib. Imbalances in the use of gefitinib after chemotherapy have been reported recently in a phase III study of Japanese patients with lung cancer who were treated with docetaxel and have been cited as a possible explanation for the prolonged median survival seen with docetaxel.[15] INTEREST (Iressa NSCLC Trial Evaluating Response and Survival against Taxotere), a worldwide phase III trial that is comparing gefitinib with docetaxel in pretreated patients who have advanced NSCLC recently demonstrated that gefitinib had statistically noninferior survival to docetaxel.[16] In contrast to V-15-32, INTEREST was larger (1,466 patients) and had subsequent therapies that were well-balanced between treatment arms.

Secondary end points, largely unaffected in this study by subsequent therapy, provided further evidence of the clinical efficacy of both gefitinib and docetaxel in Japanese patients. PFS was similar with gefitinib and docetaxel, and ORR was statistically significantly improved with gefitinib. The ORR in V-15-32 with gefitinib (22.5% *v* 12.8% with docetaxel) was consistent with a subset analysis from IDEAL 1 in Japanese patients (27.5%).[3,8,9]

A number of patient subgroups (including females, patients with adenocarcinoma, and never-smokers) have been reported

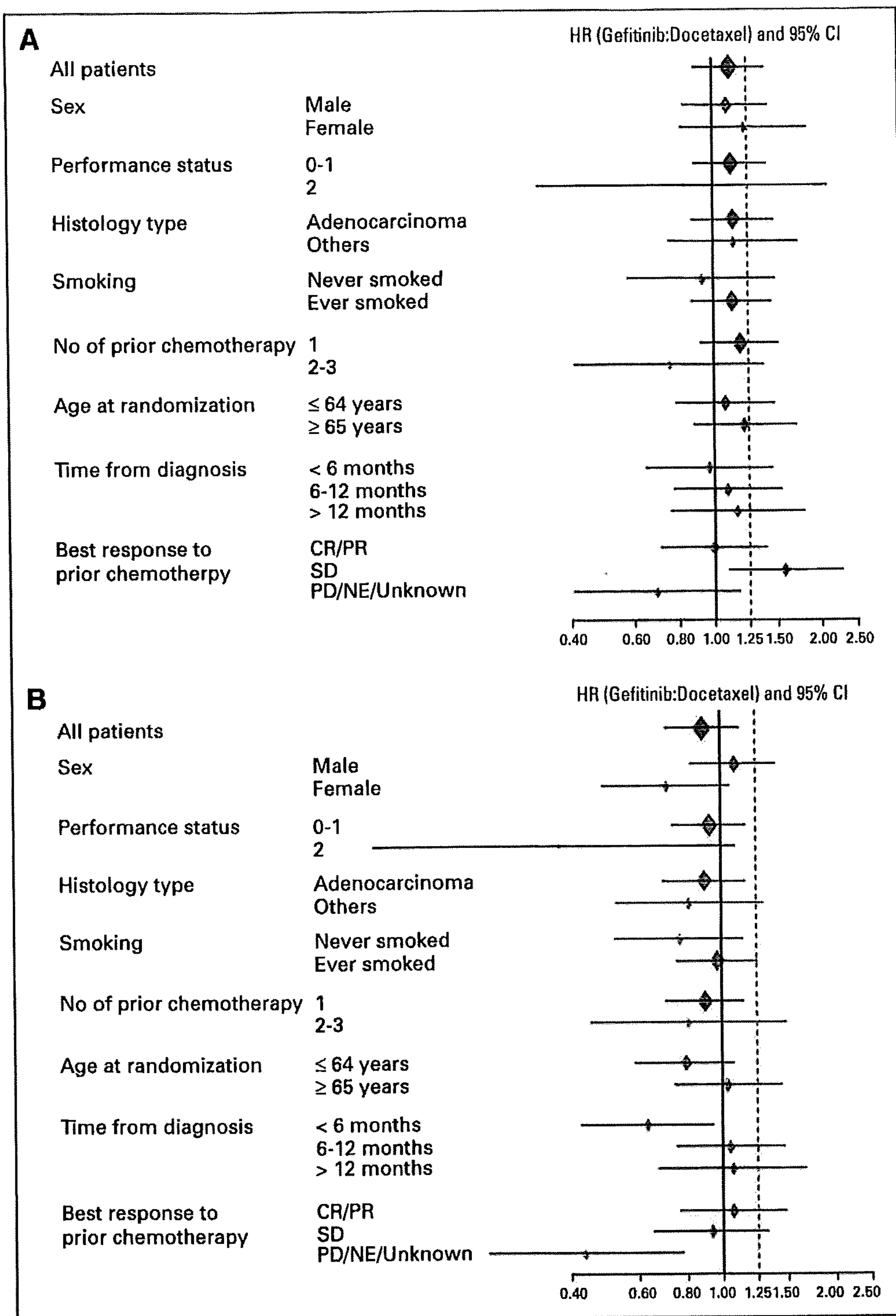

Fig 3. Forest plots of (A) overall survival and (B) progression-free survival that compare treatment groups within clinically relevant subgroups. HR, hazard ratio; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not assessable.

previously to experience improved clinical benefit with gefitinib.[2,4,7,8,10] Subgroup analyses in this study should be interpreted with caution, as the primary objective was not met, some subgroups were small, and there were imbalances in poststudy treatments. In between-treatment comparisons, no statistically significant overall survival benefit was found for gefitinib compared with docetaxel in any subgroup. However, when post hoc, within-treatment comparisons were performed, females, never-smokers, and patients with adenocarcinoma (and also patients with poor PS and > 12 months since diagnosis) had significantly longer survival than their opposite subgroups on both gefitinib and docetaxel ($P < .001$ for females *v* males, adenocarcinoma *v* others, and never-smokers *v* ever-smokers on both treatments). It appears that the subgroups typically associated with a gefitinib benefit were seen but that they also did well on docetaxel. However, the rate of subsequent gefitinib prescription in the docetaxel arm was high in

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Table 3. Summary of Adverse Event Data in the Assessable-for-Safety Population

	Patients			
	Gefitinib (n = 244)		Docetaxel (n = 239)	
Category*	No.	%	No.	%
Adverse events	242	99.2	236	98.7
Treatment-related adverse events	233	95.5	233	97.5
Treatment discontinuation because of an adverse event	33	13.5	42	17.6
NCI-CTC adverse event grades 3 to 4	99	40.6	195	81.6
Serious adverse events	42	17.2	34	14.2
Death as a result of a serious adverse event	4	1.6	0	0
ILD events	14	5.7	7	2.9

Abbreviations: NCI-CTC, National Cancer Institute Common Toxicity Criteria; ILD, interstitial lung disease.
*Participants with multiple events in the same category are counted only once in that category. Participants with events in more than one category are counted once in each of those categories.

these subgroups (eg, approximately two-thirds of docetaxel never-smokers and females had gefitinib as their first poststudy treatment); for PFS and ORR, which are largely unaffected by subsequent treatment, the benefit in these subgroups remained for gefitinib but not for docetaxel, which suggested that poststudy treatments are confounding the interpretation of overall survival in the subgroups.

AEs in our study were consistent with those previously observed, and the most commonly reported AEs were rash/acne and diarrhea for gefitinib and neutropenia for docetaxel. Docetaxel demonstrated a

Table 4. Most Common Adverse Events

	Occurrence by Treatment Arm							
	Gefitinib (n = 244)				Docetaxel (n = 239)			
	Total		Grades 3 to 4		Total		Grades 3 to 4	
Adverse Event	No.	%	No.	%	No.	%	No.	%
Rash/acne*	186	76.2	1	0.4	73	30.5	1	0.4
Diarrhea	126	51.6	5	2.0	67	28.0	2	0.8
Dry skin	90	36.9	0	0.0	13	5.4	0	0.0
Constipation	69	28.3	14	5.7	74	31.0	6	2.5
Anorexia	68	27.9	10	4.1	119	49.8	17	7.1
Nausea	61	25.0	5	2.0	92	38.5	9	3.8
Abnormal hepatic function†	59	24.2	27	11.1	13	5.4	2	0.8
Stomatitis	55	22.5	0	0.0	42	17.6	0	0.0
Nasopharyngitis	50	20.5	0	0.0	32	13.4	0	0.0
Pruritus	42	17.2	0	0.0	15	6.3	0	0.0
Vomiting	41	16.8	4	1.6	41	17.2	3	1.3
Fatigue	36	14.8	1	0.4	107	44.8	6	2.5
Paronychia	33	13.5	1	0.4	2	0.8	0	0.0
Insomnia	32	13.1	0	0.0	20	8.4	0	0.0
Neutropenia‡	24	9.8	20	8.2	190	79.5	176	73.6
Pyrexia	24	9.8	1	0.4	51	21.3	1	0.4
Alopecia	19	7.8	0	0.0	142	59.4	0	0.0
Leukopenia	18	7.4	15	6.1	136	56.9	94	39.3
Headache	12	4.9	1	0.4	25	10.5	0	0.0
Edema§	11	4.5	0	0.0	30	12.6	2	0.8
Myalgia	8	3.3	0	0.0	25	10.5	0	0.0
Dysgeusia	7	2.9	0	0.0	37	15.5	0	0.0
Febrile neutropenia	4	1.6	2	0.8	17	7.1	17	7.1

NOTE. The most common adverse events were considered those that occurred in ≥ 10% of the study population or occurred with > 5% difference between treatments.
*Includes MedDRA high-level terms of rashes, eruptions and exanthems; and of acnes and preferred terms of rash pustular, dermatitis, dermatitis exfoliative, and dermatitis exfoliative generalized.
†Includes MedDRA preferred terms of hepatic function abnormal, alanine aminotransferase increased, aspartate aminotransferase increased and liver disorder.
‡With the exception of one treatment-related adverse event, all other instances of neutropenia reported with gefitinib were in patients who had switched to docetaxel 60 mg/m^2 or other chemotherapy and were reported within the 30-day reporting period. In these other instances, no causal relationship was assigned by the investigator.
§Includes MedDRA preferred terms of edema, edema peripheral, face edema, eyelid edema, and macular edema.

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typically high incidence of neutropenia (79.5%) and febrile neutropenia (7.1%) compared with gefitinib (9.8% and 1.6%, respectively). These neutropenia levels that accompanied docetaxel treatment are consistent with previously reported studies in Japanese patients (95.4%[1] and 81.5%[4]). The incidence of interstitial lung disease reported in this study with gefitinib (5.7%) is consistent with that reported in the Japanese postmarketing study (5.8%).[17]

Although the patient numbers were too small for firm conclusions, the biomarker data from this study suggest that EGFR mutation–positive or EGFR FISH–positive patients have a greater response to both gefitinib and docetaxel compared with EGFR mutation– or FISH–negative patients. The gefitinib data are consistent with several previous reports.[18] The docetaxel data provide potential new information about EGFR biomarkers and chemotherapy; this has not been consistently seen before, because there are only a few small studies in the literature, and they have conflicting results.[19] Hence, it is difficult to say conclusively that EGFR mutation or EGFR FISH–positivity predict for docetaxel as well as gefitinib benefit.

Although the study did not prove noninferior survival for gefitinib compared with docetaxel in this patient population, the clinical efficacy and tolerability of gefitinib 250 mg/d in Japanese patients who had NSCLC, reported here, is consistent with the clinical experience reported to date, and gefitinib remains an effective treatment option for previously treated Japanese patients who have locally advanced/metastatic NSCLC.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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The novel microtubule-interfering agent TZT-1027 enhances the anticancer effect of radiation *in vitro* and *in vivo*

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Translational Therapeutics

TZT-1027 is a novel anticancer agent that inhibits microtubule polymerisation and manifests potent antitumour activity in preclinical models. We have examined the effect of TZT-1027 on cell cycle progression as well as the anticancer activity of this drug both *in vitro* and *in vivo*. With the use of tsFT210 cells, which express a temperature-sensitive mutant of Cdc2, we found that TZT-1027 arrests cell cycle progression in mitosis, the phase of the cell cycle most sensitive to radiation. A clonogenic assay indeed revealed that TZT-1027 increased the sensitivity of H460 cells to γ-radiation, with a dose enhancement factor of 1.2. Furthermore, TZT-1027 increased the radiosensitivity of H460 and A549 cells in nude mice, as revealed by a marked delay in tumour growth and an enhancement factor of 3.0 and 2.2, respectively. TZT-1027 also potentiated the induction of apoptosis in H460 cells by radiation both *in vitro* and *in vivo*. Histological evaluation of H460 tumours revealed that TZT-1027 induced morphological damage to the vascular endothelium followed by extensive central tumour necrosis. Our results thus suggest that TZT-1027 enhances the antitumour effect of ionising radiation, and that this action is attributable in part to potentiation of apoptosis induction and to an antivascular effect. Combined treatment with TZT-1027 and radiation therefore warrants investigation in clinical trials as a potential anticancer strategy.
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Keywords: TZT-1027; radiosensitisation; microtubule; mitotic arrest; apoptosis; antivascular effect

The combination of modalities of cancer treatment offers improvements in the survival of cancer patients compared with individual therapeutic approaches. Such therapeutic benefit has been achieved with combinations of chemo- and radiotherapy in a variety of cancers. The cytotoxicity of most chemotherapeutic agents as well as that of radiation is highly dependent on the phase of the cell cycle. Although various types of anticancer drugs are able to arrest cells at specific cell cycle checkpoints, the ability of antimicrotubule agents to block cell cycle progression in G_2-M phase is the biological basis for combination of these agents with radiation (Pawlik and Keyomarsi, 2004). Microtubule-interfering agents have been shown to increase the radiosensitivity of tumour cells in preclinical and clinical studies (Liebmann *et al*, 1994; Choy *et al*, 1995; Edelstein *et al*, 1996; Vokes *et al*, 1996; Kim *et al*, 2001, 2003; Hofstetter *et al*, 2005; Simoens *et al*, 2006).

TZT-1027 (Soblidotin), a novel microtubule-interfering agent synthesised from dolastatin 10 (Figure 1), exhibits greater antitumour activities and a reduced toxicity compared with its parent compound (Miyazaki *et al*, 1995). TZT-1027 inhibits microtubule assembly by binding to tubulin (Kobayashi *et al*, 1997; Natsume *et al*, 2000). *In vitro*, it inhibits the growth of various human cancer cells at low concentrations (Watanabe *et al*, 2000). *In vivo*, TZT-1027 also manifests a broad spectrum of activity against various murine tumours as well as human tumour xenografts, without inducing a pronounced reduction in body weight (Kobayashi *et al*, 1997; Watanabe *et al*, 2000, 2006a; Natsume *et al*, 2003, 2006). Furthermore, the drug exhibited a potent antivascular effect on existing vasculature in an advanced-stage tumour model (Otani *et al*, 2000). TZT-1027 is currently undergoing clinical evaluation, with a reduction in tumour size and disease stabilisation having been observed in a subset of patients (Schoffski *et al*, 2004; de Jonge *et al*, 2005; Greystoke *et al*, 2006; Tamura *et al*, 2007).

Despite its demonstrated efficacy against solid tumours, the effects of TZT-1027 in combination with radiation have not been examined. As an initial step in determining the antitumour activity of TZT-1027 in combination with radiation, we investigated the effect of this agent on cell cycle progression in synchronised tsFT210 cells (Osada *et al*, 1997), which harbour a temperature-sensitive mutant of Cdc2. We found that TZT-1027 induces arrest of cells in mitosis, the phase of the cell cycle most sensitive to radiation. We then studied the radiosensitising properties of TZT-1027 *in vitro* and *in vivo* with a human lung cancer model and elucidated the mechanism of radiosensitisation by this agent.

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MATERIALS AND METHODS

Cell lines and reagents

tsFT210 mouse mammary carcinoma cells, which express a temperature-sensitive mutant of Cdc2, were kindly provided by H Kakeya (Antibiotics Laboratory, Discovery Research Institute, RIKEN, Saitama, Japan) and were maintained under a humidified atmosphere of 5% CO_2 in air at 32.0°C in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. H460 human lung large cell carcinoma and A549 human lung adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained as for tsFT210 cells with the exception that the culture temperature was 37°C. TZT-1027 (Figure 1) was provided by Daiichi Pharmaceutical Co. Ltd (Tokyo, Japan). Nocodazole and roscovitine were obtained from Sigma.

Cell cycle analysis

Cells were harvested, washed with phosphate-buffered saline (PBS), fixed with 70% methanol, washed again with PBS, and stained with propidium iodide ($0.05\,mg\,ml^{-1}$) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A ($0.05\,mg\,ml^{-1}$). The stained cells ($\sim 1 \times 10^6$) were then analysed for DNA content with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

Measurement of mitotic index and apoptotic cells

Cells were harvested, washed with PBS, fixed with methanol : acetic acid (3 : 1, v/v), washed again with PBS, and stained with 4′,6-diamidino-2-phenylindole (DAPI) ($0.5\,\mu g\,ml^{-1}$). The stained cells ($\sim 1 \times 10^6$) were observed with a fluorescence microscope (IX71; Olympus, Tokyo, Japan). To determine the proportion of mitotic or apoptotic cells, we scored at least 300 cells in each of at least three randomly selected microscopic fields for each of three slides per sample. Cells with condensed chromosomes and no obvious nuclear membrane were regarded as mitotic cells, and the mitotic index was calculated as the percentage of mitotic cells among total viable cells. Cells with fragmented and uniformly condensed nuclei were regarded as apoptotic cells.

Clonogenic assay

Exponentially growing H460 cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm^2 flasks containing 10 ml of medium. The cells were treated with 1 nM TZT-1027 or vehicle (dimethyl sulfoxide, or DMSO; final concentration, 0.1%) for 24 h and then exposed to various doses of γ-radiation with a ^{60}Co irradiator at a rate of $\sim 0.82\,Gy\,min^{-1}$ and at room temperature. The cells were then washed with PBS, cultured in drug-free medium for 10–14 days, fixed with methanol : acetic acid (10 : 1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted. The surviving fraction was calculated as: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for nonirradiated controls. The surviving fraction for combined treatment was corrected by that for TZT-1027 treatment alone. The dose enhancement factor (DEF) was calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.1 for vehicle-treated cells divided by that for TZT-1027-treated cells (after correction for drug toxicity).

In vivo antitumour activity of TZT-1027 with or without radiation

All animal studies were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. The ethical guidelines followed meet the requirements of the UKCCCR guidelines (Workman *et al*, 1998). Tumour cells (2×10^6) were injected subcutaneously into the right hind leg of 7-week-old female athymic nude mice. Tumour volume was determined from caliper measurement of tumour length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when tumours in each group achieved an average volume of $\sim 200-250\,mm^3$. Treatment groups consisted of control, TZT-1027 alone, radiation alone, and the combination of TZT-1027 and radiation. Each treatment group contained six to eight mice. TZT-1027 was administered intravenously in a single dose of $0.5\,mg\,kg^{-1}$ of body weight; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the leg were exposed to 10 Gy of γ-radiation with a ^{60}Co irradiator at a rate of $\sim 0.32\,Gy\,min^{-1}$ immediately after drug treatment. Growth delay (GD) was calculated as the time for treated tumours to achieve an average volume of $500\,mm^3$ minus the time for control tumours to reach $500\,mm^3$. The enhancement factor was then determined as: $(GD_{combination} - GD_{TZT-1027})/(GD_{radiation})$.

TUNEL staining

Mice were killed 14 days after treatment initiation and the tumours were removed and preserved in 10% paraformaldehyde. Apoptosis in tumour sections was determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) assay with the use of an apoptosis detection kit (Chemicon, Temecula, CA, USA). The number of apoptotic cells was counted in 10 separate microscopic fields (×100) for three sections of each tumour of each group.

Histological analysis

A single dose of TZT-1027 ($2.0\,mg\,kg^{-1}$) or vehicle (physiological saline) was administered intravenously to mice when H460 tumours had achieved a volume of ~ 400 to $600\,mm^3$. Tumour tissue was extirpated 4 or 24 h after drug administration, and half of the tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The other half of the tumour tissue was fixed for 12–48 h in zinc fixative

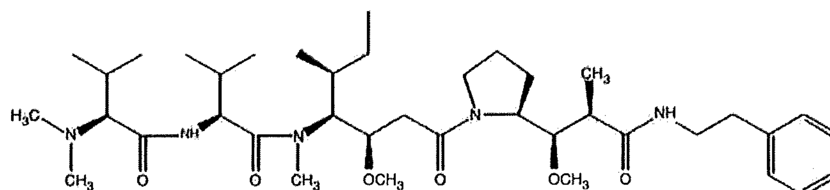

Figure 1 Chemical structure of TZT-1027.

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(BD Biosciences, San Jose, CA, USA), embedded in paraffin, sectioned, and immunostained for CD31. Endogenous peroxidase activity was blocked by incubation of the latter sections for 20 min with 0.3% H_2O_2 in methanol, and nonspecific sites were blocked with antibody diluent (Dako Japan, Kyoto, Japan). Sections were then incubated overnight at 4°C with a 1:50 dilution of a rat monoclonal antibody to mouse CD31 (BD Biosciences), washed with PBS, and processed with a Histfine Simple Stain PO (M) kit (Nichirei, Tokyo, Japan) for detection of immune complexes. Sections were counterstained with Mayer's hematoxylin, covered with a coverslip with the use of a permanent mounting medium, and examined with a light microscope (CX41; Olympus, Tokyo, Japan).

Statistical analysis

Data are presented as means ± s.d. or s.e. and were compared by the unpaired Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Induction of cell cycle arrest at M phase but not at G_1-S in tsFT210 cells by TZT-1027

To examine the effect of TZT-1027 on cell cycle progression, we performed flow cytometric analysis of tsFT210 cells, which express a temperature-sensitive mutant of Cdc2. These mammary carcinoma cells exhibit a normal cell cycle distribution when incubated at the permissive temperature of 32.0°C, but they arrest at G_2 phase as a result of Cdc2 inactivation when incubated at the nonpermissive temperature of 39.4°C (Figure 2A and B). We synchronised tsFT210 cells at G_2 phase by incubation at 39.4°C for 17 h and then cultured them at 32.0°C for 6 h in the presence of nocodazole (an inhibitor of microtubule polymerisation), TZT-1027, or vehicle (DMSO). In the presence of vehicle alone, the number of cells in G_2 phase decreased markedly and there was a corresponding increase in the number of cells in G_1 phase, indicative of re-entry of cells into the cell cycle (Figure 2C). In contrast, treatment with nocodazole or TZT-1027 prevented the cells from passing through G_2-M phase (Figure 2D and E). Given that flow cytometric analysis did not distinguish between cells in M phase and those in G_2 phase, we determined the mitotic index of cells by DAPI staining and fluorescence microscopy. Most of the cells released from temperature-induced arrest in the presence of nocodazole manifested condensed chromosomes without a nuclear membrane, yielding a mitotic index of 93%; most of the cells had thus arrested in mitosis (Figure 2D). Most of the cells released from temperature-induced arrest in the presence of TZT-1027 showed similar mitotic figures, yielding a mitotic index of 85% (Figure 2E) and demonstrating that TZT-1027 also inhibits cell cycle progression at mitosis.

We next examined whether TZT-1027 affects the G_1-S transition. We arrested tsFT210 cells at G_2 phase by incubation at 39.4°C, released the cells into G_1 phase by shifting to the permissive temperature for 6 h, and then incubated them for an additional 6 h in the presence of roscovitine (an inhibitor of CDK2 that prevents cell cycle progression at G_1 phase), TZT-1027, or vehicle (Figure 3). The cells incubated with vehicle passed through G_1 phase and yielded a broad S-phase peak (Figure 3D), whereas those treated with roscovitine did not pass through G_1 phase (Figure 3E). In contrast, TZT-1027 had no effect on passage of the synchronised tsFT210 cells through the G_1-S transition (Figure 3F). Together,

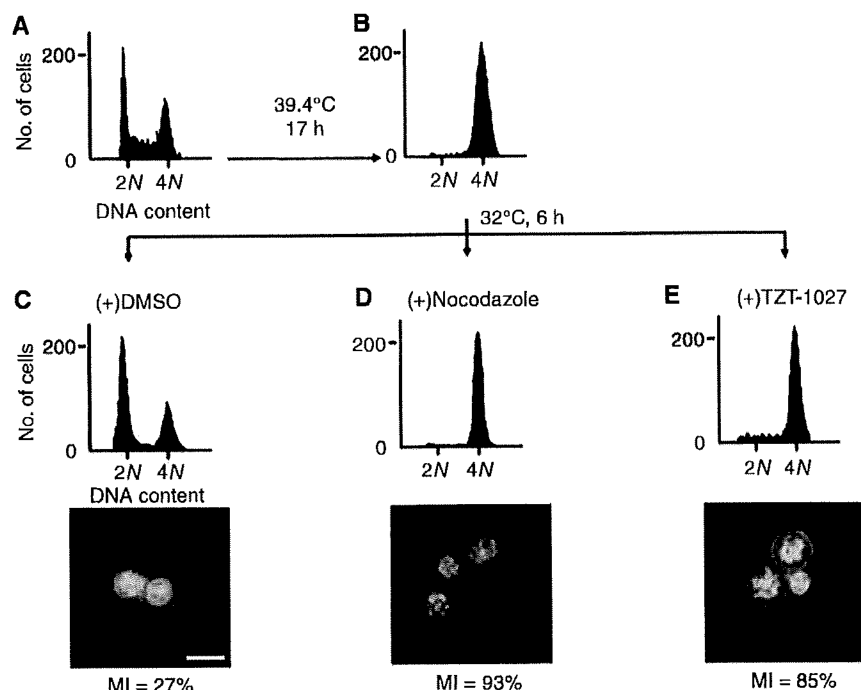

Figure 2 Inhibition of tsFT210 cell cycle progression through G_2-M by TZT-1027. Cells were cultured at the permissive temperature of 32.0°C (**A**) and then incubated for 17 h at the nonpermissive temperature of 39.4°C (**B**). They were subsequently released from G_2 arrest by incubation at 32.0°C for 6 h in the presence of DMSO (**C**), 1 μM nocodazole (**D**), or 2 nM TZT-1027 (**E**). At each stage of the protocol, cells were analysed for DNA content by staining with propidium iodide and flow cytometry. The 2N and 4N peaks indicate cells in G_0-G_1 and G_2-M phases of the cell cycle, respectively. The cells were also stained with DAPI and examined by fluorescence microscopy after treatment with DMSO, nocodazole, or TZT-1027 (lower panels), and the mitotic index (MI) was determined; scale bar, 20 μm. Data are representative of at least three independent experiments.

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these results indicate that the effect of TZT-1027 on cell cycle progression is specific to M phase.

Induction of cell cycle arrest at M phase in asynchronous H460 cells by TZT-1027

We next examined whether TZT-1027 induced mitotic arrest in asynchronous H460 human non-small cell lung cancer cells. Flow cytometric analysis revealed that treatment of H460 cells with TZT-1027 for 24 h induced a threefold increase in the proportion of cells with a DNA content of 4N compared with that apparent for vehicle-treated cells (29.1 *vs* 8.7%) (Figure 4A and B). Furthermore, DAPI staining revealed that TZT-1027 induced a significant increase in the mitotic index of H460 cells compared with that for the control cells (23.3 *vs* 4.6%) (Figure 4C and D), indicating that most of the TZT-1027-treated cells with a DNA content of 4N were arrested in M phase rather than in G_2 phase. These observations thus showed that TZT-1027 also induced mitotic arrest in asynchronous H460 cells.

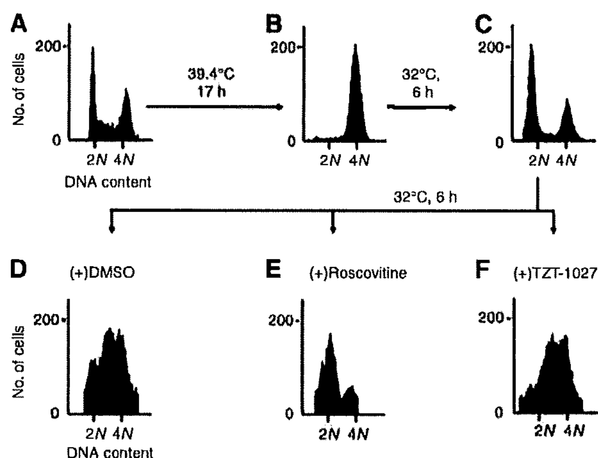

Figure 3 Lack of effect of TZT-1027 on tsFT210 cell cycle progression through G_1-S. Exponentially growing tsFT210 cells (**A**) were arrested in G_2 phase by incubation for 17 h at 39.4°C (**B**). The cells were incubated at 32.0°C first for 6 h to allow progression to G_1 phase (**C**) and then for an additional 6 h in the presence of DMSO (**D**), 50 μM roscovitine (**E**), or 2 nM TZT-1027 (**F**). At each stage of the protocol, cells were analysed for DNA content by flow cytometry. Data are representative of at least three independent experiments.

Radiosensitisation of H460 cells by TZT-1027 *in vitro*

Cells in M phase are more sensitive to radiation than are those in other phases of the cell cycle. Given that exposure of H460 cells to TZT-1027-induced mitotic arrest, we next examined whether this agent might sensitise H460 cells to γ-radiation with the use of a clonogenic assay. H460 cells were incubated for 24 h with 1 nM TZT-1027 or vehicle (DMSO) and then exposed to various doses (0, 2, 4, or 6 Gy) of γ-radiation. The cells were then allowed to form colonies in drug-free medium for 10–14 days. Survival curves revealed that TZT-1027 increased the radiosensitivity of H460 cells, with a DEF of 1.2 (Figure 5A).

To determine whether radiosensitisation by TZT-1027 was reflected by an increase in the proportion of apoptotic cells, we exposed H460 cells to 1 nM TZT-1027 or vehicle for 24 h, treated the cells with various doses (0, 2, 4, or 6 Gy) of radiation, and then incubated them in drug-free medium for an additional 24 h before quantification of apoptosis. Combined treatment with TZT-1027 and 4 or 6 Gy of radiation resulted in a significant increase in the number of apoptotic cells compared with the sum of the values for treatment with drug alone or radiation alone (Figure 5B). TZT-1027 thus promoted radiation-induced apoptosis in H460 cells.

Radiosensitisation of H460 cells and A549 cells by TZT-1027 *in vivo*

To determine whether the TZT-1027-induced increase in the radiosensitivity of tumour cells observed *in vitro* might also be apparent *in vivo*, we injected H460 cells or A549 human lung

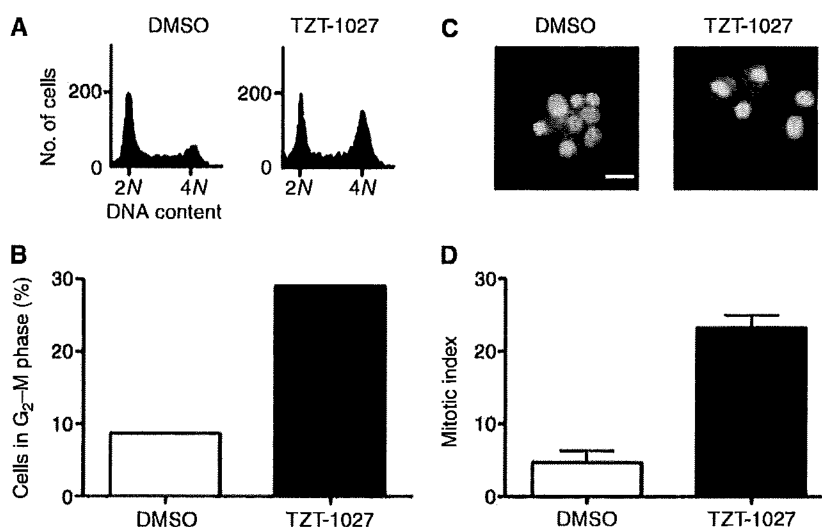

Figure 4 Induction of cell cycle arrest at M phase in H460 cells by TZT-1027. H460 cells were incubated in the presence of 1 nM TZT-1027 or vehicle (DMSO) for 24 h, after which DNA content was measured by flow cytometry (**A**) and the fraction of cells in G_2-M phase was determined (**B**). The cells were also stained with DAPI and examined by fluorescence microscopy (**C**) and the mitotic index was determined (**D**). Data in (**A**) through (**C**) are representative of at least three independent experiments; data in (**D**) are means$\pm$s.d. of values from three independent experiments. Scale bar in (**C**), 20 μm.

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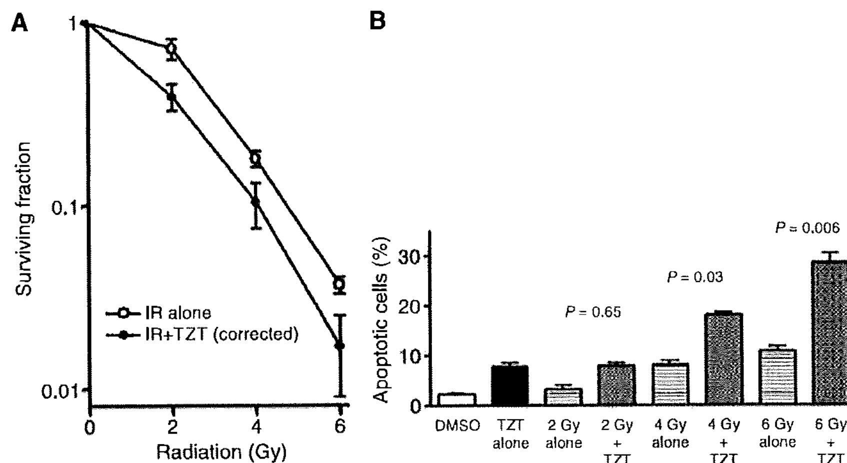

Figure 5 Sensitisation of H460 cells to γ-radiation by TZT-1027 *in vitro*. (**A**) Clonogenic assay. Cells were incubated with 1 nM TZT-1027 or vehicle (DMSO) for 24 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and TZT-1027 by that apparent for treatment with TZT-1027 alone. (**B**) Assay of apoptosis. Cells were incubated with 1 nM TZT-1027 or vehicle (DMSO) for 24 h, exposed to various doses (0, 2, 4, or 6 Gy) of γ-radiation, and then incubated for 24 h in drug-free medium. Cells were then fixed and stained with DAPI for determination of the proportion of apoptotic cells by fluorescence microscopy. Data in (**A**) and (**B**) are means ± s.d. of values from three independent experiments. *P* values in (**B**) are for comparison of the value for combined treatment with TZT-1027 and radiation *vs* the sum of the corresponding values for each treatment alone, after correction of all data by the control (DMSO) value.

adenocarcinoma cells into nude mice in order to elicit the formation of solid tumours. The mice were then treated with TZT-1027, radiation, or both modalities. Treatment with TZT-1027 alone (single dose of 0.5 mg kg^{-1}) or with radiation alone (single dose of 10 Gy) resulted in relatively small inhibitory effects on tumour growth, whereas combined treatment with both TZT-1027 and radiation exerted a markedly greater inhibitory effect (Figure 6A and B). The tumour GDs induced by treatment with TZT-1027 alone, radiation alone, or both TZT-1027 and radiation were 1.0, 2.6, and 8.8 days, respectively, for H460 cells and 1.4, 4.9, and 12.4 days, respectively, for A549 cells (Table 1). The enhancement factor for the effect of TZT-1027 on the efficacy of radiation was 3.0 for H460 cells and 2.2 for A549 cells, revealing the effect to be greater than additive. No pronounced tissue damage or toxicities such as diarrhoea or weight loss of >10% were observed in mice in any of the four treatment groups (Table 2).

We examined the effects of the treatment protocols on apoptosis in H460 tumours by TUNEL staining of tumour sections. Quantification of the number of apoptotic cells revealed that the combined treatment with radiation and TZT-1027 induced a significant increase in this parameter compared with treatment with radiation or TZT-1027 alone (Figure 6C).

Histological appearance of H460 tumours after administration of TZT-1027

Finally, we examined whether an effect of TZT-1027 on tumour vasculature might contribute to the antitumour activity of this drug *in vivo*. Mice harbouring H460 tumours were injected with TZT-1027, and the tumours were excised 4 or 24 h thereafter and examined by hematoxylin-eosin staining (Figure 7A–C) or by immunostaining for the endothelial cell marker CD31 (Figure 7D and E). Tumour capillaries appeared congested, with thrombus formation, and showed a loss of endothelial cells 4 h after administration of TZT-1027 (Figure 7B and E), whereas vessels within viable areas of control tumours were generally not congested and showed an intact normal endothelium (Figure 7A and D). The effects of TZT-1027 on the tumour vasculature appeared selective, given that neither loss of CD31 staining nor vessel congestion was apparent in the vasculature of surrounding normal tissue after drug treatment (Figure 7E). Extensive necrosis was apparent at the tumour core, with a characteristic thin rim of viable tumour cells remaining at the periphery, 24 h after TZT-1027 administration (Figure 7C). These results were thus indicative of a characteristic antivascular effect of TZT-1027 in the H460 tumour model.

DISCUSSION

TZT-1027 is a novel antitumour agent that inhibits microtubule polymerisation and exhibits potent antitumour activity in preclinical models (Miyazaki *et al*, 1995; Kobayashi *et al*, 1997; Natsume *et al*, 2000, 2003, 2006; Otani *et al*, 2000; Watanabe *et al*, 2000, 2006a). We investigated the effect of TZT-1027 on cell cycle progression with the use of tsFT210 cells, which can be synchronised in G_2 phase by incubation at 39.4°C and consequent inactivation of Cdc2 (Osada *et al*, 1997; Tamura *et al*, 1999). The use of these cells allows cell synchronisation without the need for agents that prevent DNA synthesis (such as hydroxyurea or thymidine) or that inhibit formation of the mitotic spindle (such as nocodazole). Although such agents halt cell cycle progression in specific phases of the cycle, they are also toxic and kill a proportion of the treated cells. The tsFT210 cell system is thus suited to sensitive analysis of the effects of new compounds on cell cycle progression without loss of cell viability. We have now shown that tsFT210 cells released from G_2 arrest by incubation at 32.0°C failed to pass through M phase in the presence of TZT-1027. Although previous flow cytometric analysis of exponentially growing tumour cells revealed that TZT-1027 induced a marked increase in the proportion of cells in G_2-M (Watanabe *et al*, 2000), it was uncertain whether the drug arrested cell cycle progression in G_2 or in mitosis. Our morphological data now indicate that, similar to the effect of nocodazole, TZT-1027 arrested tsFT210 cells in M phase rather than in G_2, consistent with the mode of action of this new compound. Given that microtubules contribute to various cellular functions in addition to cell division, including intracellular transport and signal transduction (Mollinedo and Gajate,

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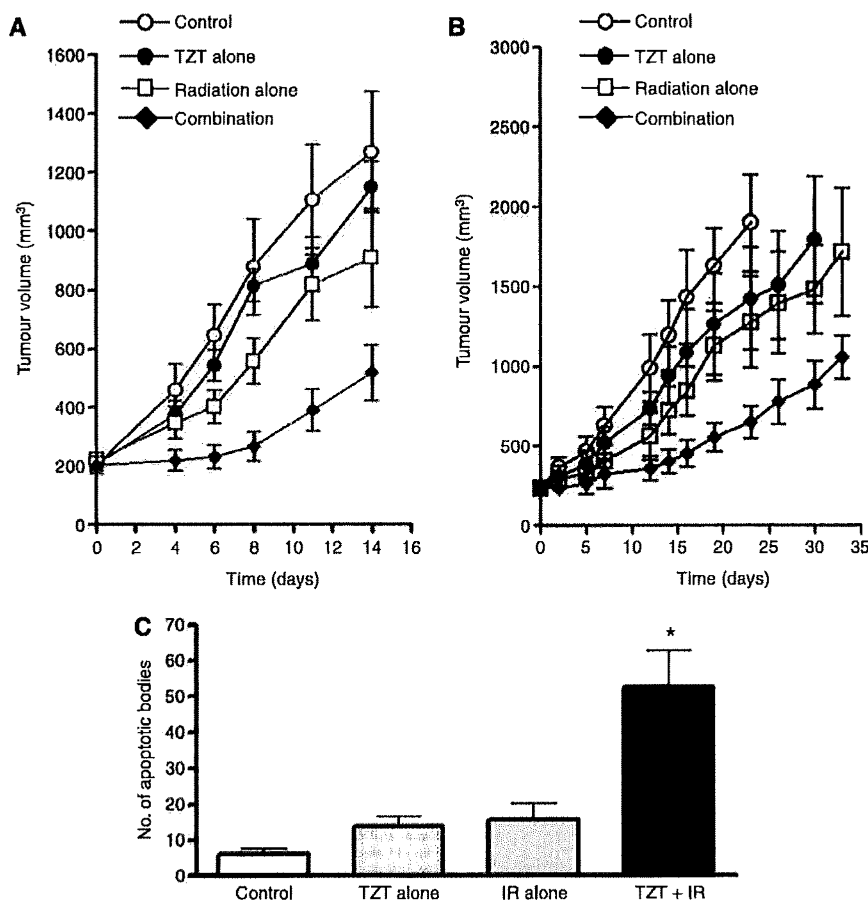

Figure 6 Sensitisation of H460 and A549 cells to γ-radiation by TZT-1027 *in vivo*. (**A** and **B**) Evaluation of tumour growth. Nude mice with H460 (**A**) or A549 (**B**) tumour xenografts (~200 to 250 mm³) were treated with a single intravenous dose of TZT-1027 (0.5 mg kg⁻¹), a single dose of γ-radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated times thereafter. Data are means ± s.e. for six to eight mice per group. (**C**) Quantification of apoptotic cells in H460 tumour sections by TUNEL staining 14 days after the initiation of treatment as in (**A**). Data are means ± s.d. *$P<0.05$ *vs* mice treated with TZT-1027 alone or radiation alone.

Table 1 Tumour growth delay value

	H460		A549	
Treatment	**Days**[a]	**GD**[b]	**Days**	**GD**
Control	4.5		5.5	
TZT-1027 alone	5.5	1	6.9	1.4
Radiation alone	7.1	2.6	10.4	4.9
TZT-1027 + Radiation	13.3	8.8	17.9	12.4
Enhancement factor	3		2.2	

[a]Days, the period needed for the sizes of xenografts in each group to reach 500 mm³; [b]GD, the additional periods needed for the sizes of xenografts in each group to reach 500 mm³ in addition to the period needed for controls to reach 500 mm³.

Table 2 Body weight loss

	% of B.W.L[a]	
	H460	**A549**
Control	3.6	1.2
TZT-1027 alone	9.9	5.2
Radiation alone	9.7	5.5
TZT-1027+Radiation	8.7	9.9

[a]% of B.W.L, relative body weight loss 7 days after the initiation of the treatment.

2003), TZT-1027 might also be expected to affect tumour cells in interphase. With the use of synchronised tsFT210 cells, however, we found that TZT-1027 had no effect on progression of cells through the G_1–S transition of the cell cycle. The effect of TZT-1027 on cell cycle progression thus appears to be specific to M phase.

Given that cells are most sensitive to radiation during mitosis (Sinclair and Morton, 1966; Sinclair, 1968; Pawlik and Keyomarsi, 2004), we next investigated the possible interaction between TZT-1027 and ionising radiation in human lung cancer cell lines. We found that TZT-1027 increased the sensitivity of H460 cells to γ-radiation *in vitro*. The proportion of H460 cells in mitotic phase at the time of irradiation was increased by TZT-1027 treatment, consistent with the notion that this effect contributes to the observed radiosensitisation induced by this drug. TZT-1027 was previously shown to induce apoptosis in several tumour cell lines (Watanabe *et al*, 2000). Although the relation between apoptosis and radiosensitivity is controversial (Lawrence *et al*, 2001; Pawlik and Keyomarsi, 2004), we showed that treatment of H460 cells with