References

1. Curt GA. Impact of fatigue on quality of life in oncology patients. *Semin Hematol* 2000;37:14–7.
2. Casadevall N. Update on the role of epoetin alfa in hematologic malignancies and myelodysplastic syndromes. *Semin Oncol* 1998;25:12–8.
3. Ludwig H, Fritz E. Anemia in cancer patients. *Semin Oncol* 1998;25:2–6.
4. Groopman JE, Itri LM. Chemotherapy-induced anemia in adults: incidence and treatment. *J Natl Cancer Inst* 1999;91:1616–34.
5. Koury MJ. Bondurant MC. Control of red cell production: the roles of programmed cell death (apoptosis) and erythropoietin. *Transfusion* 1990;30:673–4.
6. Walker RH. Transfusion risks. *Am J Clin Pathol* 1987;88:374–8.
7. Goodnough LT, Skikne B, Brugnara C. Erythropoietin, iron, and erythropoiesis. *Blood* 2000;96:823–33.
8. Cascinu S, Fedeli A, Del Ferro E, Luzi Fedeli S, Catalano G. Recombinant human erythropoietin treatment in cisplatin-associated anemia: a randomized double-blind trial with placebo. *J Clin Oncol* 1994;12:1058–62.
9. Abels RI. Use of recombinant human erythropoietin in the treatment of anemia in patients who have cancer. *Semin Oncol* 1992;19:29–35.
10. Vansteenkiste J, Pirker R, Massuti B, Barata F, Font A, Fiegl M, et al. Double-blind, placebo-controlled, randomized phase 3 trial of darbepoetin alfa in lung cancer patients receiving chemotherapy. *J Natl Cancer Inst* 2002;94:1211–20.
11. Nowrousian MR. Recombinant human erythropoietin in the treatment of cancer-related or chemotherapy-induced anaemia in patients with solid tumours. *Med Oncol* 1998;15(Suppl 1):S19–28.
12. Cazzola M, Messinger D, Battistel V, Bron D, Cimino R, Enller-Ziegler L, et al. Recombinant human erythropoietin in the anemia associated with multiple myeloma or non-Hodgkin's lymphoma: dose finding and identification of predictors of response. *Blood* 1995;86:4446–53.
13. Österborg A, Boogaerts MA, Cimino R, Essers U, Holowiecki J, Juliusson G, et al. Recombinant human erythropoietin in transfusion-dependent anemic patients with multiple myeloma and non-Hodgkin's lymphoma: a randomized multicenter study. *Blood* 1996;87:2675–82.
14. Österborg A, Brandberg Y, Molostova V, Iosava G, Abdulkadyrov K, Hedenus M, et al. Randomized, double-blind, placebo-controlled trial of recombinant human erythropoietin, epoetin beta, in hematologic malignancies. *J Clin Oncol* 2002;20:2486–94.
15. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Nephrol Dial Transplant* 2001;16(Suppl 3):3–13.
16. Macdougall IC, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, et al. Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. *J Am Soc Nephrol* 1999;10:2392–5.
17. Kotasek D, Steger G, Faught W, Underhill C, Poulsen E, Colowick AB, et al. Darbepoetin alfa administered every 3 weeks alleviates anaemia in patients with solid tumours receiving chemotherapy: results of a double-blind, placebo-controlled, randomised study. *Eur J Cancer* 2003;39:2026–34.
18. Glaspy JA, Jadeja JS, Justice G, Kessler J, Richards D, Schwartzberg L, et al. Darbepoetin alfa given every 1 or 2 weeks alleviates anaemia associated with cancer chemotherapy. *Br J Cancer* 2002;87:268–76.
19. Hedenus M, Hansen S, Taylor K, Arthur C, Emmerich B, Dewey C, et al. Randomized, dose-finding study of darbepoetin alfa in anaemic patients with lymphoproliferative malignancies. *Br J Haematol* 2002;119:79–86.
20. Demetri GD, Kris M, Wade J, Degos L, Cella D. Quality-of-life benefit in chemotherapy patients treated with epoetin alfa is independent of disease response or tumor type: results from a prospective community oncology study. *J Clin Oncol* 1998;16:3412–25.
21. Glaspy J, Bukowski R, Steinberg D, Taylor C, Tchekmedyian S, Vadhan-Raj S, et al. Impact of therapy with epoetin alfa on clinical outcomes in patients with nonmyeloid malignancies during cancer chemotherapy in community oncology practice. *J Clin Oncol* 1997;15:1218–34.
22. Gascón P. Evaluating erythropoietic agents for the treatment of anaemia in the oncology setting. *Eur J Cancer* 2005;41:2601–12.
23. Yellen SB, Cella DF, Webster K, Blendowski C, Kaplan E. Measuring fatigue and other anemia-related symptoms with the Functional Assessment of Cancer Therapy (FACT) measurement system. *J Pain Symptom Manage* 1997;13:63–74.
24. Cella D, Lai JS, Chang CH, Peterman A, Slavin M. Fatigue in cancer patients compared with fatigue in the general United States population. *Cancer* 2002;94:528–38.
25. Jones M, Schenkel B, Just J, Fallowfield L. Epoetin alfa improves quality of life in patients with cancer: results of metaanalysis. *Cancer* 2004;101:1720–32.
26. Webster K, Cella D, Yost K. The Functional Assessment of Chronic Illness Therapy (FACIT) Measurement System: properties, applications, and interpretation. *Health Qual Life Outcomes* 2003;1:79.
27. McKoy JM, Stonecash RE, Cournoyer D, Rossert J, Nissenson AR, Raisch DW, et al. Epoetin-associated pure red cell aplasia: past, present, and future considerations. *Transfusion* 2008;48:1754–62.
28. Leyland-Jones B, Semiglazov V, Pawlicki M, Pienkowski T, Tjulandin S, Manikhas G, et al. Maintaining normal hemoglobin levels with epoetin alfa in mainly nonanemic patients with metastatic breast cancer receiving first-line chemotherapy: a survival study. *J Clin Oncol* 2005;23:5960–72.
29. Henke M, Laszig R, Rübe C, Schäfer U, Haase KD, Schilcher B, et al. Erythropoietin to treat head and neck cancer patients with anaemia undergoing radiotherapy: randomised, double-blind, placebo-controlled trial. *Lancet* 2003;362:1255–60.
30. Smith RE, Jr. Aapro MS, Ludwig H, Pintér T, Smakal M, Ciuleanu TE, et al. Darbepoetin alpha for the treatment of anemia in patients with active cancer not receiving chemotherapy or radiotherapy: results of a phase III, multicenter, randomized, double-blind, placebo-controlled study. *J Clin Oncol* 2008;26:1040–50.
31. Overgaard J, Hoff C, Sand Hansen H, Specht L, Overgaard M, Grau C, et al. Randomized study of the importance of novel erythropoiesis stimulating protein (Aranesp) for the effect of radiotherapy in patients with primary squamous cell carcinoma of the head and neck (HNSCC): the Danish Head and Neck Cancer Group DAHANCA 10 randomized trial. *Eur J Cancer* 2007;5(Suppl):7.
32. Hedenus M, Adriansson M, San Miguel J, Kramer MH, Schipperus MR, Juvonen E, et al. Efficacy and safety of darbepoetin alfa in anaemic patients with lymphoproliferative malignancies: a randomized, double-blind, placebo-controlled study. *Br J Haematol* 2003;122:394–403.
33. FDA Briefing Document. May 10, 2007: Oncologic Drugs Advisory Committee. Continuing reassessment of the risks of erythropoiesis-stimulating agents (ESAs) administered for the treatment of anemia associated with cancer chemotherapy. http://www.fda.gov/ohrms/dockets/ac/07/briefing/2007-4301b2-02-FDA.pdf.
34. Wright JR, Ung YC, Julian JA, Pritchard KI, Whelan TJ, Smith C, et al. Randomized, double-blind, placebo-controlled trial of erythropoietin in non-small-cell lung cancer with disease-related anemia. *J Clin Oncol* 2007;25:1027–32.
35. Thomas G, Ali S, Hoebers FJ, Darcy KM, Rodgers WH, Patel M, et al. Phase III trial to evaluate the efficacy of maintaining hemoglobin levels above 12.0 g/dL with erythropoietin vs above 10.0 g/dL without erythropoietin in anemic patients receiving concurrent radiation and cisplatin for cervical cancer. *Gynecol Oncol* 2008;108:317–25.
36. Bohlius J, Schmidlin K, Brillant C, Schwarzer G, Trelle S, Seidenfeld J, et al. Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: a meta-analysis of randomised trials. *Lancet* 2009;373:1532–42.
37. Ludwig H, Crawford J, Österborg A, Vansteenkiste J, Henry DH, Fleishman A, et al. Pooled analysis of individual patient-level data from all randomized, double-blind, placebo-controlled trials of darbepoetin alfa in the treatment of patients with chemotherapy-induced anemia. *J Clin Oncol* 2009;27:2838–47.
38. Aapro M, Scherhag A, Burger HU. Effect of treatment with epoetin-beta on survival, tumour progression and thromboembolic events in patients with cancer: an updated meta-analysis of 12 randomised controlled studies including 2301 patients. *Br J Cancer* 2008;99:14–22.
39. Tonelli M, Hemmelgarn B, Reiman T, Manns B, Reaume MN, Lloyd A, et al. Benefits and harms of erythropoiesis-stimulating agents for anemia related to cancer: a meta-analysis. *CMAJ* 2009;180:E62–71.

40. LaMontagne KR, Butler J, Marshall DJ, Tullai J, Gechtman Z, Hall C, et al. Recombinant epoetins do not stimulate tumor growth in erythropoietin receptor-positive breast carcinoma models. *Mol Cancer Ther* 2006;5:347–55.
41. Sigounas G, Sallah S, Sigounas VY. Erythropoietin modulates the anticancer activity of chemotherapeutic drugs in a murine lung cancer model. *Cancer Lett* 2004;214:171–9.
42. Shannon AM, Bouchier-Hayes DJ, Condron CM, Toomey D. Correction of anaemia through the use of darbepoetin alfa improves chemotherapeutic outcome in a murine model of Lewis lung carcinoma. *Br J Cancer* 2005;93:224–32.
43. Aapro M, Spivak JL. Update on erythropoiesis-stimulating agents and clinical trials in oncology. *Oncologist* 2009;14(Suppl 1): 6–15.

Disease Control as a Predictor of Survival with Gefitinib and Docetaxel in a Phase III Study (V-15-32) in Advanced Non-small Cell Lung Cancer Patients

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Introduction: This post hoc analysis investigated the relationship between tumor response and overall survival (OS) in pretreated advanced non-small cell lung cancer (NSCLC).

Methods: We conducted landmark survival analyses of V-15-32, a phase III study comparing gefitinib with docetaxel in pretreated advanced NSCLC. Best response at weeks 8, 12, 16, and 20, and visit response at week 4, were evaluated.

Results: Disease control (DC; complete response [CR], partial response [PR], or stable disease) was a better predictor of OS than CR/PR at all time points. The strongest predictor of OS for both gefitinib and docetaxel was DC at week 8 (hazard ratio [HR] DC versus non-DC: 0.30, 95% confidence interval [CI] 0.20–0.45, $p < 0.001$ for both treatments). DC at week 4 was also associated with longer survival compared with non-DC for both treatments (HR 0.33, 95% CI 0.23–0.49, $p < 0.001$ for gefitinib; HR 0.30, 95% CI 0.19–0.47, $p < 0.001$ for docetaxel).

Discussion: DC is a better predictor of OS with gefitinib and docetaxel than CR/PR in advanced pretreated NSCLC, with a best response of DC at week 8 the strongest predictor.

Key Words: Docetaxel, Epidermal growth factor receptor tyrosine kinase inhibitors, Gefitinib, Landmark analysis, NSCLC.

(*J Thorac Oncol.* 2010;5: 1042–1047)

Patients with advanced/metastatic non-small cell lung cancer (NSCLC) have a poor prognosis, with less than 10% of patients experiencing tumor shrinkage equating to a complete response or partial response (CR or PR) with standard second-/third-line chemotherapy.[1–4] However, many patients do achieve nonprogression or disease control (DC; the sum of CR, PR, and stable disease [SD]), with approximately 35 to 45% of patients achieving SD in clinical trials.[1–4] In the setting of advanced NSCLC, SD may be a positive therapeutic outcome, with potential benefits including improved quality of life and prolonged survival.[5,6] In a prospective, randomized study of erlotinib in advanced NSCLC after first-/second-line chemotherapy, there was significant prolongation of survival with erlotinib compared with placebo (6.7 versus 4.7 months, respectively; hazard ratio [HR] 0.61, $p < 0.001$), despite a response rate of <10%; one possible explanation for this is that a high proportion of patients had SD during therapy and this may have contributed to the prolongation in survival.[4] In clinical trials, response based on the Response Evaluation Criteria in Solid Tumors (RECIST) is widely used to identify and quantify the antitumor activity of new agents, providing a relatively quick assessment of efficacy; however, its value as a predictor of a survival benefit remains unclear.[7]

Based on the fact that many more patients initially achieve nonprogression than CR or PR, Lara et al.[8] first hypothesized that the rate of nonprogression, or DC, is a stronger predictor of clinical benefit than CR/PR after platinum-based chemotherapy in patients with advanced NSCLC. In a pooled analysis of data from 984 patients with advanced NSCLC who entered into three randomized trials of first-line platinum-based chemotherapy, landmark survival analysis showed that DC versus non-DC at week 8 was a stronger predictor of longer survival (HR 0.45, $p < 0.0001$) than the traditional CR/PR versus non-CR/PR (HR 0.61, $p < 0.001$). A study conducted using landmark analysis also found that a decrease or no change in tumor size at week 8 was significantly associated with longer survival ($p = 0.043$) in patients with advanced NSCLC who received first-line chemotherapy in the Four-Arm Cooperative Study or pemetrexed as salvage therapy for previously treated disease.[9] A meta-analysis of the published literature that included 28 phase II/III trials in 6171 patients with advanced NSCLC receiving gefitinib or

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Disclosure: Dr. negoro was paid an honorarium from AstraZeneca and Sanofy-Aventis. Dr. Jiang and Dr. Itoh are employees of AstraZeneca. Dr. Saijo was paid an honorarium from AstaZeneca, Eli Lilly, Chugai Pharmaceutical Company Ltd., and taiho Pharmaceutical Company Ltd. Professor Fukuoka was paid an honorarium from AstraZeneca, Chugai Pharmaceutical Company Ltd., and Boehringer Ingelheim. The other authors declare no conflicts of interest.

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ISSN: 1556-0864/10/0507-1042

erlotinib found a significant correlation between both response rate and DC with survival ($p < 0.0001$ and $p = 0.003$, respectively).[10]

Gefitinib is an orally bioavailable, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor and was the first molecular-targeted drug approved for NSCLC. A recent large international phase III study (IRESSA NSCLC Trial Evaluating REsponse and Survival versus Taxotere) comparing gefitinib and docetaxel in 1466 patients with pretreated (at least one platinum-based regimen) advanced NSCLC established noninferior survival of gefitinib compared with docetaxel (HR 1.02, 96% confidence interval [CI] 0.91–1.15; noninferiority margin 1.154).[11] A similar phase III study, V-15-32, compared gefitinib with docetaxel in 489 patients in Japan with advanced NSCLC who had failed one or two chemotherapy regimens.[12] Noninferiority in overall survival (OS) was not proven (HR 1.12, 95.24% CI 0.89–1.40; noninferiority margin 1.25), although there was no significant difference in OS ($p = 0.330$) or in progression-free survival between treatments. The objective response rate was 22.5% in the gefitinib group and 12.8% in the docetaxel group (odds ratio 2.14, 95% CI 1.21–3.78, $p = 0.009$). Gefitinib also significantly improved quality of life versus docetaxel and was associated with a lower incidence of grade 3/4 adverse events (40.6% versus 81.6%, respectively).

The aim of this post hoc landmark analysis was to assess the relationship between tumor response and OS in Japanese patients with advanced, previously treated NSCLC who received gefitinib or docetaxel in the V-15-32 study.

PATIENTS AND METHODS

The methodology and overall study results for V-15-32 (clinicaltrials.gov identifier NCT00252707) have been reported in full previously.[12] Tumor responses were assessed by RECIST[13] at baseline, every 4 weeks for the first 24 weeks, and every 8 weeks thereafter.

Two types of objective tumor response were assessed: visit response (CR/PR, SD, or progressive disease [PD]) at week 4 and best overall response (CR/PR, SD, or PD) up to weeks 8, 12, 16, and 20. In the analysis of best response, patients who eventually had a CR/PR but not by the time point were classified as having SD; patients who eventually had SD but not by the time point (because of missing values or being not evaluable [NE]) were classified as NE. PD included "symptomatic deterioration." For the purposes of the analyses reported here, DC was defined as CR/PR/SD where SD is defined as SD lasting ≥6 weeks after randomization, instead of ≥12 weeks used in the previous report.[12] In the analysis of visit response at week 4, CR/PR/SD were

TABLE 1. Landmark Analysis of Overall Survival by Best Response up to Weeks 8, 12, 16, and 20, and Visit Response at Week 4 (Evaluable for Response Population)

				Best Response[a]			Comparison: Survival HR (95% CI), p	
Treatment	Week	Statistics	Evaluable for Response	CR/PR	SD	PD	CR/PR vs. non-CR/PR	DC vs. non-DC
Best response analysis								
Gefitinib	8	No. of patients (%)	182 (91.0)	28 (14.0)	52 (26.0)	102 (51.0)	0.55 (0.31–0.96)	0.30 (0.20–0.45)
		No. of deaths	119	14	23	82	0.034	<0.001
	12	No. of patients (%)	173 (86.5)	40 (20.0)	42 (21.0)	91 (45.5)	0.44 (0.26–0.73)	0.32 (0.22–0.48)
		No. of deaths	109	17	20	72	0.002	<0.001
	16	No. of patients (%)	160 (80.0)	42 (21.0)	40 (20.0)	78 (39.0)	0.47 (0.28–0.80)	0.36 (0.23–0.54)
		No. of deaths	95	17	19	59	0.005	<0.001
	20	No. of patients (%)	151 (75.5)	45 (22.5)	37 (18.5)	69 (34.5)	0.46 (0.27–0.78)	0.40 (0.26–0.62)
		No. of deaths	86	17	19	50	0.004	<0.001
Docetaxel	8	No. of patients (%)	173 (92.5)	8 (4.3)	71 (38.0)	94 (50.3)	0.64 (0.24–1.75)	0.30 (0.20–0.45)
		No. of deaths	107	4	29	74	0.386	<0.001
	12	No. of patients (%)	170 (90.9)	18 (9.6)	64 (34.2)	88 (47.1)	0.35 (0.15–0.79)	0.33 (0.22–0.50)
		No. of deaths	104	6	30	68	0.012	<0.001
	16	No. of patients (%)	160 (85.6)	21 (11.2)	61 (32.6)	78 (41.7)	0.49 (0.25–0.98)	0.37 (0.25–0.57)
		No. of deaths	94	9	27	58	0.044	<0.001
	20	No. of patients (%)	150 (80.2)	24 (12.8)	57 (30.5)	69 (36.9)	0.54 (0.28–1.05)	0.41 (0.27–0.63)
		No. of deaths	84	10	25	49	0.070	<0.001
Visit response analysis								
Gefitinib	4	No. of patients (%)	172 (86.0)	33 (16.5)	83 (41.5)	56 (28.0)	0.44 (0.26–0.76)	0.33 (0.23–0.49)
		No. of deaths	114	15	52	47	0.003	<0.001
Docetaxel	4	No. of patients (%)	156 (83.4)	11 (5.9)	105 (56.1)	40 (21.4)	0.85 (0.37–1.94)	0.30 (0.19–0.47)
		No. of deaths	92	6	54	32	0.695	<0.001

HR <1 implies a lower risk of death for those patients with CR/PR vs. non-CR/PR or DC vs. non-DC.
[a]Visit response for week 4.
CI, confidence interval; CR, complete response; DC, disease control (CR, PR, or SD); HR, hazard ratio; PD, progressive disease; PR, partial response; SD, stable disease.

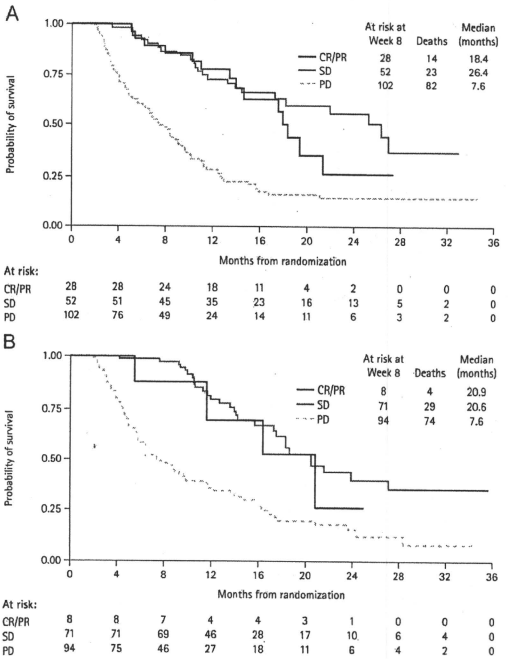


FIGURE 1. Overall survival by best response up to week 8 after treatment with (*A*) gefitinib or (*B*) docetaxel (evaluable for response population).

CR, complete response; PD, progresssive disease; PR, partial response; SD, stable disease

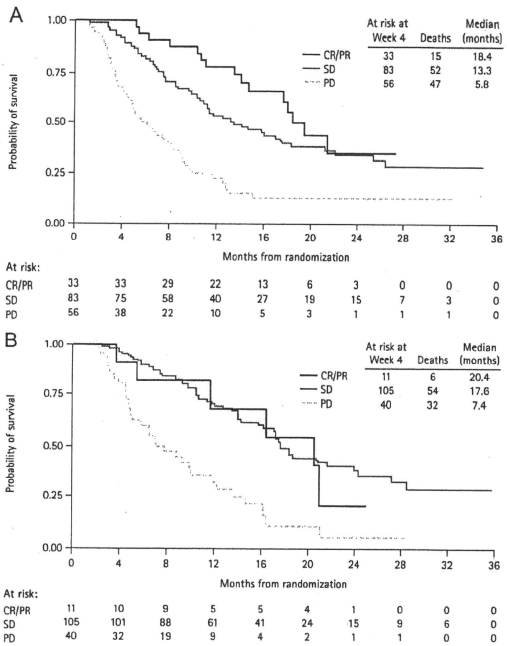
defined according to RECIST (tumor shrinkage) without considering the duration of each status. In the analysis of visit response at week 4, if a patient was judged as "symptomatic deterioration" before the time window of week 4 defined in the protocol (25–31 days), it was assumed to occur at week 4.

Landmark analysis is a valid method of comparing survival by response category[14] and determines each patient's response at a fixed time point, with survival estimates calculated from that time point and associated statistical tests being conditional on patients' landmark responses. Patients who die before the landmark time point are excluded from the analysis. In this study, landmark analysis was performed to assess the associations of best response and visit response outcomes with OS in the evaluable for response population (defined as patients with ≥1 measurable lesion at baseline by RECIST), excluding NE patients. Separate Cox regression models for each treatment group and time point in patients in the evaluable for response population who were alive at the response assessment time point were used to determine HRs for comparing response groups (CR/PR versus non-CR/PR, DC versus non-DC).

RESULTS

The evaluable for response population included 200 patients randomized to gefitinib and 187 patients randomized to docetaxel.

At each time point (best response at weeks 8, 12, 16, and 20), and for both treatments, survival was significantly longer among patients with DC compared with non-DC (Table 1). Similarly, survival was longer among patients with CR/PR compared with non-CR/PR. However, DC was a stronger predictor of survival than CR/PR, with smaller HRs for both treatments at all time points. The HR of DC versus non-DC was smallest at week 8 than at later time points in both treatment groups (HR 0.30, 95% CI 0.20–0.45, $p < 0.001$ with both gefitinib and docetaxel). Both CR/PR and SD as best response at week 8 were associated with longer survival times compared with PD for both gefitinib and docetaxel (Figure 1), although the docetaxel data are harder to interpret because of the small number of responders. In the gefitinib group, OS was similar among patients with CR/PR and those with SD as their best response at week 8 (Figure



CR, complete response; PD, progresssive disease; PR, partial response; SD, stable disease

FIGURE 2. Overall survival by visit response at week 4 after treatment with (*A*) gefitinib or (*B*) docetaxel (evaluable for response population).

1*A*). With both treatments, survival in patients with SD by week 8 tracked closer to survival in patients with CR/PR than to those with PD. A similar pattern of results was obtained by best response at weeks 12, 16, and 20 (data not shown).

Consistent with the findings for best response at later time points, at week 4, a visit response of DC was associated with longer survival compared with non-DC for both gefitinib and docetaxel, with smaller HRs than CR/PR versus non-CR/PR (Table 1). The magnitude of effect was similar at week 4 (visit response analysis) to week 8 (best response analysis) (gefitinib HRs of 0.33 [week 4] and 0.30 [week 8]; docetaxel HRs of 0.30 [week 4] and 0.30 [week 8]). In both treatment groups, the Kaplan-Meier survival curves for patients with CR/PR and for those with SD were separated from those with PD by visit response at week 4 (Figure 2). In the gefitinib group, there was also some separation in the survival curves between the CR/PR and SD subgroups at week 4. This was difficult to assess for the docetaxel group because of the small number of responders.

Comparing visit response at week 4 to best response at week 8 shows that most patients with a best response of CR/PR at week 8 also had a visit response of CR/PR at week 4 (27 of 28 [96%] on gefitinib; 8 of 8 [100%] on docetaxel). Similarly most patients with a best response of DC at week 8 also had a visit response of DC at week 4 (75 of 80 [94%] on gefitinib, 78 of 79 [99%] on docetaxel) (Table 2). Of those patients with a visit response of DC at week 4, 75 of 116 (65%) on gefitinib and 78 of 116 (67%) on docetaxel maintained DC at week 8.

DISCUSSION

In this landmark survival analysis of data from a single randomized phase III study, we found that DC was a better predictor of OS than CR/PR at all time points in previously treated Japanese patients with advanced NSCLC. The strongest predictor of OS for both gefitinib and docetaxel was DC as best response at week 8. Once a patient has PD, survival outcome is predicted to be markedly poorer than for patients without PD at that time. In addition, DC and CR/PR at the week 4 visit were also early predictors of survival for both gefitinib and docetaxel. Consistent with the later findings at week 8, DC at week 4 was a stronger predictor of survival than CR/PR. The strength of the predictive value of DC for

TABLE 2. Relationship Between Visit Response at Week 4 and Best Response at Week 8 (Evaluable for Response Population)

	Best Response at Week 8					
Treatment	CR/PR	SD	PD	NE	Dead	Total
Gefitinib						
Visit response at week 4						
CR/PR	27	6	0	0	0	33
SD	0	42	39	1	1	83
PD	0	0	53	0	3	56
NE	1	4	10	2	9	26
Dead	0	0	0	0	2	2
Total	28	52	102	3	15	200
Docetaxel						
Visit response at week 4						
CR/PR	8	1	1	1	0	11
SD	0	69	34	2	0	105
PD	0	0	40	0	0	40
NE	0	1	19	5	5	30
Dead	0	0	0	0	1	1
Total	8	71	94	8	6	187

CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease; NE, not evaluable.

survival was similar at weeks 4 and 8, suggesting that the visit response at week 4 may potentially be used as an early predictor of survival.

These results are consistent with those of Lara et al.[8] and Yamamoto et al.,[9] supporting the concept that week 8 is a landmark time point for advanced NSCLC patients for systemic therapy regardless of line of therapy. Lara et al.[8] found no substantial new findings from analyses at later time points (weeks 14 and 20), compared with week 8 of therapy. However, although in the current analysis visit response at week 4 was a predictor of survival, as well as best response at week 8, during second- or third-line treatment with either docetaxel or gefitinib, in our previous analysis of other studies employing first-line chemotherapy or pemetrexed as salvage therapy, week 4 was not a landmark time point, with no significant associations found between response and survival.[9]

Lara et al.[8] also found that DC at week 8 was a better predictor of survival than CR/PR after platinum-based chemotherapy in the first-line setting using either Southwest Oncology Group (modified World Health Organization) tumor response criteria or RECIST to define response.[8] In our earlier landmark analysis, tumor shrinkage rate (defined as a decrease or no change in tumor size) at week 8 was significantly associated with longer survival after first-line platinum-based chemotherapy or after salvage therapy with pemetrexed.[9] It has been suggested that because of the mechanism of action, it might be more important to achieve SD with EGFR tyrosine kinase inhibitors such as gefitinib than with cytotoxic chemotherapy[5]; however, the relationship between DC and survival seemed similar for docetaxel and gefitinib in the current analyses.

EGFR mutation is predictive of outcome to gefitinib[15–17] and it would have been interesting to have looked at the impact of EGFR mutation on our analysis. However, in this study, data on EGFR mutation were limited due to the fact that informed consent for tissue collection was obtained at a very late stage of the study and consequently was obtained from patients with relatively longer survival. Thus, biomarker analyses were not performed because analyzing these samples would have introduced considerable bias.

These data suggest that DC could give an early indication of OS time; whether this is a true surrogate for OS benefit is yet to be determined. If proven, DC could be used as a predictor in early phase development and as a possible end point in phase II trials. There are also implications for medical practice, raising the importance of continuing to treat patients who achieve SD because of the potential survival time; patients who achieve SD have a survival outcome similar to those patients with a CR/PR, suggesting that they should remain on the same treatment rather than switch to an alternative therapy because they are considered not to have responded to treatment. Further validation of DC as a predictor or surrogate of survival benefit in a prospective study is required.

In conclusion, our data suggest that DC is a stronger predictor of subsequent survival than the traditional CR/PR in patients receiving second- or third-line gefitinib or docetaxel for advanced NSCLC, as previously shown for patients who received first-line platinum-based chemotherapy. These results suggest that patients with DC should remain on their current treatment rather than switching. The strongest predictor of OS for both gefitinib and docetaxel was DC as best response at week 8.

ACKNOWLEDGMENTS

The authors thank Annette Smith, PhD, from Complete Medical Communications, who provided medical writing support funded by AstraZeneca.

REFERENCES

1. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. *J Clin Oncol* 2000;18:2095–2103.
2. Fossella FV, Devore R, Kerr RN, et al. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-Small Cell Lung Cancer Study Group. *J Clin Oncol* 2000;18:2354–2362.
3. Hanna N, Shepherd FA, Fossella FV, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *J Clin Oncol* 2004;22:1589–1597.
4. Shepherd FA, Rodrigues PJ, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005;353:123–132.
5. Kelly K. The benefits of achieving stable disease in advanced lung cancer. *Oncology (Williston Park)* 2003;17:957–963.
6. Kurata T, Matsuo K, Takada M, et al. Is the importance of achieving stable disease different between epidermal growth factor receptor tyrosine kinase inhibitors and cytotoxic agents in the second-line setting for advanced non-small cell lung cancer? *J Thorac Oncol* 2006;1:684–691.
7. Therasse P. Measuring the clinical response. What does it mean? *Eur J Cancer* 2002;38:1817–1823.
8. Lara PN Jr, Redman MW, Kelly K, et al. Disease control rate at 8 weeks predicts clinical benefit in advanced non-small-cell lung cancer: results from Southwest Oncology Group randomized trials. *J Clin Oncol* 2008; 26:463–467.
9. Yamamoto N, Nambu Y, Fujimoto T, et al. A Landmark Point Analysis with Cytotoxic Agents for Advanced NSCLC. *J Thorac Oncol* 2009;4: 697–701.
10. Tsujino K, Kawaguchi T, Kubo A, et al. Response rate is associated with prolonged survival in patients with advanced non-small cell lung cancer treated with gefitinib or erlotinib. *J Thorac Oncol* 2009;4:994–1001.
11. Kim ES, Hirsch V, Mok T, et al. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer: a randomised phase III trial (INTEREST). *Lancet* 2008;372:1809–1818.
12. Maruyama R, Nishiwaki Y, Tamura T, et al. Phase III study, V-15-32, of gefitinib versus docetaxel in previously treated Japanese patients with non-small-cell lung cancer. *J Clin Oncol* 2008;26:4244–4252.
13. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000;92:205–216.
14. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response and other comparisons of time-to-event by outcome variables. *J Clin Oncol* 2008;26:3913–3915.
15. Morita S, Okamoto I, Kobayashi K, et al. Combined survival analysis of prospective clinical trials of gefitinib for non-small cell lung cancer with EGFR mutations. *Clin Cancer Res* 2009;15:4493–4498.
16. Sequist LV, Martins RG, Spigel D, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. *J Clin Oncol* 2008;26:2442–2449.
17. Mok TS, Wu Y-L, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 2009;361:947–957.

Cancer Science
The official journal of the Japanese Cancer Association

Bortezomib potentially inhibits cellular growth of vascular endothelial cells through suppression of G2/M transition

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(Received January 13, 2010/Revised February 12, 2010/Accepted February 21, 2010/Accepted manuscript online February 27, 2010/Article first published online March 29, 2010)

Bortezomib, a selective 26S proteasome inhibitor, has shown clinical benefits against refractory multiple myeloma. The indirect anti-angiogenic activity of bortezomib has been widely recognized; however, the growth-inhibitory mechanism of bortezomib on vascular endothelial cells remains unclear, especially on the cell cycle. Here, we showed that bortezomib (2 nM of the IC_{50} value) potently inhibited the cellular growth of human umbilical vascular endothelial cells (HUVECs) via a vascular endothelial growth factor receptor (VEGFR)-independent mechanism resulting in the induction of apoptosis. Bortezomib significantly increased the vascular permeability of HUVECs, whereas a VEGFR-2 tyrosine kinase inhibitor decreased it. Interestingly, a cell cycle analysis using flow cytometry, the immunostaining of phospho-histone H3, and Giemsa staining revealed that bortezomib suppressed the G2/M transition of HUVECs, whereas the mitotic inhibitor paclitaxel induced M-phase accumulation. A further analysis of cell cycle-related proteins revealed that bortezomib increased the expression levels of cyclin B1, the cdc2/cyclin B complex, and the phosphorylation of all T14, Y15, and T161 residues on cdc2. Bortezomib also increased the ubiquitination of cyclin B1 and wee1, but inhibited the kinase activity of the cdc2/cyclin B complex. These protein modifications support the concept that bortezomib suppresses the G2/M transition, rather than causing M-phase arrest. In conclusion, we demonstrated that bortezomib potently inhibits cell growth by suppressing the G2/M transition, modifying G2/M-phase-related cycle regulators, and increasing the vascular permeability of vascular endothelial cells. Our findings reveal a cell cycle-related mode of action and strongly suggest that bortezomib exerts an additional unique vascular disrupting effect as a vascular targeting drug. (*Cancer Sci* 2010; 101: 1403–1408)

The proteasome is an essential enzyme complex for nonlysosomal and ATP-dependent proteolytic pathways. The ubiquitin–proteasome pathway plays an important role in the intracellular degradation of damaged, oxidized, or misfolded proteins[1–4] as well as in the cell cycle progression. Such damaged, oxidized, or misfolded proteins have been identified as substrates for the ubiquitin/proteasome system.[1,5–7] In addition, this system has been implicated in the regulation of cell proliferation, differentiation, survival, apoptosis, and angiogenesis.[8,9] Because of these unique effects of the proteasome/ubiquitin system on cellular regulation, the proteasome is a novel and promising target for cancer therapy.[10–12]

Bortezomib (Velcade, PS-341), a selective 26S proteasome inhibitor, demonstrates potent antitumor activity against several human cancers and has been clinically used mainly in patients with refractory multiple myeloma.[13–15] The main mechanism of action of this drug was initially thought to be the inhibition of nuclear factor-κB (NF-κB), which acts as a transcription factor for anti-apoptotic proteins, such as Bcl-2, c-IAP2, and survivin. Accumulating data indicates that bortezomib disrupts the cell cycle by modifying cyclins and inhibits the up-regulation of interleukin-6 (IL-6), which plays an important role in the proliferation of myeloma cells, by inhibiting NF-κB and stabilizing p53, p21, and p27, resulting in its anticancer activity.[1,16–18]

Bortezomib exerts an anti-angiogenic effect by decreasing the secretion of vascular endothelial growth factor (VEGF) from myeloma cells.[19,20] This anti-angiogenic effect of bortezomib is considered an indirect effect on vascular endothelial cells resulting from ligand depletion. Meanwhile, direct negative proliferative effects of bortezomib on vascular endothelial cells have emerged which play an important role in its anti-angiogenic activity. Roccaro *et al.*[9] reported that bortezomib induces inhibition of angiogenesis in functional assays of angiogenesis, including chemotaxis, adhesion to fibronectin, capillary formation on Matrigel, and chick embryo chorioallantoic membrane assay using multiple myeloma patient-derived endothelial cells and human umbilical vein endothelial cells (HUVECs). Podar *et al.*[21] reported that Caveolin-1 is a molecular target of bortezomib in multiple myeloma cells and HUVECs and this is required for VEGF-triggered multiple myeloma. However the underlying mechanism responsible for the direct negative proliferative effect of bortezomib on vascular endothelial cells remains unclear, especially with regard to its effect on the cell cycle.

To gain insight into the direct anti-angiogenic effects of bortezomib on HUVECs, we examined cellular proliferation, tube formation, VEGF receptor-2 (VEGFR-2) signaling, the apoptotic pathway, vascular permeability, cell cycle analysis, and effects of drugs on cell cycle-related proteins.

Materials and Methods

Anticancer agents. Bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). The VEGFR-2 tyrosine kinase inhibitor (VEGFR-2-TKI) Ki8751 (IC_{50} value for VEGFR-2 kinase inhibition = 0.90 nM) was purchased from Sigma (St. Louis, MO, USA). Paclitaxel was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each chemical agent was dissolved in dimethylsulfoxide for use in the *in vitro* experiments.

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doi: 10.1111/j.1349-7006.2010.01544.x

Cell cultures. HUVECs were maintained in Humedia-EG2 (Kurabo, Tokyo, Japan) with 2% fetal bovine serum and 0.1% gentamicin-amphotericinB with the addition of 10 ng/mL of epidermal growth factor, 5 ng/mL of fibroblast growth factor, and 2 ng/mL of VEGF (R&D Systems, Minneapolis, MN, USA). All the cell lines were incubated at 37°C with humidified 5% CO_2.

***In vitro* growth inhibition assay.** Growth inhibition was evaluated using the MTT assay, as described previously.[22] The experiment was performed in triplicate.

Western blotting. The antibodies used for western blotting were anti-phospho-VEGFR-2 (Tyr1175), anti-VEGFR-2, anti-MAPK, anti-phospho-MAPK, anti-β-actin, anti-cleaved or non-cleaved-caspase3, anti-cleaved or non-cleaved-poly ADP-ribose polymerase (PARP), anti-cyclin B1, anti-phospho-cdc2, anti-cdc2, anti-phospho-wee1, anti-wee1, anti-phospho-cdc25C, anti-cdc25C, anti-phospho-chk1 and -2, and anti-chk1 and -2 (Cell Signaling, Beverly, MA, USA). HUVECs were cultured overnight in serum-starved medium and then exposed to the indicated concentrations of bortezomib or Ki8751 for 3 h before the addition of 10 ng/mL of VEGF for 5 min. The western blot analysis was performed as described previously.[23] The experiment was performed in duplicate.

Immunoprecipitation. Total cell lysates were immunoprecipitated with anti-wee1, cdc2 antibodies (Cell Signaling), or anti-cyclin B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The protein complex was incubated with protein G-agarose (Invitrogen, San Diego, CA, USA) for 1 h at 4°C and washed three times with lysis buffer. After sequential centrifugation and washing, the pellets were resuspended in 1.5 × sample loading buffer and subjected to immunoblot analyses.

Cell cycle analysis. Cells were treated with the indicated concentrations of bortezomib for 24 h. The cells were then harvested, washed with PBS, fixed with 70% ethanol at −20°C overnight, washed again with PBS, and then stained with 5 μg/mL of propidium iodide containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase I (BD Bioscience, San Jose, CA, USA). The stained cells were then analyzed for DNA content using a FACS Calibur flow cytometer (BD Biosciences) and the cell cycle distributions were calculated using ModFit LT software. The experiment was performed in triplicate.

Giemsa staining. Morphological changes in mitotic cells were evaluated using Giemsa staining. HUVECs treated with bortezomib (1 μM) or paclitaxel (1 μM) for 24 h were fixed with 10% neutral-buffered formaldehyde before staining and were stained for 30 min, then washed with tap water for 5 min. The morphological changes were evaluated using a light microscope (×40).

Immunofluorescence staining of phospho-histone H3. HUVECs were treated with 1 μM of bortezomib or paclitaxel for 24 h and were then fixed and permeabilized with 4% formaldehyde/PBS for 15 min. The cells were blocked with 5% normal goat serum in PBS for 60 min. After washing, anti-phospho-histone H3 antibody (Cell Signaling) was diluted 1:200 in PBS/Triton and incubated for 1 h at room temperature, followed by detection using Alexa Fluor 594 goat antirabbit IgG antibody (Invitrogen) for 1 h. After washing, the cells were counterstained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min. Images were obtained using fluorescence microscopy (IX71; Olympus, Tokyo, Japan). The mitotic index was calculated by dividing the number of p-Histone H3-positive cells by the total number of treated cells (DAPI-positive cells). At least 100 cells were scored per low-power field, and the cells were counted over three fields. The experiment was performed in triplicate.

cdc2/cyclinB1 kinase assay. The cdc2/cyclinB1 kinase activity in the cells was quantified using a Cyclex Cdc2-CylinB Kinase Assay Kit (Cyclex, Nagano, Japan) according to the manufacturer's instructions. The experiment was performed in triplicate.

***In vitro* permeability assay.** Transwell permeability assays were performed using monolayers of HUVECs and an *in vitro* vascular permeability assay kit (Chemicon, Temecula, CA, USA). Briefly, HUVECs seeded onto collagen-coated inserts were pretreated with or without bortezomib (1, 0.1 μM) or VEGFR-2-TKI (1 μM) for 6 h, and VEGF (20 ng/mL) was added, except in the control sample, 4 h thereafter. Two hours after the addition of VEGF, fluorescein isothiocyanate dextran (FITC dextran) was added on the top of the cells and the extent of FITC dextran permeation was determined by measuring the fluorescence of the plate well solution, according to the supplier's instructions. The experiment was performed in triplicate.

Results

Bortezomib potentially inhibited the cellular growth of HUVECs independent of VEGF signaling. To evaluate the growth inhibitory activity of bortezomib *in vitro*, we performed MTT assays on HUVECs under the 20 ng/mL of VEGF or without it. Bortezomib exhibited a potent growth inhibitory activity on HUVECs with an IC_{50} of 2 nM; however, VEGF stimulation did not influence the growth inhibitory activity of bortezomib (Fig. 1a).

To address the question whether the growth inhibitory activity of bortezomib involves VEGFR-2 signaling, we compared the inhibitory effects of bortezomib with that of a VEGFR-2-TKI, Ki8751, on the phosphorylation levels of VEGFR and MAPK. Bortezomib did not inhibit the phosphorylation level of VEGFR-2, whereas Ki8751 (0.01–1 μM) completely inhibited VEGFR-2 phosphorylation (Fig. 1b). Similar results were observed for MAPK phosphorylation. These results indicate that the growth inhibitory activity of bortezomib is induced via a VEGFR-2 signaling-independent mechanism.

Bortezomib increases vascular permeability *in vitro*. Generally, the characteristics of vascular disrupting agents include a potent anti-proliferative effect. Microtubule-binding drugs (MBD) are widely used in cancer chemotherapy and also have clinically relevant vascular-disrupting properties. The disruption of adherens junctions contributes to the rounding of endothelial cells, leading to a direct increase in vasculature permeability.[24] Therefore, we examined the effect of bortezomib on vasculature permeability to gain an insight into its vascular-disrupting properties. As expected, Ki8751 significantly decreased vasculature permeability during VEGF stimulation, in contrast to the situation in untreated controls. On the other hand, bortezomib significantly increased the vasculature permeability of vasculature endothelial cells in a dose-dependent manner (Fig. 1c). This result supports the hypothesis that bortezomib has vascular-disrupting properties in HUVECs in addition to its potent growth inhibitory effect.

Bortezomib induces apoptosis of HUVECs. We speculated that the potent growth inhibitory activity of bortezomib was based on the induction of apoptosis; thus, we evaluated the expression levels of cleaved caspase 3, cleaved PARP, and ubiquitinated protein from whole cell lysates. The expression levels of cleaved caspase 3 and PARP showed that bortezomib induced the activation of caspase 3 at a dose of 0.1 μM and subsequent PARP cleavage in HUVECs in a dose- and time-dependent manner (Fig. 2). The accumulation of ubiquitinated proteins, which represents a direct effect of bortezomib, was observed at 0.01 μM in a time-dependent manner. These findings indicate that bortezomib is capable of inducing the apoptosis of HUVECs at a relatively low concentration.

Bortezomib inhibits G2/M transition. An analysis of the cell cycle distribution of HUVECs revealed that bortezomib significantly increased the population of cells in the G2/M phase

doi: 10.1111/j.1349-7006.2010.01544.x

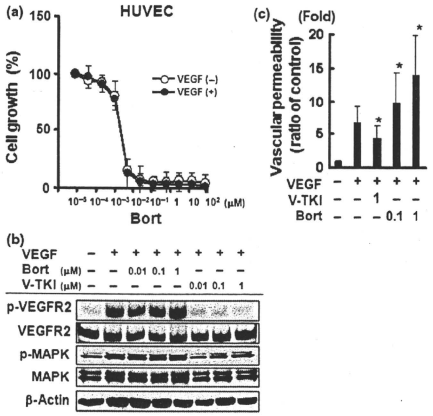

Fig. 1. Bortezomib potently inhibited the cellular growth and increased the vascular permeability of HUVECs. (a) *In vitro* growth-inhibitory effect of bortezomib on HUVECs using an MTT assay with 10 ng/mL vascular endothelial growth factor (VEGF) or without it. The data shown represents the average ± SD of three independent experiments. (b) Effects of bortezomib on VEGF signaling in HUVECs. Western blot analysis was performed for the expression and phosphorylation levels of VEGF receptor-2 (VEGFR-2) and MAPK. HUVECs were cultured under serum-starved conditions and exposed to bortezomib or Ki8751 at the indicated concentrations for 3 h. After 10 ng/mL VEGF stimulation for 5 min, the cells were analyzed. (c) Effect of bortezomib on vascular permeability *in vitro*. HUVECs were seeded onto collagen-coated inserts and were pretreated with or without bortezomib (0.1 and 1 μM) or VEGFR-2 tyrosine kinase inhibitor (VEGFR-2-TKI) (1 μM) for 6 h. After 20 ng/mL of VEGF stimulation for 2 h, fluorescein isothiocyanate dextran (FITC dextran) was added on the top of the inserts and the extent of FITC dextran permeation was determined by measuring the fluorescence of the plate well solution. The relative vascular permeability was calculated using the ratio to the permeability in the control cells (untreated). The data shown represents the average ± SD of three independent experiments. *$P < 0.05$. Bort, bortezomib; V-TKI, VEGFR-2-TKI.

(Fig. 3a). This effect was observed when the cells were exposed to 0.01 μM of bortezomib. Generally, morphological changes, including the disappearance of the nuclear membrane, chromosomal condensation, and cytoplasmic round formation, are observed in mitotic cells. Therefore, we evaluated whether bortezomib induced morphological changes in HUVECs specific to mitotic cells. Paclitaxel, a well-known tubulin binder and mitotic inhibitor, was used as a control. Paclitaxel clearly induced these morphological changes specific to mitotic cells; however, bortezomib did not induce these changes with Giemsa staining (Fig. 3b). Further analysis using phospho-histone H3 immunostaining, an M-phase-specific marker, demonstrated that bortezomib significantly decreased the number of mitotic cells while paclitaxel markedly increased it (Fig. 3c,d). Together, these results indicated that both bortezomib and paclitaxel induced cell cycle arrest at the G2/M phase; however, bortezomib did not increase the number of mitotic cells unlike paclitaxel. These results suggest that bortezomib inhibits the G2/M transition in HUVECs.

Bortezomib decreases cdc2/cyclinB kinase activity. Cell cycle progression at the G2/M transition is regulated by cdc2/cyclin B complex activity, and the activation of this complex is controlled as a consecutive process as follows: (i) the levels of cyclin B protein are increased during late S and G2 phases; (ii) cyclin B binds to unphosphorylated cdc2 and forms an inactive cdc2/cyclinB complex; (iii) cdc2 is phosphorylated at its T14, Y15, and T161 residues during the G2 phase; and (iv) the dephosphorylation of T14 and Y15 on cdc2 by phosphatase cdc25 activates the cdc2/cyclin B complex and introduces the cells to mitosis.

Bortezomib increased the expression of cyclin B1 in a dose- and time-dependent manner, and an immunoprecipitation analysis showed that bortezomib also increased the production of cdc2/cyclin B complexes (Fig. 4a). Bortezomib markedly increased the phosphorylation status of the T14, Y15, and T161 residues on cdc2 in a dose- and time-dependent manner, suggesting that bortezomib promoted the presence of the inactive form of the cdc2/cyclin B complex (Fig. 4b). These results showed that bortezomib inhibits the G2/M transition. In addition, we examined the effects on a competing kinase, wee1, and the phosphatase cdc25C. Increased expression and phosphorylation levels of wee1 were observed after bortezomib treatment, whereas no remarkable changes in cdc25C expression or phosphorylation were observed (Fig. 4b). Regarding the effects of bortezomib on the proteasome–ubiquitin pathway, we found that the ubiquitination of wee1 and cyclin B protein was increased by bortezomib in a dose-dependent manner, suggesting that the increase in the ubiquitination of wee1 and cyclin B may be at least partially involved in the suppression of the G2/M transition and the mode of action of this drug (Fig. 5a). Finally, a kinase assay of the cdc2/cyclin B complex showed that bortezomib (0.01 μM) significantly inhibited the kinase activity of the complex, indicating that the inhibition of kinase activity might suppress the G2/M transition (Fig. 5b).

Together, these results revealed that bortezomib increases the expression levels of cyclin B1, the formation of the cdc2/cyclin

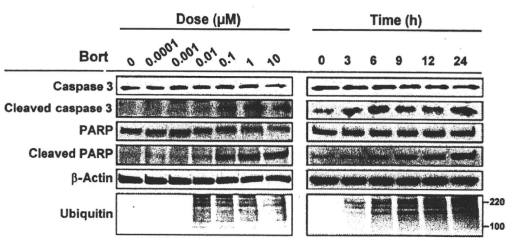

Fig. 2. Bortezomib induces apoptosis of HUVECs. Western blot analysis was performed for the cleaved form and the expression levels of caspase 3, poly ADP-ribose polymerase (PARP), and whole ubiquitinated-protein. HUVECs were treated with bortezomib at the indicated concentrations for 24 h and analyzed (left panel), or they were treated with bortezomib at 0.1 μM for the indicated hours (right panel). Protein size markers are shown at 100 and 220 kDa.

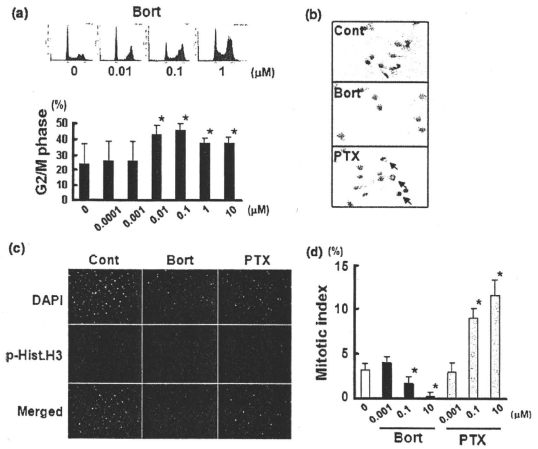

Fig. 3. Bortezomib suppress the G2/M transition. (a) DNA histogram of HUVECs treated with bortezomib. HUVECs were treated with bortezomib at the indicated concentrations. The upper panel represents the result of a flow cytometry analysis, and the lower panel shows the population at the G2/M phase. $*P < 0.05$. (b) Giemsa staining of HUVECs treated with bortezomib or paclitaxel at 1 μM. The arrows indicate cells with mitotic changes (disappearance of the nuclear membrane, chromosomal condensation, and cytoplasmic round formation). (c) Immunostaining for phospho-histone H3 (p-Hist.H3) and 4′,6-diamidino-2-phenylindole (DAPI) observed with fluorescence microscopy. p-Hist.H3 was used as an M-phase-specific molecular marker. Note that both bortezomib and paclitaxel induced cell cycle arrest at the G2/M phase, but unlike paclitaxel, bortezomib did not increase the number of cells in the M phase. (d) Mitotic index after treatment with bortezomib or paclitaxel at the indicated concentrations in HUVECs. The mitotic index was calculated using the number of p.Hist.H3-positive cells per the total number of cells (DAPI-positive cells). The columns indicate the average ± SD of three independent experiments. $*P < 0.05$. Bort, bortezomib; Cont, untreated control; PTX, paclitaxel.

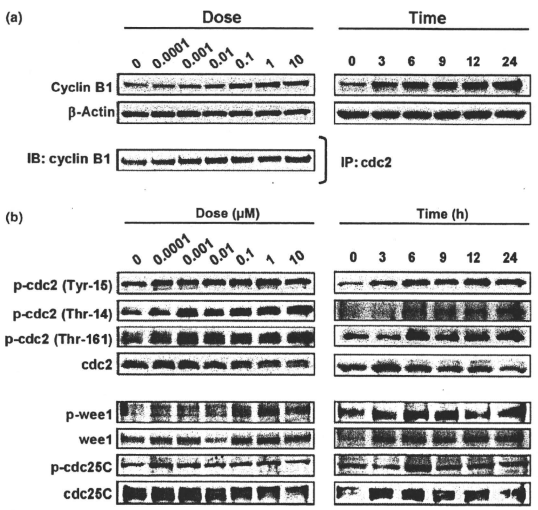

Fig. 4. Bortezomib increases the expression levels of cyclin B1, the production of the cdc2/cyclin B complex, and the phosphorylation of the T14, Y15, and T161 residues on cdc2 in HUVECs. (a) Western blots of the cyclin B1 expression levels in whole protein (upper panel) and samples immunoprecipitated with a cdc2 antibody (lower panel). (b) Western blots for G2/M-phase-related cell cycle regulators. HUVECs were treated with bortezomib at the indicated concentrations for 24 h and for the indicated hours at 0.1 μM. IB, immunoblots; IP, immunoprecipitation.

B complex, the phosphorylation of T14, Y15 and T161 residues on cdc2, and the ubiquitination of cyclin B1 and wee1. Bortezomib also significantly inhibited the kinase activity of cdc2/cyclin B. These modifications of G2/M-phase-related cell cycle regulators suggest that bortezomib suppresses the G2/M transition (Fig. 5c). We concluded that bortezomib potently inhibits cell growth of vascular endothelial cells by suppressing the G2/M transition through modifying G2/M-phase-related cycle regulators.

Discussion

Inhibition of the 26S proteasome results in the accumulation of cyclins A, B, D, E, p21, and p27, thereby disrupting the cell

doi: 10.1111/j.1349-7006.2010.01544.x

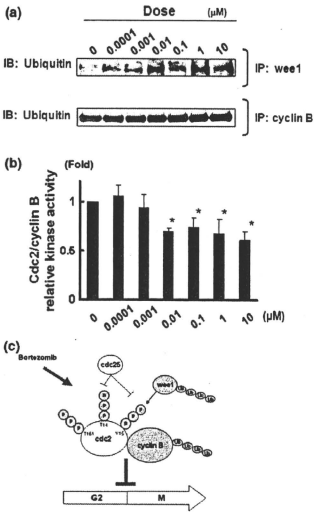

Fig. 5. Bortezomib increases the ubiquitination of cyclin B1 and wee1 and inhibits the kinase activity of cdc2/cyclin B. (a) Western blots for the ubiquitination levels in the samples immunoprecipitated with wee1 antibody (lower panel) or cyclin B1 antibody (lower panel). (b) Kinase activity of cdc2/cyclin B in HUVECs treated with bortezomib at the indicated concentrations. Whole cell lysates were used for the analysis. The relative kinase activity was calculated using the ratio to the activity level in the control (untreated). The data shown represents the average ± SD of three independent experiments. $*P < 0.05$. (c) Schematic diagram of the effects of bortezomib on G2/M-phase cell cycle progression in vascular endothelial cells. Bortezomib increases the expression levels of cyclin B1, the production of the cdc2/cyclin B complex, and the phosphorylation of the T14, Y15, and T161 residues on cdc2. Bortezomib also increases the ubiquitination of cyclin B1 and wee1. Changes in the expression or phosphorylation levels of cdc2 were not detected. These modifications of G2/M-phase-related cell cycle regulators suggest that bortezomib suppresses the G2/M transition.

cycle and promoting cell death via multiple pathways.[25] In cancer cells, bortezomib leads to an increase in the accumulation and activation of G2/M-phase-related cycle regulators cyclin A and cyclin B1, and also leads to cell cycle blockade at the G2/M phase.[1] However, whether bortezomib inhibits G2/M transition or induces M-phase arrest has been uncertain. In addition, no data on the effects of bortezomib on the cell cycle arrest at the G2 phase in vascular endothelial cells has been available. Our data showed that bortezomib caused cell cycle arrest at the G2 phase, not at the M phase, using Giemsa staining and immunofluorescence staining of the phospho-histone H3 in HUVECs (Fig. 3). Because bortezomib inhibits the G2/M transition, we focused on changes in G2/M-phase-related cell cycle regulators, such as cyclin B and cdc2. We found that bortezomib increased the expression levels of cyclin B, ubiquitinated cyclin B, and the cyclin B/cdc2 complex in dose-and time-dependent manners (Figs 4 and 5a). Further analysis revealed that the phosphorylation statuses of the T14, Y15, and T161 residues on cdc2 were markedly increased, indicating the presence of the inactive form of cdc2 that occurs during G2 arrest; the kinase activity of the cyclin B/cdc2 complex was also inhibited by this treatment (Figs 4b,5b). These data indicate that bortezomib inhibits the G2/M transition, rather than causing M-phase arrest. Since few anticancer drugs are known to suppress the G2/M transition, our results provide an insight to the unique mode of action of bortezomib. The expression, ubiquitination, and phosphorylation of wee1 were markedly increased after bortezomib treatment. Wee1 degrades via the proteasome–ubiquitin pathway, similar to cyclin B, and activated wee1 inhibits cdc2 kinase activity. Therefore, these results raise the possibility that wee1 is involved in the mode of action of bortezomib.

Vascular targeting agents (VTAs) including VEGFR-TKIs target the development of new vessels and have a preventative action, require chronic administration, and are likely to be of particular benefit in early stage or asymptomatic metastatic disease. Meanwhile, vascular disrupting agents (VDAs) target established tumor blood vessels,[26] causing a rapid collapse in tumor blood flow leading to a prolonged period of vascular shutdown and culminating in the extensive necrosis of tumor cells.[27] VDAs are therefore given acutely, show immediate effects, and may have particular efficacy against advanced disease. Thus, VDAs are considered to be different from VTAs in some key aspects including the type or extent of disease which has sensitivity to the agents and the treatment scheduling.[26]

Generally, the characteristics of VDAs include a potent antiproliferative effect, the induction of G2/M-phase arrest, and an increase in vascular permeability.[28] Our results indicate that bortezomib exerts similar effects on vascular endothelial cells. Thus, we speculate that bortezomib could be categorized as a VDA and that the vascular disrupting effect of bortezomib might be at least partly responsible for its antitumor activity.

In conclusion, we demonstrated that bortezomib potently inhibits cellular growth by suppressing the G2/M transition in vascular endothelial cells. Our findings strongly suggest that bortezomib has a unique additional vascular disrupting effect.

Acknowledgments

We thank Mr Shinji Kurashimo, Mr Yoshihiro Mine, and Ms Tomoko Kitayama for their technical assistance. This work was supported in part by the Third-Term Comprehensive 10-Year Strategy for Cancer Control, the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio), funds for Health and Labor Scientific Research Grants, and a Grant-in-Aid for Scientific Research (A).

References

1 Ling YH, Liebes L, Jiang JD *et al.* Mechanisms of proteasome inhibitor PS-341-induced G(2)-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines. *Clin Cancer Res* 2003; **9**: 1145–54.
2 Hochstrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr Opin Cell Biol* 1995; **7**: 215–23.
3 Jentsch S, Schlenker S. Selective protein degradation: a journey's end within the proteasome. *Cell* 1995; **82**: 881–4.
4 Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998; **67**: 425–79.
5 Desai SD, Liu LF, Vazquez-Abad D, D'Arpa P. Ubiquitin-dependent destruction of topoisomerase I is stimulated by the antitumor drug camptothecin. *J Biol Chem* 1997; **272**: 24159–64.
6 Ciechanover A, DiGiuseppe JA, Bercovich B *et al.* Degradation of nuclear oncoproteins by the ubiquitin system in vitro. *Proc Natl Acad Sci U S A* 1991; **88**: 139–43.

7 Treier M, Staszewski LM, Bohmann D. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell* 1994; **78**: 787–98.
8 Grimm LM, Goldberg AL, Poirier GG, Schwartz LM, Osborne BA. Proteasomes play an essential role in thymocyte apoptosis. *EMBO J* 1996; **15**: 3835–44.
9 Roccaro AM, Hideshima T, Raje N *et al.* Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. *Cancer Res* 2006; **66**: 184–91.
10 Rajkumar SV, Richardson PG, Hideshima T, Anderson KC. Proteasome inhibition as a novel therapeutic target in human cancer. *J Clin Oncol* 2005; **23**: 630–9.
11 Mani A, Gelmann EP. The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* 2005; **23**: 4776–89.
12 Genini D, Carbone GM, Catapano CV Multiple Interactions between Peroxisome Proliferators-Activated Receptors and the Ubiquitin-Proteasome System and Implications for Cancer Pathogenesis. *PPAR Res* 2008; **2008**: 195065.
13 APEX (Assessment of Proteasome inhibition for Extending remissions) trial: phase III randomized, multicenter, placebo-controlled trial to evaluate the efficacy and safety of bortezomib versus dexamethasone in patients with recurrent or treatment-resistant multiple myeloma. *Clin Adv Hematol Oncol* 2003; **1**: 190.
14 Jung L, Holle L, Dalton WS. Discovery, Development, and clinical applications of bortezomib. *Oncology (Williston Park)* 2004; **18**: 4–13.
15 Richardson PG, Sonneveld P, Schuster MW *et al.* Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med* 2005; **352**: 2487–98.
16 Pham LV, Tamayo AT, Yoshimura LC, Lo P, Ford RJ. Inhibition of constitutive NF-kappa B activation in mantle cell lymphoma B cells leads to induction of cell cycle arrest and apoptosis. *J Immunol* 2003; **171**: 88–95.
17 Hideshima T, Richardson P, Chauhan D *et al.* The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Res* 2001; **61**: 3071–6.
18 Strauss SJ, Higginbottom K, Juliger S *et al.* The proteasome inhibitor bortezomib acts independently of p53 and induces cell death via apoptosis and mitotic catastrophe in B-cell lymphoma cell lines. *Cancer Res* 2007; **67**: 2783–90.
19 Hayashi T, Hideshima T, Anderson KC. Novel therapies for multiple myeloma. *Br J Haematol* 2003; **120**: 10–7.
20 Hideshima T, Chauhan D, Podar K, Schlossman RL, Richardson P, Anderson KC. Novel therapies targeting the myeloma cell and its bone marrow microenvironment. *Semin Oncol* 2001; **28**: 607–12.
21 Podar K, Shringarpure R, Tai YT *et al.* Caveolin-1 is required for vascular endothelial growth factor-triggered multiple myeloma cell migration and is targeted by bortezomib. *Cancer Res* 2004; **64**: 7500–6.
22 Takeda M, Arao T, Yokote H *et al.* AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. *Clin Cancer Res* 2007; **13**: 3051–7.
23 Matsumoto K, Arao T, Tanaka K *et al.* mTOR signal and hypoxia-inducible factor-1 alpha regulate CD133 expression in cancer cells. *Cancer Res* 2009; **69**: 7160–4.
24 Schwartz EL. Antivascular actions of microtubule-binding drugs. *Clin Cancer Res* 2009; **15**: 2594–601.
25 Leonard JP, Furman RR, Coleman M. Proteasome inhibition with bortezomib: a new therapeutic strategy for non-Hodgkin's lymphoma. *Int J Cancer* 2006; **119**: 971–9.
26 Lippert JW III. Vascular disrupting agents. *Bioorg Med Chem* 2007; **15**: 605–15.
27 Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nat Rev Cancer* 2005; **5**: 423–35.
28 Siemann DW, Bibby MC, Dark GG *et al.* Differentiation and definition of vascular-targeted therapies. *Clin Cancer Res* 2005; **11**: 416–20.

doi: 10.1111/j.1349-7006.2010.01544.x

Performing Phase I Clinical Trials of Anticancer Agents: Perspectives from within the European Union and Japan

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Abstract

Drug discovery and early clinical development is an international endeavor, conducted in partnership between commercial entities such as biotechnology and pharmaceutical companies and academic investigators. Although once considered quite disparate, early clinical trials requirements and conduct are largely harmonized between the European Union, Japan, and the United States, increasing the opportunities for productive commercial-academic collaborations. *Clin Cancer Res; 16(6); 1737–44.* ©*2010 AACR.*

Cancer drug discovery and development is an international activity. Many of first-in-human studies of anticancer drugs are conducted in two sites in different countries, usually in North America (United States or Canada) and the other in Europe. Additional phase I studies including Japanese patients are often required for later marketing approvals in Japan. Phase I capabilities exist worldwide, including Australia, Asia, and South America. Although there are differences in attitudes between different countries in general, the concerns and approaches of phase I investigators are similar across the world. In particular there is emphasis on trial designs that minimize the number of patients treated with ineffective doses and maximize the possibility of eliciting therapeutic signals. We summarize here early clinical trial activities in Europe and Japan, highlighting key opportunities and challenges as part of this issue of *CCR Focus*, which examines the phase I clinical trial process (1).

European Union

In terms of organization, there is no overarching organization for European countries or even for one individual country. Rather, the tendency has been for potential sponsors to negotiate individually with individual centers. This has led to the focusing of many studies in relatively few centers where the expertise and the reputation for the conduct of these studies have been established. Of note, privately owned and commercially operated phase I clinical trials centers are emerging in Europe.

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doi: 10.1158/1078-0432.CCR-09-2228

Marketing approval for new drugs within the European Union is the responsibility of the European Medicines Agency, which is a decentralized body of the European Union with headquarters in London. The agency is responsible for the scientific evaluation of applications for European marketing authorization for medicinal products using a centralized procedure. Companies submit a single marketing authorization application to the agency. Once granted by the European Commission, this authorization is valid in all European Union (EU) and EEA-EFTA states (Iceland, Liechtenstein, and Norway). In contrast, approval for clinical trials of new agents is the responsibility of each member state, with each country providing its own regulatory authority.

First in human trials are clinical experiments and are therefore governed by a number of regulations and guidelines. Patients who participate are given an intervention that they would not normally receive, with an unknown efficacy and toxicity profile, and often undergo additional clinical assessments and tests. It is therefore essential to protect their safety and well being, which is the primary role of the regulatory guidelines. Adherence to these guidelines is particularly relevant in phase I studies, in which the investigational medicinal product may have been administered to few, if any, humans previously. The regulations also aim to ensure that the clinical trial data produced are robust and accurately represent the activities of the Investigational Medicinal Product (IMP).

The first internationally recognized guidelines were the Nuremberg Code, developed in 1948, following the inhumane experimentation on subjects without appropriate consent during the Second World War (2). The Nuremberg Code formed the basis for the Declaration of Helsinki, first declared by the World Health Authority in 1964, which has undergone a number of subsequent revisions (latest in 2008: ref. 3). All clinical studies need to follow its guidelines, although it is not legally binding in international law, and it has formed the backbone to the Code of Federal Regulations (title 45, volume 46) in the United States (4) and the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guidelines.

Table 1. Europe: example of documents required for a clinical trial application

Clinical Trial Application required documents
EudraCT number
IB
IMPD
IND application (in the United States) if available
Information of investigators, recruiting sites, and analysis laboratories
Details of drug manufacturing and distribution
Trial production and sample consent form
Completed application form
Information from independent ethics committee
Fee

NOTE: From the Medicines and Healthcare products Regulatory Agency (9).

International conference on harmonization

In 1996, the ICH guidelines were developed, in an attempt to harmonize the requirements for registering medicines across the European Union, the United States, and Japan and to allow data produced from one country to be accepted by another (5). The tripartite guidelines expand on the Declaration of Helsinki to advise on quality, efficacy, patient safety, and other miscellaneous aspects of clinical research. They focus on the core principles of Good Clinical Practice (GCP), which cover trial design, conduct, and analyses, with strict pharmacovigilance and thorough informed subject consent (6). Quality topics describe IMP production and evaluation to Good Manufacturing Practice (GMP) and there are extensive guidelines on preclinical safety evaluation and the training and responsibilities of trial staff. ICH GCP provides international guidelines to standardize the conduct of clinical trials, but their implementation has been variable between different researchers and countries.

EU clinical trials directive

The variability in interpretation of ICH GCP led the European Union to develop the EU Clinical Trials and GCP Directives (2001/20/EC and 2005/28/EC, respectively), implemented from 2004. The directives formed a legal framework for clinical trial research, including phase I trials, and required incorporation into the legal systems of member states.

Directive (7) includes 24 articles required to be met, covering core areas including:

- The safety and well-being of clinical trial subjects,
- The procedures for independent ethical committee review and approval,
- The procedures to give regulatory approval before a trial starts recruitment,
- The procedures for authorities to inspect trial conduct to GCP standards,
- The standards for the manufacture and handling of IMPs,
- The procedures for reporting and processing adverse events.

In common with other EU directives, the Clinical Trials Directive (CTD) provides the principles, which are interpreted and converted to law separately by each member state. The interpretation and incorporation by individual states may be influenced by other laws already in place, such as the EU Data Protection Directive (95/46/EC). In addition subsequent rulings in individual states may also affect the implementation of the directive. An example would be the Human Tissue Act introduced to the United Kingdom in 2004 for studies involving clinical samples. This act was introduced following an event in which tissues and organs of children who had died were retained without parental consent, resulting in a very high-profile scandal featured in the media. The result is a series of stringent measures to control the acquisition, storage, and experimentation on human tissues of all kinds.

Differences in the existing national legislation and differing legal concerns within the different member states of the EU mean that cost, timelines, ease of setting up, and the conduct of new studies may vary significantly between different countries.

Trial set up and approval. ICH and EU CTD advise about preclinical evaluation and regulatory toxicology assessments following which a phase I protocol can be developed. An IMP dossier is also required, outlining the quality, safety, and use of IMPs in the study. This dossier forms the EU equivalent to the Investigational New Drug (IND) application required in the United States (requirements for the latter are discussed in this issue of *CCR Focus*; ref. 8). In parallel with the Investigational Medicinal Product Dossier (IMPD), an Investigator's Brochure (IB) is required, which outlines all available preclinical and clinical data (6). Both the U.S. Food and Drug Administration (FDA) and Committee for Human Medicinal Products and competent authorities from each individual European country are available for consultation to discuss development strategy and the pathway to registration for an IMP.

Regulatory authorization. As noted above, each country has its own regulatory authority, such as the Medicines and Healthcare products Regulatory Agency (MHRA) in the United Kingdom or the Ministry of Health in Italy and Germany, responsible for allowing a study to be conducted. Application requirements to gain regulatory approval differ a little between EU countries but the core documents for a Clinical Trial Application (equivalent to IND application in the United States) are listed in Table 1 (9), and regulatory approval is required for each country involved. EU regulatory authorities aim to assess applications within 30 days, extended up to 60 days if further details are required. The trial is then able to be registered onto an international clinical trials register, such as ClinicalTrials.gov.

Although the authorization for clinical trials is done at a national level, all European Clinical IMP trials are required to be issued with a EU Drug Regulating Authorities Clinical Trials (EudraCT) number, which is issued by the European Medicines Agency (10).

EudraCT is the European Clinical Trials Database of all clinical trials commencing in the European Union from May 1, 2004 onwards. The EudraCT database was established in accordance with Directive 2001/20/EC. Each clinical trial with at least one site in the European Union receives a unique number for identification, the EudraCT number. The EudraCT number must be included on all clinical trial applications within the European Union and as needed on other documents relating to the trials (e.g., suspected unexpected serious adverse reaction reports).

Patient information and consent. There is strong emphasis on the protection of clinical trial patients in ICH GCP and EU CTD, leading to the generation of patient information sheets, which extensively explain the rationale of the study and possible risk of harm from the therapy. Although written in plain language these are often long and require careful explanation and time allowed to the subject for consideration, prior to informed consent being given. Patient information sheets may be required in multiple languages and within the European Union are submitted, along with the trial protocol, for independent ethical approval.

Independent ethical review. Ethical review is required to be done by an independent committee, but procedures depend on the number of sites involved and differ in different EU states. A single-center study may be reviewed by a local committee, whereas a national review may be possible in some countries (such as the United Kingdom or the Netherlands). A committee with particular expertise may be required for phase I trials and additional expert opinion may also be required if vulnerable adults and/or children are involved. This independent review differs from the Institutional Review Board (IRB) review required for trial registration within the United States. An ethics committee has a 60-day time limit to approve or decline a study, but applications may occur in parallel with the assessment of the scientific merit of the study and therefore need not add a time delay to trial initiation. It does, however, often allow easier subsequent amendments to be accepted either by chairman's review or formal committee reassessment.

In the United Kingdom, the Integrated Research Application System form has recently been introduced in an attempt to reduce the trial administrative burden. The regulatory applications are centralized to a single electronic form minimizing repetitive core data entry. Also in the United Kingdom, studies involving biomarkers on clinical samples are also required to comply with the Human Tissue Act. A Case Report Form is developed to gather data relevant to the study and individual site assessments, agreements, and approvals obtained prior to patient recruitment.

Trial conduct and completion. The ICH guidelines define GMP quality IMP production for clinical use and handling during trial evaluation (11). The procedures for drug labeling, supply, and distribution require implementation by sponsors, and differ between the European Union and United States. The EU regulations require a manufacturing authorization for the manufacturer or importers of an IMP, and one or more "qualified person(s)" to undertake responsibility for the quality assurance of each batch of unlicensed product. The qualified person needs to be based in the European Union and is therefore generally independent of the sponsor, who is able to do this role within the United States.

ICH GCP defines the documentation required to be collected in trial files, but there are several systems accepted for data storage, and the level of data monitoring and source data verification varies between studies in the European Union as it also does in the United States. Efforts to ensure data quality have been steadily increasing in all academic centers (12). Database systems validated in the European Union offer centralized statistical monitoring and automated data validation. These systems are often used in academic studies, thus requiring reduced source data verification thereby reducing monitor time and saving expense. Pharmaceutically sponsored studies often extensively monitor source data entry at added significant expense.

The EU CTD requires countries to have a system to independently inspect sponsor or participating trial institutions by regulatory bodies. Routine inspections can be preplanned or can be triggered by specific safety concerns. The EU CTD also allows for "urgent safety measures" to be taken by the sponsor before regulatory review if there are serious safety concerns.

A system for identifying and reporting adverse events is required by the EU CTD. Toxicity grades are usually standardized by the use of the CTCAE grading system and procedures, and reporting timelines are similar across the United States, the European Union, and Japan. The Eudra Vigilance Database contains safety data about all IMPs in EU clinical trials allowing information to be exchanged more easily between participating countries (13).

Acquisition of drugs

The advances in biology and in particular the advances in the understanding of the molecular biology of various different types of cancer have led to an exponential increase in the number of interesting anticancer targets for drug design. The concurrent advances in analytical technology, antibody development, and medicinal chemistry have enabled the discovery of agents with potential activity against these targets at an unprecedented rate. Potential new anticancer drugs are being developed by large pharmaceutical companies, smaller biotech companies, and academic groups, often supported by charitable funding. This increase in available agents has also led to efforts to improve clinical trial design to maximize efficiency and to

understand whether the presumed drug target has been affected (14, 15).

Numerically by far the largest proportion of new drugs undergoing phase I trials are sponsored by the pharmaceutical industry, but some notable drugs have had their origins in academic institutions. Examples are carboplatin [Institute of Cancer Research (ICR) and Bristol-Myers Squibb], raltitrexed (ICR and AstraZeneca), and temozolomide (University of Aston and Schering Plough).

Phase I trials groups and networks

Working under the regulatory framework outlined above, a number of different organizations have been set up to conduct or promote the trials of innovative new cancer agents within the EU countries. The list below is not a comprehensive list, as many countries have phase I capabilities or networks, including Germany, Scandinavia, and Italy.

European Organization for Research on the Treatment of Cancer. Anticancer drugs of the 1970s and 1980s were usually toxic and expertise in the conduct of phase I studies with them was confined to a few centers. The Early Clinical Trials Group of the European Organization for Research on the Treatment of Cancer (EORTC) was very successful in bringing European expertise together and formed a valuable point of contact for the pharmaceutical industry. This group (which later became the Early Clinical Studies Group) conducted a large number of phase I trials on agents that are now in common use. However changes in the environment eventually led to the disbandment of this group in 2004. As oncology products became mainstream, the pharmaceutical industry acquired in-house expertise in conducting these trials. A number of centers developed a high level of specialization in phase I cancer trials and therefore could offer local expertise and a high throughput directly to sponsors. This evolution led to a gradual reduction in the number of new agents being available to the cooperative group, which eventually led the EORTC to focus on its highly successful phase III programs.

This pattern is now common in most European countries. There a large number of capable and active phase I centers in most European countries that are negotiating and conducting trials on a case-by-case basis with the sponsors, most of whom are pharmaceutical companies. Many of the previously Eastern European countries now have expanding and robust clinical trials activities and compete effectively with the longer established Western European operations. Some of the better known organizations are highlighted below and play a valuable role in coordinating activity and providing a collaborative and educational platform. Much of the actual phase I–II activity, however, takes place by direct negotiation of the sponsor (or Clinical Research Organization) with the centers.

Cancer Research UK (formerly Cancer Research Campaign – UK). In 1981 the Cancer Research Campaign set up a phase I committee of oncologists with an interest in early studies of investigational cancer agents with translational scientists, chemistry and formulation expertise. This group gradually transformed into the Cancer Research UK Drug Development Office and a funding committee called the New Agents Committee. The New Agents Committee is able to consider drug candidates at any stage of their development and can organize and fund bulk synthesis, formulation, and toxicology as well as phase I–II clinical trials. Phase I clinical trials are managed by the Drug Development Office, which can handle all the requirements for a GCP trial, including sponsorship, monitoring, and reporting. This is an unusual, if not unique facility, because it can take a drug from concept stage to clinical trial entirely within the charitable and/or academic arena. It has been instrumental in developing a number of

Table 2. Factors contributing to "Lag" for approval of new oncology therapeutics in Japan

Stage in approval process	Causes of delays
Delay in the start of development	Concerns about cost and delays
	Requests for additional data unique to Japan
	Slow review and/or consultation times
	Preference for positive phase II data prior to initiating
Prolonged trial conduct	Lack of protected time for clinical research, high clinical workload
	Lack of reimbursement and/or recognition for investigators
	Inexperience in clinical research, limited support staff
	Infrequent IRB meetings
	Additional paperwork and/or requirements unique to Japan (J-GCP)
	Patient reluctance (strong national healthcare system; concerns about safety; negative media releases; expectations about a positive outcome)
Prolonged J-NDA review	Limited number of reviewers
	Inconsistency in reviews and/or requirements

Abbreviations: J-GCP, Japan Good Clinical Practice; J-NDA, Japan New Drug Application.

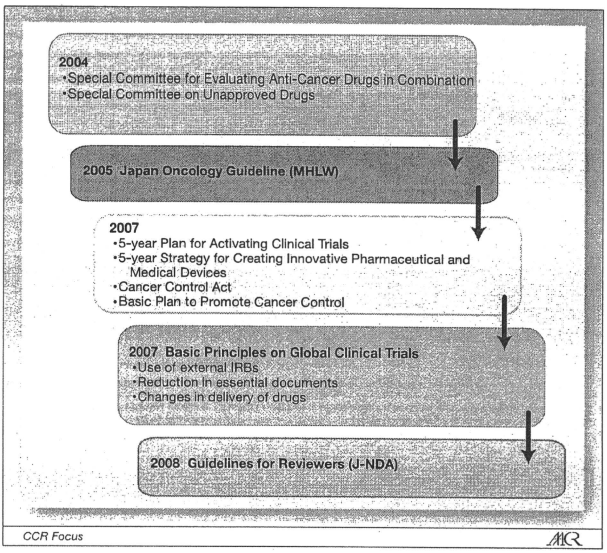

Fig. 1. Strategic changes initiated by the Ministry of Health, Labor and Welfare (MHLW). J-NDA, Japan New Drug Application.

licensed drugs, including temozolomide (16). The conduct of early clinical studies in cancer in the United Kingdom has also been greatly facilitated by the Experimental Cancer Medicine Network (17), which provides competitively allocated infrastructure funding to 19 centers around the United Kingdom.

However, it has become increasingly expensive to set up and conduct clinical trials, including early phase trials, largely owing to increased regulations and governance responsibilities. Phase I trial design has also evolved, expanding beyond simple toxicity evaluation to include multiple, often expensive, secondary and exploratory endpoints including predictive biomarkers, pharmacodynamic markers to show target inhibition in tumor or surrogate tissue, and preliminary efficacy outcomes. This means that the budget available to organizations such as CR UK will support far fewer trials than it would before the current regulations came into force. Similar budgetary considerations also apply to Clinical Development Partnerships (see below), which means that there are many interesting and potentially valuable new agents for which the clinical trials cannot be done.

The extraordinary success of modern drug development techniques, coupled with the increasing cost and complexity of clinical development, has resulted in many pharmaceutical companies having more promising agents in their pipeline than they have resources to develop. Acquisitions and mergers between these companies compound the situation often resulting in the pipelines of the merged company having several representatives of each class of drugs. The fact that many potentially useful drugs do not undergo clinical development is a potential loss to the oncology patient, because it is frequently not possible to make an accurate judgment of the clinical utility of a new agent without clinical data. Cancer Research UK Clinical Development Partnerships are designed to address this problem by offering to undertake early clinical development at the expense of CR UK. The collaborating company has an option to continue development with the drug if it looks successful in exchange for a revenue sharing agreement (18).

Southern Europe New Drug Organization. Southern Europe New Drug Organization (SENDO) was founded in 1997 to promote and coordinate transnational research and early clinical trials in Southern Europe with the aim of boosting research on new anticancer drugs using modern up-to-date methodology. It is a not-for-profit organization with centers in Switzerland, Italy, and Barcelona. It has all the expertise necessary for early clinical cancer drug development and a good network of collaborators for preclinical development (19).

Central European Society for Anticancer Drug Research. Central European Society for Anticancer Drug Research

(CESAR) was founded in 2001 with a focus on research into identifying new anticancer agents, the development of new agents, and fostering the translation of laboratory research into the clinic. CESAR comprises scientists from basic research and preclinical and clinical oncology in Austria, Germany, and Switzerland. In addition, the CESAR has created a network of study centers experienced in oncology of solid tumors in a number of Central and Eastern European (CEE) countries with the aim to foster international cooperation between oncologists and study centers in this area. CESAR has a portfolio of phase I and translational studies open and organizes meetings to promote and coordinate research (20).

Phase I–II cancer trials in France. The French National Federation of Cancer Centers has established a group ("Essais précoces") coordinating early phase cancer trials in a number of French centers. The Institut National du Cancer (INCa) has also set up an agreement with U.S. National Cancer Institute Cancer Therapy Evaluation Program (CTEP) permitting a number of French centers to be selected for phase I–II trials sponsored by CTEP. The new French Cancer Plan has identified a need to establish a funded network of cancer centers for phase I–II trials.

Phase I–II cancer trials in Spain. There is substantial amount of phase I activity in Spain but no single overarching organization. A number of Spanish centers undertake a significant number of phase I trials and some of them receive governmental support as part of the Cooperative Network of Cancer Centers.

Phase I–II cancer trials in Switzerland. Within the SAKK group (the Swiss national group for clinical studies in cancer) there is an independent phase I group.

Japan

Despite Japan's leadership role in basic research and discovery, its prominence in early clinical research is less established, although separate trials have often been required because of the differing pharmacology of drugs in Asian patients. In recent years polymorphic variations in proteins involved in drug clearance have been identified that begin to explain these differences (21). Marketing

Table 3. Requirements for phase I studies in the European Union, Japan, and the United States

Study component	Requirements	Japan	EU	United States
Preclinical data and starting dose	ICH Guidelines for safety (S3, S4, S6, S7, S9, M3)	In Japan, ICH S9 is at Step 4; May use data from other phase I studies; level at which no DLT reported. If no ethic difference in metabolism and/or safety suspected, phase I data from other countries may be acceptable to support phase II studies	One species (rodent) acceptable	Two species (one nonrodent) required
Pharmacokinetics	ADME defined	Required	Desirable	Desirable
	Assays developed and available			
Facilities and Personnel	Adequate knowledge of preclinical data			
	Investigators knowledgeable in clinical pharmacology and oncology therapy			
	Number of centers	Single center preferred; if multicenter, good communication channels must be in place		Single center studies recommended
Patients	Usually cancer patients unless minimal toxicity (volunteers may be acceptable); Patients with poor performance status (ECOG 3 or 4) excluded			
	No standard options known to prolong life			
	Hospitalization?	Hospitalization may be required by PMDA		May be treated as inpatients or outpatients
	May include multiple tumor types or be tumor specific if appropriate			
	Criteria for organ function and/or eligibility	Additional criteria may be required		Standard
Design and conduct	Must evaluate more than a single dose			
	Pharmacokinetics	Especially for combination studies		Recommended
	Independent data monitoring committee	May be required		Not mandated

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; ECOG, Eastern Co-operative Group.