した。また，Eguchi ら[19]は，細胞周期関連蛋白であるp27の発現増強を介してIFNの効果が発揮されることを報告している。5FUの中間代謝物質（FdMP）の細胞内農道上昇効果，あるいはthymidylate syntase阻害率の上昇などの報告もある[20,21]。また，IFN/5FUあるいはIFN/FP療法の臨床的効果からは，今回のわれわれの結果が示すように肝癌細胞の着床・転移抑制効果も推測され，今後のさらなる研究が期待される。

結　語

われわれが行っているIFP療法は，肉眼的門脈侵襲陽性肝癌術後の制御不能な再発を予防し，予後を改善する可能性がある有用な治療戦略と考えられる。

文　献

1）Imamura, H., Matsuyama, Y., Tanaka, E., Ohkubo, T., *et al.*: Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J. Hepatol., 38：200-207, 2003

2）Llovet, J. M., Fuster, J., Bruix, J.: Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. Hepatology, 30：1434-1440, 1999

3）Poon, R. T., Fan, S. T., Ng, I. O., Lo, C. M., *et al.*: Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. Cancer, 89：500-507, 2000

4）日本肝臓学会　編：科学的根拠に基づく肝癌診療ガイドライン2009年度版．金原出版．

5）Arii, S., Sata, M., Sakamoto, M., Shimada, M., *et al.*: Management of hepatocellular carcinoma: Report of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). Hepatol. Res., 40(7)：667-685, 2010

6）Kanematsu, M., Hoshi, H., Yamada, T., Murakami, T., *et al.*: Small hepatic nodules in cirrhosis: ultrasonographic, CT, and MR imaging findings. Abdom. Imaging, 24：47-55, 1999

7）Poon, R. T., Fan, S. T., Tsang, F. H., Wong, J., Locoregional therapies for hepatocellular carcinoma: a critical review from the surgeon's perspective. Ann. Surg., 235：466-486, 2002

8）Shimada, M., Yamashita, Y., Hamatsu, T., Rikimaru, T., *et al.*: Surgical indications for advanced hepatocellular carcinoma. Hepatogastroenterology, 47：1095-1099, 2000

9）Chung, Y. H., Song, I. H., Song, B. C., Lee, G. C., *et al.*: Combined therapy consisting of intraarterial cisplatin infusion and systemic interferon-alpha for hepatocellular carcinoma patients with major portal vein thrombosis or distant metastasis. Cancer, 88(9)：1986-1891, 2000

10）Ji, S. K., Park, N. H., Choi, H. M., *et al.*: Combined cisplatinum and alpha interferon therapy of advanced hepatocellular carcinoma. Korean J. Intern. Med., 11(1)：58-68, 1996

11）Ota, H., Nagano, H., Sakon, M., Eguchi, H., *et al.*: Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. Br. J. Cancer, 93：557-564, 2005

12）Obi, S., Yoshida, H., Toune, R., Unuma, T., *et al.*: Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. Cancer, 106：1990-1997, 2006

13）Llovet, J. M., Sala, M., Castells, L., Suarez, Y., *et al.*: Randomized controlled trial of interferon treatment for advanced hepatocellular carcinoma. Hepatology, 31：54-58, 2000

14）Sachs, E., Di Bisceglie, A. M., Dusheiko, G. M., Song, E., *et al.*: Treatment of hepatocellular carcinoma with recombinant leucocyte interferon: a pilot study. Br.

J. Cancer, 52 : 105-109, 1985

15) Feun, L. G., Savaraj, N., Hung, S., Reddy, R., *et al.*: A phase II trial of recombinant leukocyte interferon plus doxorubicin in patients with hepatocellular carcinoma. Am. J. Clin. Oncol., 17 : 393-395, 1994

16) Colleoni, M., Buzzoni, R., Bajetta, E., Bochicchio, A. M., *et al.*: A phase II study of mitoxantrone combined with beta-interferon in unresectable hepatocellular carcinoma. Cancer, 72 : 3196-3201, 1993

17) Arakawa, Y., Shimada, M., Utsunomiya, T., Imura, S., *et al.*: Effects of pegylated interferon α2b on metastasis of hepatocellular carcinoma. J. Surg. Res.(in press)

18) Takaoka, A., Hayakawa, S., Yanai, H., Stoiber, D., *et al.*: Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature, 424 : 516-523, 2003

19) Eguchi, H., Nagano, H., Yamamoto, H., Miyamoto, A., *et al.*: Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. Clin. Cancer Res., 6 : 2881-2890, 2000

20) Schwartz, E. L., Hoffman, M., O'Connor, C. J, Wadler, S.: Stimulation of 5-fluorouracil metabolic activation by interferon-alpha in human colon carcinoma cells. Biochem. Biophys. Res. Commun., 182 : 1232-1239, 1992

21) Wadler, S., Schwartz, E. L.: Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies : a review. Cancer Res., 50 : 3473-3486, 1990

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Strong anti-tumor effect of NVP-AUY922, a novel Hsp90 inhibitor, on non-small cell lung cancer

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ABSTRACT

The anti-tumor activity of a newly developed Hsp90 inhibitor, NVP-AUY922 (AUY922), against non-small cell lung cancer (NSCLC) was examined. Twenty-one NSCLC cell lines were used, the somatic alterations of which were characterized. Cell proliferation was analyzed using a modified MTS assay. Expression of the client proteins was assessed using Western blotting. The cell cycle was analyzed using flow cytometry. The IC_{50} value of AUY922 for the NSCLC cell lines ranged from 5.2 to 860 nM (median, 20.4 nM). Based on previous data, cells with an IC_{50} of less than 50 nM were classified as sensitive cells and 19 of the 21 NSCLC cell lines were judged to be sensitive. The IC_{50} of five malignant pleural mesothelioma (MPM) cell lines revealed that the MPM cells had a significantly higher IC_{50} value (median, 89.2 nM; range, 22.2–24,100 nM) than the NSCLC cells ($p = 0.015$). There was significant depletion of both the total and phosphorylated client proteins – EGFR, MET, HER2 and AKT – at low drug concentrations (50–100 nM) in drug-sensitive cell lines. Cell-cycle analysis was performed for two sensitive cell lines, H1975 and H838. Following AUY922 treatment, an increase in the sub-G_0-G_1 cell population, as well as appearance of cleaved PARP expression, indicated the induction of apoptosis. In conclusion, AUY922 was effective against most NSCLC cell lines, independent of the type of known molecular alteration, and appears to be a promising new drug for the treatment of NSCLC.

1. Introduction

Lung cancer is associated with various types of molecular alteration, including epidermal growth factor receptor (EGFR) mutation, *K-ras* mutation, *HER2* amplification and, as recently found, *EMK4-ALK* gene fusion [1–3]. Improvements in our understanding of the molecular alterations involved in lung cancer have brought significant advancements in molecular-targeted therapy [4]. Among these alterations, *EGFR* mutations, which are frequent alterations in lung adenocarcinoma, are a predictive factor for the efficacy of EGFR-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib [1,2]. These EGFR-TKIs have a marked anti-tumor effect on NSCLCs with common *EGFR* mutations. However, acquired resistance from, for example, a secondary *EGFR* T790M mutation or *MET* amplification is a major problem that is responsible for treatment failure [5–7].

The heat-shock protein 90 (Hsp90) complex is a chaperone protein that facilitates the refolding of unfolded or misfolded proteins. It plays a pivotal role in cancer cell survival, as it stabilizes a large set of proteins, so-called client proteins, many of which are essential for apoptosis, cell-cycle regulation, proliferation, and other characteristic properties of cancer cells [8,9]. In NSCLC, Hsp90 stabilizes oncogenic proteins such as EGFR, MET, HER2 and AKT [9,10]. We and some other studies have shown that geldanamycin (GM) and its analogues, the benzoquinone ansamycin class (17 allylamino-17demethoxygeldanamycin [17-AAG] and 17 dimethylaminoethylamino-17-demethoxygeldanamycin [17-DMAG]), are effective against *EGFR*-mutated cell lines, even those that contain the *EGFR* T790M mutation that causes resistance to EGFR-TKI [11–14]. However, the results of clinical trials for 17-AAG and 17-DMAG were somewhat disappointing [15–19] and new potent Hsp90 inhibitors have therefore been pharmacologically designed and synthesized to offer improved efficacy and acceptable toxicity. NVP-AUY922 (AUY922) is one of these newly designed small-molecule Hsp90 inhibitors based on the 4,5-diarylisoxazole scaffold; it has a much higher affinity for Hsp90 than previous GM

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analogues [20]. AUY922 is bound to the ATP binding site of Hsp90α at the N-terminal domain, and its X-ray crystal structure confirms a crucial network of hydrogen bonding interactions. It exhibits the tightest binding of any small-molecule Hsp90 ligand because the entropy of binding to Hsp90 is almost negligible. Indeed, preclinical data from various types of human cancer have shown an anti-proliferative effect of AUY922, with low nanomolar potency both *in vivo* and *in vitro*, with no major adverse effects being observed in mice [20–24]. In these studies, AUY922 suppressed the client proteins (EGFR, MET, HER2 and AKT) that participate in the progression of various cancer cells, and AUY922 is considered to be a promising agent for NSCLC. However, to our knowledge, the efficacy of AUY992 has been reported in only one NSCLC cell line (A549) to date [25], although Phase II clinical trials for patients with advanced NSCLC have recently started.

In this study, we examined the anti-tumor effect of AUY922 against NSCLC cell lines containing several known genetic alterations, including *EGFR* mutations.

2. Materials and methods

2.1. Drugs and cell lines

AUY922 was obtained from Novartis (Nuremberg, Germany) and dissolved in dimethyl sulfide (DMSO) at stocked concentrations of 10 mM and stored at −20 °C. Working dilutions were always freshly prepared. Most of NSCLC and MPM cell lines used in this study were established at two institutions. The prefix NCI-H- (abbreviated as H-) indicates cell lines established at the National Cancer Institute-Navy Medical Oncology Branch, National Naval Medical Center, Bethesda, MD, and the prefix HCC- indicates lines established at the Hamon Center for Therapeutic Oncology Research, the University of Texas Southwestern Medical Center at Dallas, Dallas, TX. These cell lines were kindly provided by Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA). A549 was purchased from American Type Culture Collection (Manassas, VA). NCI-H3255 was provided from Dr. Bruce Johnson (Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, MA). PC-9 was provided from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). Gefitinib-resistant PC-9 cell line (RPC-9) was provided from the Department of Hematology, Oncology, and Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Japan [26]. All the cancer cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Determination of cell proliferation

Cell proliferation was determined by a modified MTS assay with CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI). Cells were seeded on 96-well flat-bottomed tissue culture plates (Becton Dickinson, San Jose, CA) at a concentration of 3×10^3 cells/well with complete culture medium and allowed to adhere to the plate for 24 h. Then the cells were incubated in the presence of the drug of each concentration ranging from 0 (control) to 10 μM of for another 72 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the treatment, 20 μL of CellTiter 96® AQueous One Solution Reagent was dropped into each well of plates. After the incubation of another 60 min, the optical densities (ODs) of these samples were directly measured using an Immuno Mini NJ-2300 (Nalge Nunc International, Rochester, NY). A reference wavelength at 490 nm was used to subtract background contributed by excess cell debris, fingerprints and other nonspecific absorbance. The OD of control samples was regarded as 100 and others were compared to the control. Each drug concentration was distributed in 4-replicate wells and each experiment was repeated thrice. The anti-proliferative activity of AUY922 was shown as IC_{50}, which is the concentration of the drug required to inhibit cell proliferation by 50%.

2.3. Western blot analysis and immunoprecipitation

Protein expression analysis was assessed by Western blotting. The lysate was extracted and 20 μg of total protein was then separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with anti-EGFR, anti-phospho-EGFR (Ty1068), anti-Met (25H2), anti-phospho-Met (3D7, Tyr1234/1235), anti-HER2, anti-phospho-HER2 (Tyr877), anti-Akt, anti-phosphor-Akt (Ser473), anti-p44/42 mitogen-activated protein kinases (MAPK), anti-phosphor-MAPK (Thr202/Tyr204), anti-Cyclin D1, anti-cdc2 and anti-cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) (19F4) antibodies (Cell Signaling Technology, Beverly, MA), anti-Hsp90 (Novocastra, Newcastle, UK), anti-Hsp70 (Stressgen Bioreagents, Ann Arbor, MI), anti-CDK4 (C-22) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Actin (used as loading control, Millipore, Billerica, MA) and then with goat anti-rabbit and goat anti-mouse IgG-HRP coupled to horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After the incubation with antibodies, the membranes were developed by ECL Plus Western Blotting Detection Reagents (Amersham Biosciences UK Limited, Buckinghamshire, UK).

2.4. Flow cytometric analysis

Cells were harvested and resuspended in PBS containing 0.2% Triton X-100 and 1 mg/mL RNase for 5 min at room temperature and then stained with propidium iodide at 50 μg/mL to determine subdiploid DNA content using a FACScan. Doublets, cell debris, and fixation artifacts were gated out, and cell cycle analysis was done using CellQuest version 3.3 software.

3. Results

3.1. Anti-proliferative effect of AUY922 in NSCLC cell lines

The concentrations of AUY922 at IC_{50} in each cell line are shown in Table 1 and Fig. 1. The molecular characteristics of NSCLC cell lines are also described (Table 1). The IC_{50} values in the NSCLC cell lines ranged from 5.2 to 860 nM, whereas those in the MPM cell lines ranged from 22.2 to 24,100 nM ($p = 0.015$), indicating a significant difference in AUY922 sensitivity between NSCLC and MPM cell lines. For NSCLC, AUY922 exhibited a strong anti-proliferative effect in cell lines with *EGFR* mutations that were either sensitive to EGFR-TKI or that had acquired resistance to EGFR-TKI, similar to the effects of GM analogues. Furthermore, AUY922 also exhibited anti-proliferative effects on cell lines with wild-type *EGFR*, a *K-ras* mutation, *EML4-ALK* fusion gene, or other genetic alterations.

We also determined the IC_{50} value of the SKBR3 breast cancer cell line to validate the IC_{50} value determined with our MTS assay by comparing it with published data [22]. Our and previously published IC_{50} values were 9.7 ± 3.5 nM and 3.3 ± 0.9 nM, respectively, suggesting that the IC_{50} value measured using our system was not remarkably different from the published data [22]. Thus, in accordance with the published criteria, an IC_{50} value of less than 50 nM was regarded as being a sensitive cell line [22]. On the basis of this criteria, 19 of the 21 NSCLC cell lines and two of the five MPM cell lines were classified as being sensitive ($p = 0.034$).

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Table 1
IC_{50} inhibition values for NVP-AUY922 in NSCLC and MPM cell lines.

Cancer type	Cell lines	Histological subtypes	AUY922		Genetic alterations	
			Sensitivity[a]	IC_{50} (nM)		
NSCLC	PC-9	AD	Sensitive	8.6 ± 0.5	*EGFR* mutation	Exon19 del.
	HCC2935	AD	Sensitive	9.2 ± 0.1		Exon19 del.
	HCC827	AD	Sensitive	16.9 ± 0.4		Exon19 del.
	HCC2279	AD	Sensitive	26.3 ± 3.6		Exon19 del.
	HCC4011	AD	Sensitive	17.9 ± 0.1		L858R
	H3255	AD	Sensitive	29.5 ± 5.8		L858R
	RPC-9	AD	Sensitive	20.4 ± 1.4		Exon19 del. ± T790M
	H1975	AD	Sensitive	5.2 ± 0.3		L858R ± T790M
	H1650	AD	Sensitive	23.5 ± 2.9		Exon19 del. ± PTEN del.
	H1299	LC	Sensitive	32.4 ± 0.1	*N-ras* mutation	
	A549	AD	Sensitive	16.3 ± 0.6	*K-ras* mutation	
	H2009	AD	Sensitive	21.4 ± 0.8	*K-ras* mutation	
	H358	AD	Sensitive	28.1 ± 4.1	*K-ras* mutation	
	H2170	SQ	Sensitive	9.1 ± 0.3	*HER2* amplification	
	H1648	AD	Sensitive	9.6 ± 0.1	*HER2* amplification	
	H1819	AD	Sensitive	23.9 ± 1.0	*HER2* amplification	
	Calu3	AD	Resistant	248 ± 8.5	*HER2* amplification	
	H1993	AD	Sensitive	7.7 ± 0.2	*MET* amplification	
	H1395	AD	Resistant	860 ± 7.1	*B-raf* mutation	
	H2228	AD	Sensitive	20.4 ± 6.5	*EML4-ALK* fusion gene variant E6a/b;A20	
	H838	AD	Sensitive	17.1 ± 0.6	None	
MPM	H211	Biphasic	Sensitive	22.2 ± 3.8		
	H290	Epithelial	Sensitive	27.3 ± 3.8		
	H28	Sarcomatoid	Resistant	89.2 ± 8.2		
	HP1	Biphasic	Resistant	1070 ± 10		
	H2052	Epithelial	Resistant	24,100 ± 4900		
BC	SKBR3		Sensitive	9.7 ± 3.5		

NSCLC, non-small cell lung cancer; MPM, malignant pleural mesothelioma; BC, breast cancer; AD, adenocarcinoma; LC, large cell carcinoma; SQ, squamous cell carcinoma.
[a] Sensitivity: sensitive cell lines, IC_{50} value ≤ 50 nM; resistant cell lines, IC_{50} value > 50 nM; del, deletion; NVP-AUY922 exhibited strong effects to most NSCLC cell lines with *EGFR* and *K-ras* mutation or *HER2* and *MET* amplification.

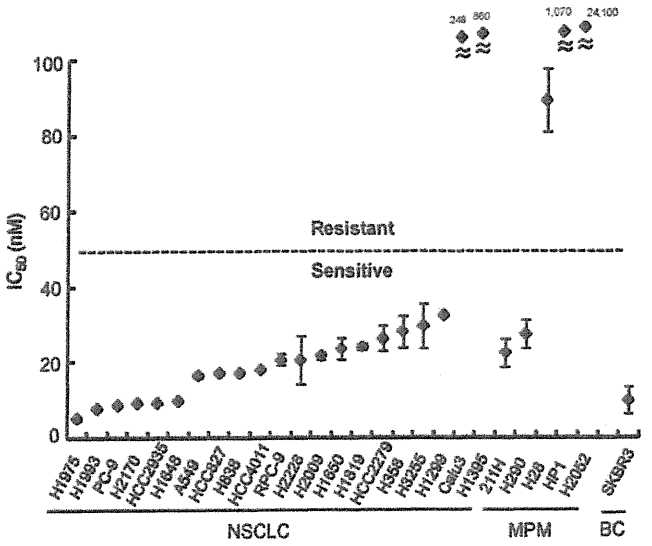

Fig. 1. IC_{50} values of non-small cell lung cancer and malignant pleural mesothelioma cell lines.

Two cell lines, H1395 and Calu3, were considered to be resistant. H1395 contains a *B-raf* mutation as a known molecular alteration, while Calu3 has a strong amplification of *HER2* and increased copy numbers of *EGFR* and *PIK3CA*. However, the H2170 cell line, which also exhibited strong *HER2* amplification and an increased copy number of *EGFR*, was classified as a sensitive cell line (IC_{50} = 9.1 ± 0.3), suggesting that amplification of *HER2* or *EGFR* is not the factor that causes resistance to AUY922.

3.2. Effects of AUY922 on molecular signature in NSCLC cell lines

Subsequent experiments focused on NSCLC. The effect of AUY922 on protein expression was examined according to concentration and exposure time in three sensitive cell lines (H1975, A549, and H838) and two resistant cell lines (H1395 and Calu3). Cells were harvested 24 h after drug treatment in a concentration gradient experiment (Fig. 2 and Supplementary Fig. 1). In sensitive cell lines, the depletion of both the total and the phosphorylated client proteins, such as EGFR, MET, HER2, AKT, and Cyclin D1 (CCND1), was observed after treatment with 50 nM of AUY922. Suppression of phospho-MAPK (p-MAPK) but not total-MAPK (t-MAPK) may be caused by down-regulation of its upstream molecules, which are the client proteins of Hsp90. Although inhibition of Hsp90 activity with drugs is generally correlated with Hsp70 protein levels after treatment [22,27], Hsp70 expression increased in both sensitive and resistant cell lines. In terms of the resistant cell lines, although expression of the client proteins was not depleted after treatment with a high concentration of AUY922 in Calu3 (IC_{50} = 248 nM), H1395 – another resistant cell line (IC_{50} = 850 nM) – showed depletion of client proteins after treatment with AUY922 at a low concentration (Fig. 2 and Supplementary Fig. 1).

For exposure time analysis, each cell line except H1395 was treated with the AUY922 concentration, which was five times as high as each IC_{50}. H1395, the IC_{50} of which was 850 nM, was exposed to 100 nM of AUY922. Although variation of protein depletion and recovery was observed according to proteins or cell lines, decreased expression of the majority of proteins was observed from 12 to 72 h (Fig. 3 and Supplementary Fig. 2). Of note, there was no major difference in the pattern of the protein expression profile time course between sensitive cell lines and H1395-resistant cell lines.

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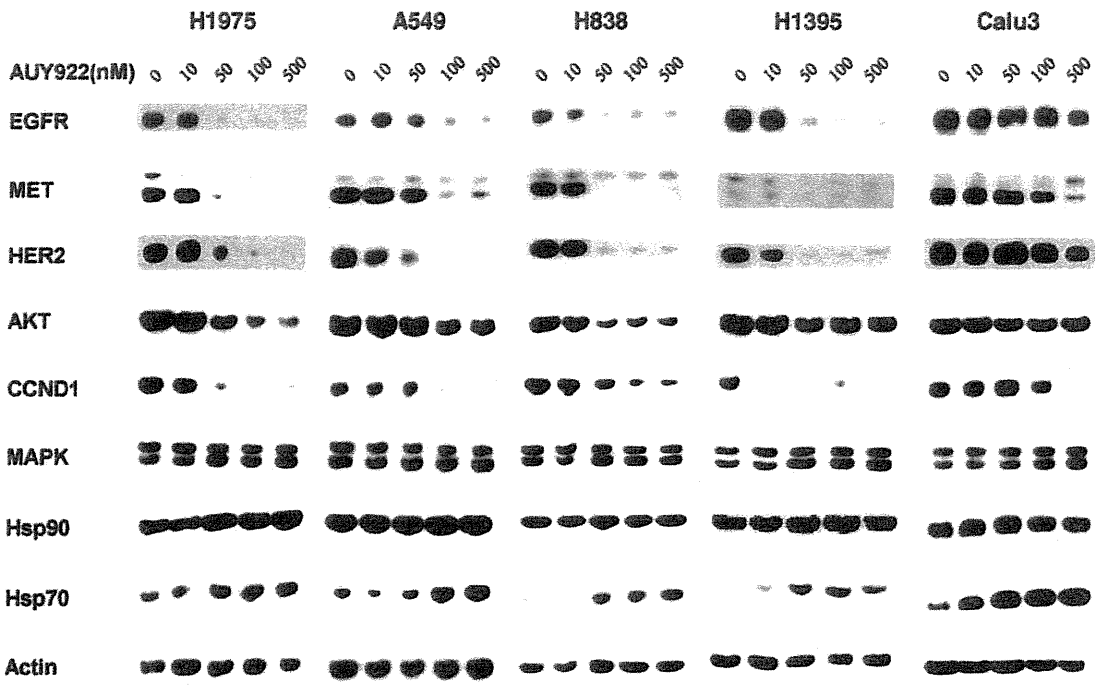

Fig. 2. The profiles of protein expression under the treatment of different AUY922 concentrations for 24 h.

3.3. *Effects of AUY922 on cell cycle and apoptosis*

We analyzed the cell cycle in two sensitive cell lines (H1975 and H838) to examine the impact of AUY922 on cell-cycle distribution, especially induction of apoptosis. Whereas the pattern of cell-cycle distribution after treatment of AUY922 was different between two cell lines, sub-G_0-G_1 DNA content increased in a time-dependent manner for both cell lines. Cleaved PARP also increased with AUY922 treatment, indicating that AUY922 induced apoptosis in these two cell lines (Fig. 4).

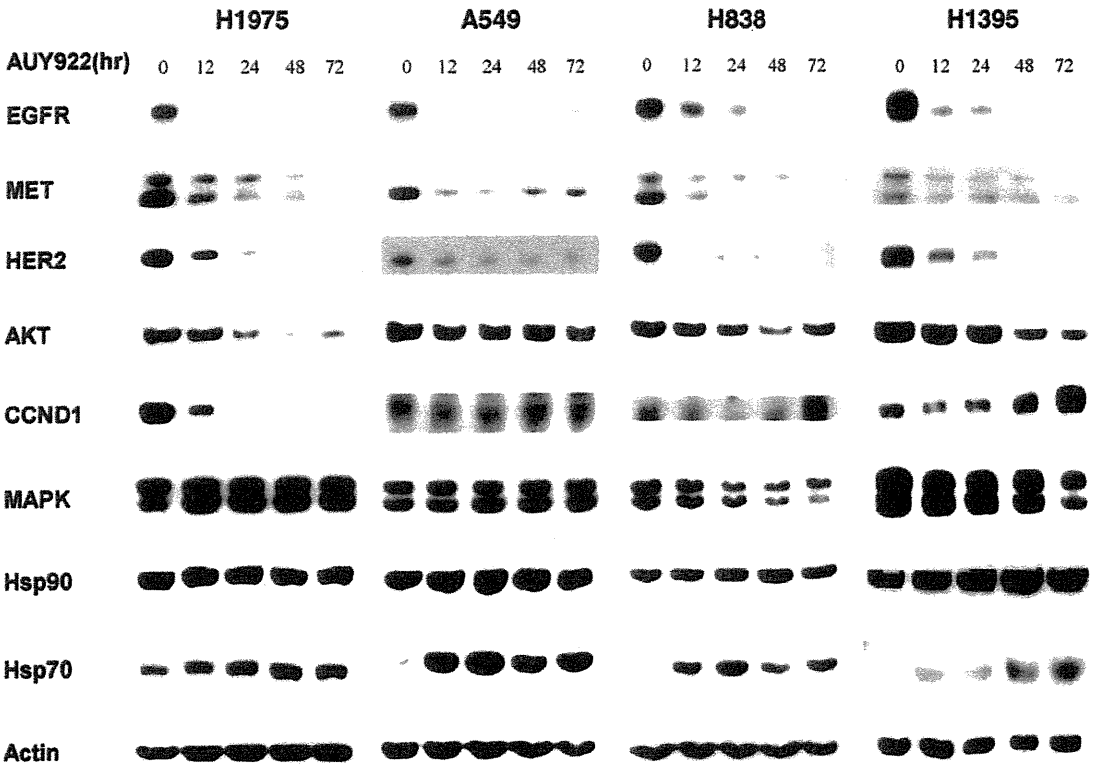

Fig. 3. The profiles of protein expression according to exposure time with AUY922. Each NSCLC cell line (H1975, A549, and H838) was treated with AUY922 of which concentration was five times as high as each IC_{50}. H1395 was exposed to 100 nM of AUY922.

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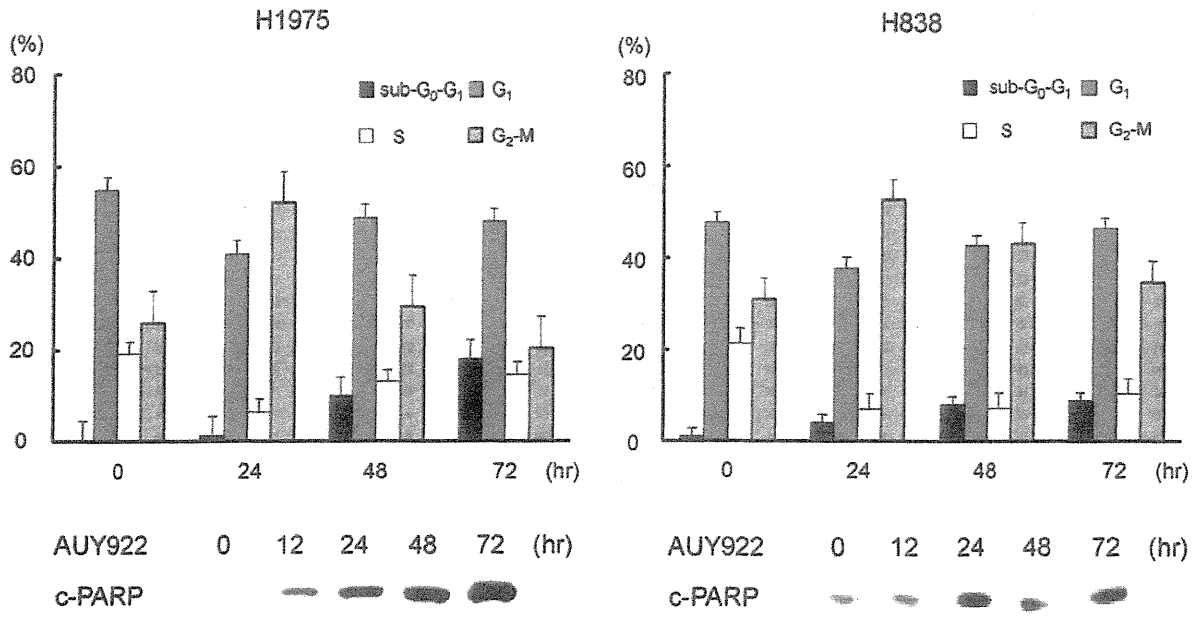

Fig. 4. The impact of AUY922 on cell cycle distribution and induction of apoptosis. Using two sensitive cell lines, cell cycle distribution was analyzed using flow cytometry and cleaved PARP expression was examined using Western blotting. After treatment of AUY922, sub-G_0-G_1 DNA content increased in a time-dependent manner and cleaved PARP also increased with AUY922 treatment.

4. Discussion

In this study, we found that AUY922 had a strong anti-proliferative effect on most NSCLC cell lines. Previous studies have indicated that GM analogue Hsp90 inhibitors have an anti-tumor effect on *EGFR* mutant NSCLC cell lines, including acquired TKI-resistant NSCLC. This suggests that Hsp90 inhibitors are promising agents for resistance to EGFR-TKI in the treatment of NSCLC [12]. However, a recent clinical trial for IPI-504, an analogue of 17-AAG, failed to show its significant effectiveness for *EGFR* mutant NSCLC patients [17]. On the other hand, IPI-504 showed response to 2 of 3 NSCLC patients with *EML4-ALK* fusion gene. One of the reasons is that enrolled patients with *EGFR* mutation had been treated at least two prior EGFR-TKI agents, suggesting that the biological features of these *EGFR* mutant tumors might be different from those of untreated tumors with single oncogene addicted status. In addition, cancer cell lines with *EML4-ALK* might be more sensitive for 17-AAG than those with *EGFR* mutation [17]. Unlike GM analogues including 17-AAG, AUY992 exhibited similar anti-tumor effect not only in *EGFR* mutant tumors, but also in wild-type *EGFR* tumors with various molecular alterations including *K-ras* mutation, *EML4-ALK* fusion gene, or *MET* or *HER2* amplification. One reason is that AUY922 has a much higher affinity for the N-terminal nucleotide-binding site of human Hsp90 than other Hsp90 inhibitors and can strongly suppress the expression of many client proteins at low concentrations [20].

Cell-cycle distribution was examined in two cell lines to assess the induction of apoptosis, but the pattern of distributions was not identical. Many client proteins of Hsp90 are thought to be involved in the pathogenesis of cancers. The degree and manner of involvement of each client protein should vary according to the cancer type, resulting in variation of the cellular response, such as cell-cycle distribution and degree of apoptosis. This would account for the difference in pattern of cell distribution and degree of apoptosis even in the sensitive cell lines.

In our series, the two cell lines Calu3 and H1395 were regarded as being resistant to AUY922. The client proteins in Calu3 were not depleted with AUY922 treatment as much. The fact that expression of Hsp70 was induced in Calu3 confirmed the inhibition of Hsp90 with AUY922, which suggested that drug transporters or metabolic activity might not be responsible for the resistance of Calu3. The cause of preserved expression of client proteins is unclear. In contrast, H1395 showed decreased expression of the client proteins at a low concentration of AUY922, which was similar to the response in sensitive cell lines. As early recovery of client proteins under AUY922 treatment was related to drug resistance in glioblastoma [28], we examined whether there was a difference in the recovery time of depleted proteins between sensitive and resistant cell lines. However, there was no difference between them in NSCLC and the mechanism of resistance was unclear. One possible explanation for the observed resistance is that although Hsp90 has many client proteins that are generally essential for tumor proliferation and survival in the majority of cancers, when cancer cells do not depend on these client proteins for survival, the inhibition of Hsp90 may not be effective. Of clinical relevance, this point may suggest that the selection of patients suited to AUY922 treatment based on molecular properties is difficult. Further investigation to identify the factors that can predict sensitivity or resistance to AUY922 is necessary.

Our results suggest that AUY922 is not effective in MPM compared to NSCLC. Although the precise mechanism of resistance is not clear, the molecular characteristics of MPM are different from those of NSCLC [29,30]. Regarding the clinical use of AUY922, Phase I/II trials of intravenously administered AUY922 are currently ongoing (http://clinicaltrials.gov/) for patients with various types of cancer. From February 2011 to present, two interesting clinical trials have begun for advanced NSCLC. The NCT01124864 trial is for patients who have received at least two lines of prior chemotherapy, and the patients are stratified according to *K-ras* and *EGFR* mutation status. The NCT01259089 trial is for patients with lung adenocarcinoma with "acquired resistance" to EGFR-TKI. It is noteworthy that our data strongly support the use of AUY922 for the treatment of NSCLC patients with various somatic alterations or with acquired resistance to EGFR-TKI.

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In conclusion, our study suggests that AUY922 is a potent candidate for the treatment of the majority of NSCLCs, independent of the major known genetic alterations.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2011.09.011.

References

[1] Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
[2] Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
[3] Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561–6.
[4] Toyooka S, Mitsudomi T, Soh J, Aokage K, Yamane M, Oto T, et al. Molecular oncology of lung cancer. Gen Thorac Cardiovasc Surg 2011;59:527–37.
[5] Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
[6] Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
[7] Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
[8] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
[9] Solit DB, Chiosis G. Development and application of Hsp90 inhibitors. Drug Discov Today 2008;13:38–43.
[10] Neckers L. Heat shock protein 90: the cancer chaperone. J Biosci 2007;32:517–30.
[11] Kobayashi N, Toyooka S, Soh J, Yamamoto H, Dote H, Kawasaki K, et al. The anti-proliferative effect of heat shock protein 90 inhibitor, 17-DMAG, on non-small-cell lung cancers being resistant to EGFR tyrosine kinase inhibitor. Lung Cancer 2011, in press, Epub ahead of print.
[12] Shimamura T, Lowell AM, Engelman JA, Shapiro GI. Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. Cancer Res 2005;65:6401–8.
[13] Shimamura T, Li D, Ji H, Haringsma HJ, Liniker E, Borgman CL, et al. Hsp90 inhibition suppresses mutant EGFR-T790M signaling and overcomes kinase inhibitor resistance. Cancer Res 2008;68:5827–38.
[14] Sawai A, Chandarlapaty S, Greulich H, Gonen M, Ye Q, Arteaga CL, et al. Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel. Cancer Res 2008;68:589–96.
[15] Nowakowski GS, McCollum AK, Ames MM, Mandrekar SJ, Reid JM, Adjei AA, et al. A phase I trial of twice-weekly 17-allylamino-demethoxy-geldanamycin in patients with advanced cancer. Clin Cancer Res 2006;12:6087–93.
[16] Sharp S, Workman P. Inhibitors of the HSP90 molecular chaperone: current status. Adv Cancer Res 2006;95:323–48.
[17] Sequist LV, Gettinger S, Senzer NN, Martins RG, Janne PA, Lilenbaum R, et al. Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer. J Clin Oncol 2010;28:4953–60.
[18] Holzbeierlein JM, Windsperger A, Vielhauer G. Hsp90: a drug target? Curr Oncol Rep 2010;12:95–101.
[19] Ramanathan RK, Egorin MJ, Erlichman C, Remick SC, Ramalingam SS, Naret C, et al. Phase I pharmacokinetic and pharmacodynamic study of 17-dimethylaminoethylamino-17-demethoxygeldanamycin, an inhibitor of heat-shock protein 90, in patients with advanced solid tumors. J Clin Oncol 2010;28:1520–6.
[20] Brough PA, Aherne W, Barril X, Borgognoni J, Boxall K, Cansfield JE, et al. 4,5-Diarylisoxazole Hsp90 chaperone inhibitors: potential therapeutic agents for the treatment of cancer. J Med Chem 2008;51:196–218.
[21] Eccles SA, Massey A, Raynaud FI, Sharp SY, Box G, Valenti M, et al. NVP-AUY922: a novel heat shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis. Cancer Res 2008;68:2850–60.
[22] Jensen MR, Schoepfer J, Radimerski T, Massey A, Guy CT, Brueggen J, et al. NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models. Breast Cancer Res 2008;10:R33.
[23] Lee KH, Lee JH, Han SW, Im SA, Kim TY, Oh DY, et al. Antitumor activity of NVP-AUY922, a novel heat shock protein 90 inhibitor, in human gastric cancer cells is mediated through proteasomal degradation of client proteins. Cancer Sci 2011;102:1388–95.
[24] Okui T, Shimo T, Hassan NM, Fukazawa T, Kurio N, Takaoka M, et al. Antitumor effect of novel HSP90 inhibitor NVP-AUY922 against oral squamous cell carcinoma. Anticancer Res 2011;31:1197–204.
[25] Stingl L, Stuhmer T, Chatterjee M, Jensen MR, Flentje M, Djuzenova CS. Novel HSP90 inhibitors, NVP-AUY922 and NVP-BEP800, radiosensitise tumour cells through cell-cycle impairment, increased DNA damage and repair protraction. Br J Cancer 2010;102:1578–91.
[26] Ogino A, Kitao H, Hirano S, Uchida A, Ishiai M, Kozuki T, et al. Emergence of epidermal growth factor receptor T790M mutation during chronic exposure to gefitinib in a non small cell lung cancer cell line. Cancer Res 2007;67:7807–14.
[27] Beere HM. "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. J Cell Sci 2004;117:2641–51.
[28] Gaspar N, Sharp SY, Eccles SA, Gowan S, Popov S, Jones C, et al. Mechanistic evaluation of the novel HSP90 inhibitor NVP-AUY922 in adult and pediatric glioblastoma. Mol Cancer Ther 2010;9:1219–33.
[29] Toyooka S, Pass HI, Shivapurkar N, Fukuyama Y, Maruyama R, Toyooka KO, et al. Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. Cancer Res 2001;61:5727–30.
[30] Toyooka S, Kishimoto T, Date H. Advances in the molecular biology of malignant mesothelioma. Acta Med Okayama 2008;62:1–7.

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Inhibition of mTOR by temsirolimus contributes to prolonged survival of mice with pleural dissemination of non-small-cell lung cancer cells

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Temsirolimus (CCI-779), a recently synthesized analogue of rapamycin, specifically inhibits mTOR and has been approved for clinical use in renal cell carcinoma. Recent reports have indicated the growth inhibitory effect of temsirolimus in some cancers including non-small-cell lung carcinoma (NSCLC). In this study, we aimed to explore the potential therapeutic use of temsirolimus as a treatment for NSCLC. Using cultured NSCLC cells (A549, H1299, and H358), we determined the effect of temsirolimus on cell proliferation and its antitumor effects on subcutaneous tumors, as well as its contribution to the survival of mice having pleural dissemination of cancer cells, mimicking advanced NSCLC. Temsirolimus suppressed proliferation of NSCLC cells in a dose-dependent manner, with an IC_{50} of <1 nM. Western blot analysis revealed that temsirolimus treatment specifically inhibited the phosphorylation of mTOR and its downstream effectors in 1 h, accompanied by an increased cell population in the G_0/G_1 phase, but according to flow cytometry, the cell population did not increase in the sub-G_0 phase. When NSCLC subcutaneous tumor-bearing mice were treated with temsirolimus, tumor volume was significantly reduced (tumor volume on day 35: vehicle *vs* temsirolimus = 1239 *vs* 698 cm^3; $P < 0.05$). Furthermore, prolonged survival was observed in pleural disseminated tumor-bearing mice with temsirolimus treatment (median survival: vehicle *vs* temsirolimus = 53.5 *vs* 72.5 days; $P < 0.05$). These results suggest that temsirolimus could be useful for NSCLC treatment, due to its antiproliferative effect, and could be a potential treatment for advanced NSCLC, giving prolonged survival. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.01967.x, 2011)

Lung cancer is one of the most aggressive malignancies with poor prognosis. It is estimated that more than 160 000 and 65 000 lung cancer patients in the USA and Japan, respectively, die each year.[1,2] A wide variety of new chemotherapy medicines have been developed and introduced in clinical practice, but the mortality rate has not been improved.[1] Recently, the strategy of drug development has focused on targeting particular molecules that are supposed to be critical for cancer progression. Several molecules in the growth factor receptor pathway are specifically targeted because those molecules are well recognized as being aberrantly regulated in cancers. For example, epidermal growth factor receptor (EGFR) and its downstream molecules are often upregulated due to gene amplification or mutation;[3,4] therefore, targeting EGFR is a major therapeutic strategy for non-small-cell lung carcinoma (NSCLC).[5] Gefitinib is a well-known small molecule inhibitor that selectively suppresses EGFR tyrosine kinase activity[6] and has been applied in the treatment of NSCLC.[7] Several studies have shown that gefitinib treatment has a drastic antitumor effect in a subset of NSCLC which had acquired certain types of EGFR mutation.[8,9] Since the appearance of gefitinib, several selective EGFR inhibitors have been developed. However, these drugs only revealed a minimal effectiveness due to the aberrant regulation of molecules located downstream from the receptor tyrosine kinase pathways including Ras-Raf-MAPK and phosphatidylinositol 3′-kinase (PI3K)-Akt.[10,11] Among them, mammalian target of rapamycin (mTOR) is one of the major effectors regulated by the PI3K-Akt signaling pathway and plays a central role in this stimulated growth.[12,13] Moreover, there is an upregulation of mTOR activity in many types of cancers including NSCLC.[14,15] Therefore, several compounds that selectively inhibit mTOR activity have been developed for clinical use.[16,17] Temsirolimus (CCI-779), an analogue of rapamycin, was recently synthesized to specifically inhibit mTOR and has provided prolonged survival of patients with renal cell carcinoma. It was also reported that temsirolimus showed a certain antitumor effect on other types of cancers including breast cancer,[18] glioblastoma,[19] neuroendocrine carcinomas,[20] and mantle cell lymphoma.[21] Moreover, temsirolimus has antitumor effects in other diseases such as lymphangioleiomyomatosis.[22] Based on these observations, we questioned whether temsirolimus treatment could be a potential therapeutic option for NSCLC. In this study, we evaluated the antiproliferative and antitumor effects of temsirolimus in NSCLC *in vitro* and *in vivo*, with an assessment of its survival advantage in an animal model of advanced NSCLC.

Materials and Methods

Cell lines and cultures. Three cancer cell lines that were established from human NSCLC (A549, H1299, and H358) were used in this study. A549 was cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) and H1299 and H358 were cultured in RPMI-1640 medium (Sigma-Aldrich) at 37°C in humidified air with 5% CO_2 These media were supplemented with 10% FCS (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich).

Reagents. Temsirolimus, commercialized as Tricel by Wyeth K.K. (Madison, NJ, USA), was purchased from OZ International (Tokyo, Japan). The temsirolimus was diluted to the final concentration with culture media before an *in vitro* experiment. When temsirolimus was used *in vivo*, it was dissolved and diluted to a final concentration of 10 mg/kg with 0.9% sodium chloride.

Trypan blue exclusion assay. Cancer cells (5.0×10^3 per well) were plated directly in 24-well dishes with culture medium.

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After the cells entered into an exponential growth phase, they were treated with different concentrations of temsirolimus (0, 0.1, 1, 10, 100, or 1000 nM) for 48 h, stained with Trypan blue, and the number of viable cells was counted using a hemacytometer.

Apoptosis assay. Cells in apoptosis were determined by TUNEL assay using a MEBSTAIN Apoptosis kit II (MBL International, Woburn, MA, USA) according to the manufacturer's protocol. Briefly, cells (1.0×10^4 per well) were seeded on Lab-Tek 8-well permanox chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA) and were treated with 10 nM/L of temsirolimus or with an equivalent volume of diluted DMSO (final concentration, 0.005%) as a control for 48 h. The TUNEL-positive cells were counted with a fluorescence microscope.

Cell cycle analysis by flow cytometry. For cell cycle analysis, cancer cells were plated in six well tissue culture plates and treated with different concentrations of temsirolimus (0, 1, 10, or 100 nM/L). After a 24-h treatment, the cells were harvested and stained with 20 mg/mL propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (FACScan; Becton Dickinson) using CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Whole cell lysates and nuclear protein were extracted using M-PER buffer (Thermo Fisher Scientific, Rockford, IL, USA) and NE-PER buffer (Thermo Fisher Scientific) supplemented with protease inhibitors and phosphatase inhibitors. The protein concentration of the collected supernatants was determined and equal amounts of protein were electrophoresed under a reducing condition in gradient polyacrylamide gels (ATTO, Tokyo, Japan) and were then transferred onto PVDF filter membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 h. An Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA) was used for signal detection. The antibodies used for Western blotting were phospho-mTOR (Ser2448), mTOR, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-S6 ribosomal protein (Ser235/236), and hydroxy-HIF-1α (Pro564) (D43B5). All of them were obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin was obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was obtained from Dako Cytomation (Glostrup, Denmark). Goat anti-rabbit IgG was obtained from American Qualex Antibodies (La Mirada, CA, USA).

Animal experiments. The protocol for the animal experiments was approved by the Ethics Review Committee for Animal Experiments of Okayama University (Okayama, Japan). Mice used in this study were purchased from Clea (Tokyo, Japan). A549 s.c. xenografts were produced on the backs of 6-week-old male BALB/c nu/nu mice by injecting 3×10^6 cells mixed with Matrigel (BD Biosciences) at a 1:1 ratio. After 7 days, the tumor-bearing animals were randomized into two groups that consisted of seven mice each: (i) temsirolimus (10 mg/kg given i.v. once/week for 5 weeks); and (ii) saline alone as a vehicle (given i.v. once/week for 5 weeks). Tumor volume was measured weekly (length × width × height). To create the A549 pleural dissemination model, 4×10^6 cancer cells were intrathoracically injected into the pleural cavity of 6-week-old male BALB/c nu/nu mice. After 7 days, the animals were randomized into two groups that consisted of eight mice each; (i) temsirolimus (10 mg/kg given i.p. once/week for 5 weeks); and (ii) saline alone as a vehicle. The drug was given once a week and lasted until the mice expired. Each animal experiment was repeated three times and the representative data is shown. The dose and schedule of temsirolimus treatment (10 mg/kg/week) in these animal experiments was decided based upon previous reports where the researchers used a range of 8–20 mg/kg/week.[23–25]

Immunohistochemistry. Surgically resected pleural membrane tissues from mice with disseminated pleural tumors from A549 cells were used for immunohistochemical study following procedures described previously.[26] Deparaffinized tissue sections were immersed in methanol containing 3% hydrogen peroxide to block endogenous peroxide activity. An autoclave pretreatment in citrate buffer was done for antigen retrieval. After incubation with a blocking buffer the sections were treated with an anti-phospho-mTOR rabbit mAb (Cell Signaling Technology) for 6 h at room temperature followed by immunobridging with Avidin DH-biotinylated HRP complex (Nichirei, Tokyo, Japan). Signal detection was done for 2–5 min using 3,3′-diaminobenzidine tetrahydrochloride dissolved to 50 mM/L Tris–HCl (pH 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with Mayer-hematoxylin. Monoclonal anti-human mouse Ki-67 antibody (MIB-1; Dako Cytomation) was used to calculate the Ki-67 labeling index by counting the number of positively stained cells per 1000 cancer cell nuclei for each section.

Statistical analysis. Student's *t*-test was used to compare data between two groups. Data represent the mean ± SD. Overall survival was calculated using the Kaplan–Meier method and compared by the log–rank test. $P < 0.05$ was considered statistically significant.

Results

Inhibition of mTOR by temsirolimus suppresses cell growth of NSCLC cells. First, we examined how effective temsirolimus was at inhibiting the proliferation of cultured NSCLC cells using a Trypan blue exclusion assay. As shown in Figure 1, temsirolimus suppressed the cell proliferation of A549, H1299, and H358 cells in a dose-dependent manner. The IC_{50} values were measured to examine the suppression of cell proliferation by temsirolimus in NSCLC cell lines. The IC_{50} for A549 cells was 0.76 nM and those for H1299 and H358 were 0.75 nM and 0.64 nM, respectively (Fig. 1). These data indicated that temsirolimus effectively inhibited the viability of NSCLC cells at a low concentration of <1 nM.

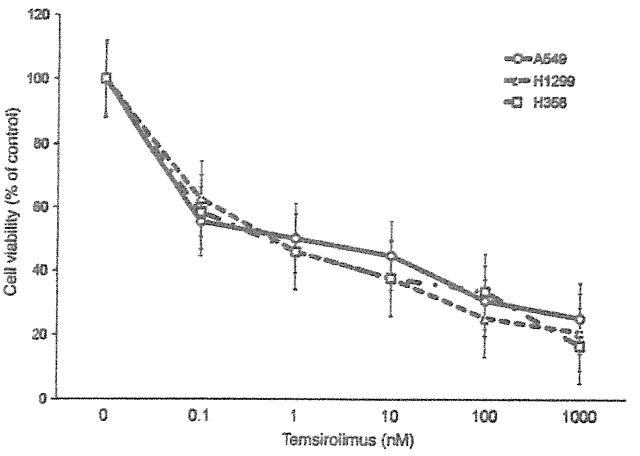

Fig. 1. Temsirolimus suppresses cell proliferation of non-small-cell lung carcinoma cells in a dose-dependent manner. Cultured cells were treated with the indicated concentrations of temsirolimus for 48 h and the number of viable cells was counted by the Trypan blue exclusion method. The IC_{50} values of A549, H1299, and H358 cells were 0.76, 0.75, and 0.64 nM, respectively.

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Temsirolimus inhibited mTOR pathway in a dose- and time-dependent manner. Next, in order to evaluate the effect of temsirolimus on regulating activation in the mTOR pathway, we examined the phosphorylation of mTOR and its downstream effectors by Western blot analysis. As expected, the treatment with temsirolimus suppressed the activations of mTOR, p70 ribosomal S6 kinase, and S6 in a dose-dependent fashion in A549 (Fig. 2A). This inhibitory effect occurred after 1 h, and lasted at least 4 h (Fig. 2B). Similar results were obtained in another NSCLC cell line, H1299 (Fig. 2C,D). Furthermore, we assessed the expression status of cell cycle markers including $p21^{cip1}$, $p27^{kip1}$, and cyclinD1, whose expression is often modified by the inactivation of p70 S6 kinase and S6.[27] Interestingly, $p21^{cip1}$ was apparently induced by temsirolimus treatment, but we did not observe any change in cyclinD1 or $p27^{kip1}$ (Fig. S1). Because the upregulation of $p21^{cip1}$ is known to contribute to cell cycle arrest, these data suggest that the inhibition of the mTOR pathway using temsirolimus is a promising strategy to diminish the proliferation of NSCLC cells.

Temsirolimus treatment leads to G_1 cell cycle arrest but not cell death. Our next question was whether temsirolimus treatment is lethal to NSCLC cells. In order to answer this question, we carried out flow cytometry to analyze the cell cycle distribution in A549 and H1299 cell lines under temsirolimus treatment. Interestingly, temsirolimus treatment increased the cell population in the G_0/G_1 phase, but not in the sub-G_0 phase, which accounted for dead cells (Fig. 3). Furthermore, when we examined the amount of apoptosis by TUNEL assay, we did not observe a significant number of apoptotic cells (data not shown). Taken together, these results suggested that temsirolimus suppressed NSCLC cell proliferation by its cytostatic effect, not by cytotoxicity.

Temsirolimus reduces s.c. tumor growth of NSCLC cells. Next, we investigated the effect of temsirolimus on *in vivo* tumor growth. A549 s.c. xenografts were made. When 10 mg/kg temsirolimus was given weekly i.v. to the mice bearing the s.c. tumor, a significant delay of s.c. tumor growth was observed on day 35 (tumor volume: vehicle *vs* temsirolimus = 1239 *vs* 698 cm^3; $P < 0.05$) (Fig. 4). None of the mice died of drug-induced toxicity and no other significant adverse events were observed. Moreover, during the observation period (up to 35 days after cell inoculation), there was no significant change in body weight in either group (data not shown).

Temsirolimus treatment prolonged survival of mice with disseminated pleural tumors of NSCLC cells. As shown above, we found that temsirolimus had a cytostatic effect on NSCLC cells and showed a delay of s.c. tumor growth. Based on these results, we predicted that a major advantage of temsirolimus treatment would be an improvement in the survival of patients bearing NSCLC tumors, similar to renal cell carcinoma.[28] To investigate the effect of temsirolimus on survival, we made a pleural dissemination animal model by injecting A549 cells into the intrapleural cavity, to mimic an advanced clinical stage of NSCLC. A weekly i.p. injection of 10 mg/kg temsirolimus significantly prolonged the survival period of these pleural disseminated tumor-bearing mice (median survival: vehicle *vs* temsirolimus = 53.5 *vs* 72.5 days; $P < 0.05$) (Fig. 5A,B).

Macroscopic observation by opening the thoracic cavity of the mice showed that temsirolimus treatment obviously reduced the number and the volume of pleural disseminated tumors on day 21 after the inoculation of A549 cells in the thoracic cavity (Fig. 5C,D), although tumors were recognized in a bilateral thoracic cavity regardless of temsirolimus treatment. This result led us to speculate that temsirolimus reduced the growth of pleural disseminated tumors, leading to the prolonged survival of the tumor-bearing mice. Furthermore, immunohistochemical analysis revealed that phosphorylation of mTOR was strongly suppressed in the tumor tissues of the temsirolimus-treated mice (Fig. 5E,F).

The immunohistochemical analysis for Ki-67 using disseminated pleural tumor tissues revealed a significant decrease in the number of proliferating cells (determined by calculating the

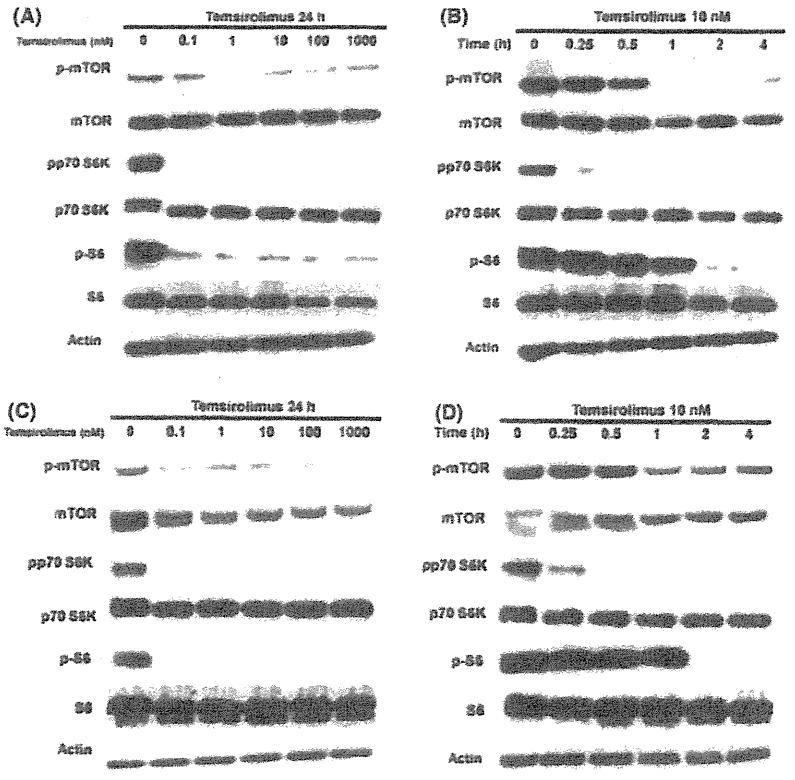

Fig. 2. Temsirolimus suppresses the activation of mTOR and its downstream effectors. Whole cell lysates of A549 (A,B) and H1299 (C,D) non-small-cell lung carcinoma cells that were treated with the indicated concentrations of temsirolimus were used for Western blot to determine the inhibitory effects on mTOR and its downstream effectors in dose-dependent (A,C) and time-course (B,D) studies. p-mTOR, phospho-mTOR; pp70 S6K, phospho-p70 S6 kinase; p-S6, phospho-S6 ribosomal protein.

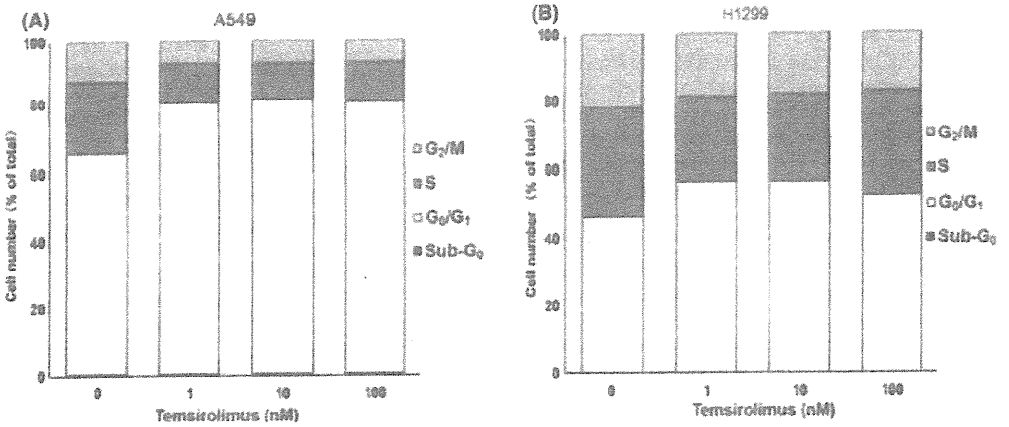

Fig. 3. Temsirolimus induces cell cycle arrest rather than cell death. A549 (A) and H1299 (B) non-small-cell lung carcinoma cells were treated with 10 nM temsirolimus for 24 h and the cell cycle distribution was analyzed by flow cytometry.

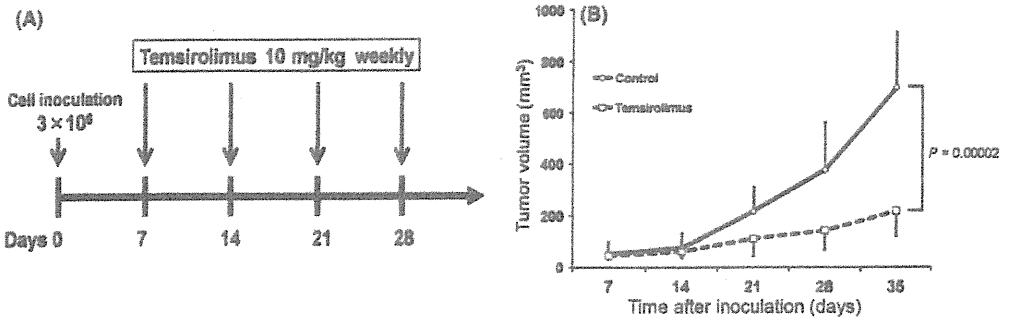

Fig. 4. Temsirolimus reduces the growth of s.c. tumors of A549 non-small-cell lung carcinoma cells. A549 cells were inoculated s.c. in the dorsum of nude mice (day 0) and i.v. injections of either temsirolimus (10 mg/kg) or saline as a vehicle were started from day 7 and continued once a week (A). Tumor volume was measured as a cube (length × width × height) and was tracked for up to 5 weeks (B). The representative data were taken from three independent experiments.

Ki-labeling index, defined in Materials and Methods) in the tissues treated with temsirolimus (temsirolimus, 0.106 ± 0.019; control, 0.191 ± 0.044; $P < 0.05$) (Fig. S2A). However, temsirolimus treatment did not increase the incidence of apoptosis in the tumor tissues, as checked by immunohistochemistry for cleaved caspase-3 (temsirolimus, 0.004 ± 0.002; control, 0.004 ± 0.002; $P > 0.05$) (Fig. S2B). These results were similar to our *in vitro* data, supporting our conclusion that the primary effect of temsirolimus is antiproliferative rather than cytotoxic. Thus, the advantage of *in vivo* temsirolimus treatment was to provide prolonged survival in advanced NSCLC tumor-bearing mice by suppressing tumor growth.

Inhibition of mTOR by temsirolimus suppresses the action of hypoxia inducible factor 1α (HIF-1α). Finally, we assessed the inhibition of mTOR by temsirolimus in NSCLC cells and tumors. Because recent reports have shown that the action of HIF-1α, a major transcriptional activator for angiogenesis and oncogenes, is regulated by the mTOR pathway,[29] and is therefore inhibited by temsirolimus *in vitro* and *in vivo*,[25,30] we also determined the effect of temsirolimus on the expression status of HIF-1α in the nuclei, where activated HIF-1α normally translocates.[31] Temsirolimus treatment suppressed the translocation of HIF-1α to the nucleus in all of NSCLC cells (Fig. S3A). As HIF-1α is known to play a critical role in cell proliferation and angiogenesis,[32] this inhibition of HIF-1α action by temsirolimus should at least partially contribute to its antiproliferative effect.

Regarding the antiangiogenic effect of temsirolimus by negatively regulating HIF-1α, we additionally determined the expression of vascular endothelial cell growth factor (VEGF), a known transcriptional target of HIF-1α. In cultured NSCLC cells, the amount of VEGF protein secreted in the culture medium was suppressed by temsirolimus treatment in a dose-dependent manner (Fig. S3B,C). Similarly, the production of VEGF mRNA expression, especially the 572-bp form of VEGF, was decreased in the pleural disseminated tumors of the mice that had temsirolimus treatment (Fig. S3D). The inhibition of HIF-1α/VEGF-mediated angiogenesis might also contribute to slowing tumor growth by temsirolimus treatment.

Discussion

Temsirolimus, an analogue of rapamycin, is a new molecular targeted agent and was first approved for the treatment of renal cell carcinoma. In terms of NSCLC, it was reported that inhibiting mTOR with rapamycin revealed a growth inhibitory effect in some NSCLC cell lines.[33] Temsirolimus was developed as an improved derivative of rapamycin,[34] and our data indicated its effectiveness by showing its potent inhibitory effect on cell proliferation of cultured NSCLC cells at a low concentration (as low as 1 nM). Concerning the antiproliferative effect of temsirolimus, our results reproduced the results of a previous report using rapamycin, which induced cell cycle arrest at the G_1 checkpoint and inhibited cell proliferation of murine NSCLC without inducing apoptosis.[23] In this study, temsirolimus suppressed the phosphorylations of p70 S6 kinase and S6 (Fig. 2). As the action of p70 S6 kinase and S6 is critical for

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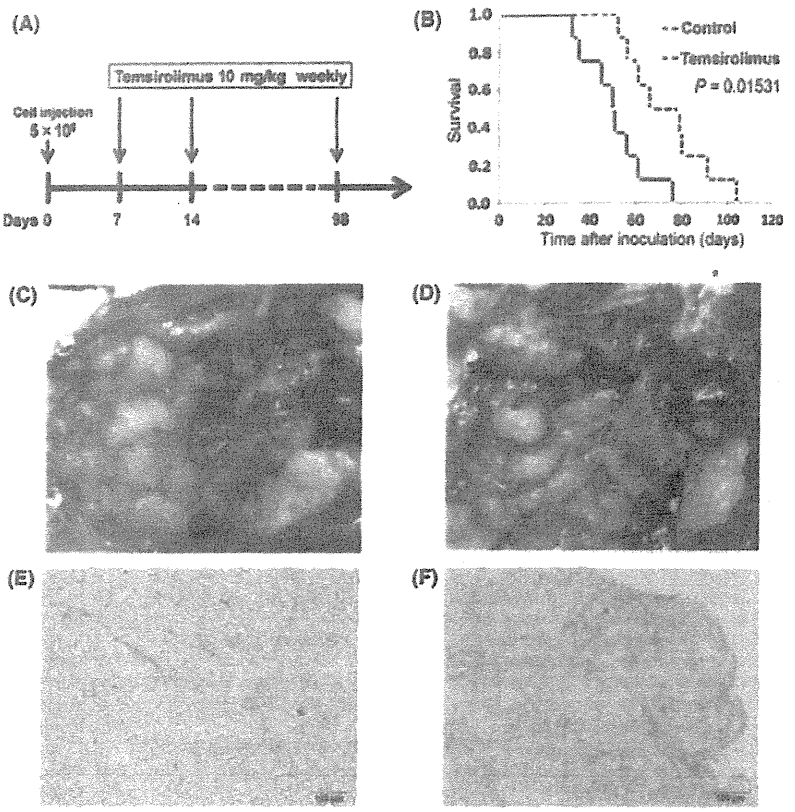

Fig. 5. Temsirolimus prolongs the survival of pleural disseminated tumor-bearing mice. A549 non-small-cell lung carcinoma cells were injected into the thoracic cavity of mice (day 0) and i.p. injections of either temsirolimus (10 mg/kg) or saline as a vehicle were started from day 7 and continued once a week (A). Cell survival periods were tracked to draw a survival curve by the Kaplan–Meier method (B). Representative images of macroscopic observation in the thoracic cavity on day 21 are shown (C, vehicle only; D, temsirolimus). Immunohistochemical examination of resected disseminated tumor tissues from the control mice (E) and temsirolimus treated mice (F) was carried out to assess the expression status of phosphorylated mTOR (day 21). Each photograph was taken at high magnification (×200). The experiment was repeated three times and the representative data are shown.

cell cycle progression,[27,35] the cytostatic effect of temsirolimus can be at least partially explained by the importance of p70 S6 kinase to cell cycle progression. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Akt are also interesting molecules related to cell proliferation signals. A recent study using rapamycin[33] showed that the inhibition of mTOR by temsirolimus appeared to regain Akt activity (Fig. S1). According to a previous report,[36] PTEN was lost in H1299 cells by its promoter methylation, whereas it remained intact in A549 cells. Regardless of their PTEN expression, our data indicated the similar potent antiproliferative effects of temsirolimus on those cell lines (Fig. 1).

Using an animal model of pleural dissemination, a condition for human lung cancer patients with one of the worst survival rates, we observed that temsirolimus reduced the growth of both s.c. tumors and pleural disseminated tumors of NSCLC cells, and that the treatment significantly prolonged the survival of mice bearing disseminated pleural tumors (Fig. 5). It is noteworthy that the dose and schedule of temsirolimus treatment in this study followed those currently in clinical use for renal cell carcinoma, with no apparent adverse effects in the mice. Because this regimen has also been tolerated in several clinical studies for other cancers,[18–21] temsirolimus treatment might safely provide prolonged survival for advanced NSCLC patients, possibly due to its cytostatic effect.

One immunohistochemical study showed that there were differences in mTOR signaling activation depending on histological type.[14] According to that study, adenocarcinoma had more frequent ctivation of phosphorylated mTOR than squamous cell carcinoma. However, it was unclear what histological type of NSCLC temsirolimus treatment would be effective in clinical use. mTOR is frequently activated in adenocarcinoma, but the outcome differs depending on the mTOR expression.[37] In a future study, it would be intriguing to establish a more effective combination therapy with temsirolimus,[38] because mTOR activity can be modified by other effectors, such as growth factors[39] and nutrition.[40,41]

In conclusion, our data suggests that temsirolimus, with a cytostatic effect on cell proliferation, may be useful for NSCLC treatment in general and could give prolonged survival to advanced NSCLC cases with pleural dissemination specifically.

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Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Jemal A, Siegel R, Ward E *et al.* Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71–96.
2 Vital Statistics Japan (Ministry of Health, Labour and Welfare). Number of cancer deaths by age and site 2007. [Cited 21 Mar 2010.] Available from URL: http://ganjoho.ncc.go.jp/professional/index.html.
3 Suzuki S, Dobashi Y, Sakurai H, Nishikawa K, Hanawa M, Ooi A. Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas. An immunohistochemical and fluorescence in situ hybridization study. *Cancer* 2005; **103**: 1265–73.
4 Yatabe Y, Takahashi T, Mitsudomi T. Epidermal growth factor receptor gene amplification is acquired in association with tumor progression of EGFR-mutated lung cancer. *Cancer Res* 2008; **68**: 2106–11.

5 El-Rayes BF, LoRusso PM. Targeting the epidermal growth factor receptor. *Br J Cancer* 2004; **91**: 418–24.
6 Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin Cancer Res* 2000; **6**: 4885–92.
7 Herbst RS, Kies MS. ZD1839 (Iressa) in non-small cell lung cancer. *Oncologist* 2002; **7**: 9–15.
8 Okabe T, Okamoto I, Tamura K *et al.* Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells bearing EGFR gene mutation and amplification. *Cancer Res* 2007; **67**: 2046–53.
9 Asahina H, Yamazaki K, Kinoshita I. A phase II trial of gefitinib as first-line therapy for advanced non-small cell lung cancer with epidermal growth factor receptor mutations. *Br J Cancer* 2006; **95**: 998–1004.
10 Das AK, Sato M, Story MD *et al.* Non-small-cell lung cancers with kinase domain mutations in the epidermal growth factor receptor are sensitive to ionizing radiation. *Cancer Res* 2006; **66**: 9601–8.
11 Balak MN, Gong Y, Riely GJ *et al.* Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin Cancer Res* 2006; **12**: 6494–501.
12 Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004; **18**: 1926–45.
13 Inoki K, Ouyang H, Li Y, Guan KL. Signaling by target of rapamycin proteins in cell growth control. *Microbiol Mol Biol Rev* 2005; **69**: 79–100.
14 Dobashi Y, Suzuki S, Matsubara H, Kimura M, Endo S, Ooi A. Critical and diverse involvement of Akt/mammalian target of rapamycin signaling in human lung carcinomas. *Cancer* 2009; **115**: 107–18.
15 Pal SK, Figlin RA, Reckamp KL. The role of targeting mammalian target of rapamycin in lung cancer. *Clin Lung Cancer* 2008; **9**: 340–5.
16 Campone M, Levy V, Bourbouloux E *et al.* Safety and pharmacokinetics of paclitaxel and the oral mTOR inhibitor everolimus in advanced solid tumours. *Br J Cancer* 2009; **100**: 315–21.
17 Wolpin BM, Hezel AF, Abrams T *et al.* Oral mTOR inhibitor everolimus in patients with gemcitabine-refractory metastatic pancreatic cancer. *J Clin Oncol* 2009; **27**: 193–8.
18 Chan S, Scheulen ME, Johnston S *et al.* Phase II study of Temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer. *J Clin Oncol* 2005; **23**: 5314–22.
19 Chang SM, Wen P, Cloughesy T *et al.* Phase II study of CCI-779 in patients with recurrent glioblastoma multiforme. *Invest New Drugs* 2005; **23** (4): 357–61.
20 Duran I, Kortmansky J, Singh D *et al.* A phase II clinical and pharmacodynamic study of Temsirolimus in advanced neuroendocrine carcinomas. *Br J Cancer* 2006; **95**: 1148–54.
21 Hess G, Herbrecht R, Romaguera J *et al.* Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. *J Clin Oncol* 2009; **27**: 3822–9.
22 Ohara T, Oto K, Miyoshi K *et al.* Sirolimus ameliorated post lung transplant chylothorax in lymphangioleiomyomatosis. *Ann Thorac Surg* 2008; **86**: e7–8.
23 Dudkin L, Dilling MB, Cheshire PJ *et al.* Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition. *Clin Cancer Res* 2001; **7**: 1758–64.
24 deGraffenried LA, Friedrichs WE, Russell DH *et al.* Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. *Clin Cancer Res.* 2004;**10**: 8059–67.
25 Wan X, Shen N, Mendoza A, Khanna C, Helman LJ. CCI-779 inhibits rhabdomyosarcoma xenograft growth by an antiangiogenic mechanism linked to the targeting of mTOR/Hif-1a/VEGF signaling. *Neoplasia* 2006; **8**: 394–401.
26 Shirakawa Y, Naomoto Y, Kimura M *et al.* Topological analysis of p21WAF1/CIP1 expression in esophageal squamous dysplasia. *Clin Cancer Res* 2000; **6**: 541–50.
27 Berven LA, Crouch MF. Cellular function of p70S6K: a role in regulating cell motility. *Immunol Cell Biol* 2000; **78**: 447–51.
28 Hudes G, Carducci M, Tomczak P *et al.* Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. *N Engl J Med* 2007; **356**: 2271–81.
29 Fang J, Ding M, Yang L, Liu LZ, Jiang BH. PI3K/PTEN/AKT signaling regulates prostate tumor angiogenesis. *Cell Signal* 2007; **19**: 2487–97.
30 Del Bufalo D, Ciuffreda L, Trisciuoglio D *et al.* Antiangiogenic potential of the mammalian target of rapamycin inhibitor temsirolimus. *Cancer Res* 2006; **66**: 5549–54.
31 Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. Regulation of the Hypoxia-inducible Transcription Factor 1α by the Ubiquitin-Proteasome Pathway. *J Biol Chem* 1999; **274**: 6519–25.
32 Zhong H, De Marzo AM, Laughner E *et al.* Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 1999; **59**: 5830–5.
33 Sun SY, Rosenberg LM, Wang X *et al.* Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 2005; **65**: 7052–8.
34 Boffa DJ, Luan F, Thomas D *et al.* Rapamycin inhibits the growth and metastatic progression of non-small cell lung cancer. *Clin Cancer Res* 2004; **10**: 293–300.
35 Pullen N, Thomas G. The modular phosphorylation and activation of p70s6k. *FEBS Lett* 1997; **410**: 78–82.
36 Soria JC, Lee HY, Lee JI *et al.* Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. *Clin Cancer Res* 2002; **8**: 1178–84.
37 Anagnostou VK, Bepler G, Syrigos KN *et al.* High expression of mammalian target of rapamycin is associated with better outcome for patients with early stage lung adenocarcinoma. *Clin Cancer Res* 2009; **15**: 4157–64.
38 Dancey J. mTOR signaling and drug development in cancer. *Nat Rev Clin Oncol* 2010;**7**:209–19.
39 Kodama Y, Baxter RC, Martin JL. Insulin-like growth factor-I inhibits cell growth in the a549 non-small lung cancer cell line. *Am J Respir Cell Mol Biol* 2002; **27**: 336–44.
40 Cohen A, Hall MN. An amino acid shuffle activates mTORC1. *Cell* 2009; **136**: 399–400.
41 Kleijn M, Proud CG. Glucose and amino acids modulate translation factor activation by growth factors in PC12 cells. *Biochem J* 2000; **347**: 399–406.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Temsirolimus induces $p21^{cip1}$ expression in none-small-cell lung carcinoma cells.

Fig. S2. Proliferation and apoptosis in tumor tissues after temsirolimus treatment in mice.

Fig. S3. Inhibition of mTOR by temsirolimus decreases the expression of proangiogenic effectors.

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Cediranib in combination with mFOLFOX6 in Japanese patients with metastatic colorectal cancer: results from the randomised phase II part of a phase I/II study

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Background: Colorectal cancer (CRC) is the second most common malignancy in Japan. Treatment with inhibitors of the vascular endothelial growth factor (VEGF) signalling pathway has proven benefit in metastatic CRC. Cediranib is an oral highly potent VEGF signalling inhibitor that inhibits all three VEGF receptors.

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Patients and methods: In this phase II, double-blind, placebo-controlled study, 172 patients with metastatic CRC were randomised to receive once-daily cediranib (20 or 30 mg) or placebo, each combined with modified FOLFOX6 (mFOLFOX6). The primary objective was comparison of progression-free survival (PFS).
Results: The comparison of cediranib 20 mg versus placebo met the primary objective of PFS prolongation [hazard ratio = 0.70 (95% confidence interval 0.44–1.11), $P = 0.167$], which met the protocol-defined criterion of $P < 0.2$. Median PFS was 10.2 versus 8.3 months, respectively. The PFS comparison for cediranib 30 mg versus placebo did not meet the criterion. The most common adverse events (AEs) in the cediranib-containing groups were diarrhoea and hypertension.
Conclusions: Cediranib 20 mg plus mFOLFOX6 met the predefined criteria in terms of improved PFS compared with placebo plus mFOLFOX6. Cediranib 20 mg was generally well tolerated and the AE profile was consistent with previous studies.
Key words: cediranib, colorectal cancer, mFOLFOX6, placebo, progression-free survival

introduction

In Japan, the incidence of colorectal cancer (CRC) has increased nearly fivefold in the last 25 years, owing primarily to changing Japanese dietary habits, which are becoming increasingly similar to those of Western countries. In 2008, there were 101 656 new cases of CRC in Japan and 43 349 deaths attributed to this disease [1]. CRC is now the second most common malignancy in Japan and is predicted to become the most common by 2015. Fluorouracil (5-FU) was one of the first chemotherapies used for the treatment of CRC, and the combination of 5-FU with leucovorin and oxaliplatin (FOLFOX) has improved outcomes. Treatment with these components (plus irinotecan in some regimens) can provide a median overall survival (OS) of up to 20 months, compared with ~6 months with best supportive care [2]. Japanese clinical guidelines recommend FOLFOX as standard treatment of metastatic colorectal cancer (mCRC) [3]. To reduce toxicity associated with the FOLFOX regimen, a number of modifications have been tried [4, 5]; the current standard is modified FOLFOX6 (mFOLFOX6).

Inhibition of the vascular endothelial growth factor (VEGF) signalling pathway with bevacizumab has demonstrated additional clinical benefit in CRC when used with 5-FU-based regimens in the first-line setting in mCRC [6, 7]. Cediranib is an oral highly potent VEGF tyrosine kinase inhibitor (TKI) that inhibits all three VEGF receptors [8, 9]. Cediranib is suitable for once-daily dosing and has demonstrated antitumour activity during early phase clinical evaluation in patients with advanced cancer [10]. Further studies demonstrated that cediranib was generally well tolerated as monotherapy [11–15] and in combination with various anticancer agents at doses ≤30 mg/day [16–21].

The efficacy of cediranib in combination with chemotherapy has been investigated in two phase III studies—HORIZON II [22] and HORIZON III [23]—in Western patients with previously untreated mCRC. Two cediranib doses were initially selected for investigation in the HORIZON programme: 20 (lowest biologically active dose) and 30 mg/day (maximum dose suitable for chronic dosing in combination with chemotherapy). The decision to investigate cediranib 20 and 30 mg/day doses in this study was taken before an end-of-phase II decision from the HORIZON programme to proceed with only the 20 mg/day dose. As such, this two-part phase I/II study, which mirrored HORIZON II, investigated cediranib, at the same doses used initially in the Western studies, plus mFOLFOX6 in Japanese patients with previously untreated mCRC (ClinicalTrials.gov identifier NCT00494221; AstraZeneca study code D8480C00039). The phase I part of this study demonstrated that both doses of cediranib were generally well tolerated in combination with mFOLFOX6 [24]. Here, we report the results of the randomised, double-blind, phase II part of this study, which assessed the efficacy of cediranib (20 or 30 mg/day) plus mFOLFOX6 compared with mFOLFOX6 alone.

patients and methods

eligibility

Eligible patients were aged ≥18 years with histological or cytological confirmation of carcinoma of the colon or rectum. Patients required chemotherapy for stage IV (metastatic) disease, had a World Health Organisation (WHO) performance status (PS) of zero or one, and one or more measurable lesions according to the RECIST (version 1.0). Any adjuvant oxaliplatin or 5-FU therapy must have been completed >12 and >6 months, respectively, before study entry. Patients with brain or meningeal metastases were considered eligible if they were clinically stable and had not required corticosteroid treatment of 10 days. Exclusion criteria included prior systemic therapy for metastatic disease and prior therapy with monoclonal antibodies or small molecule inhibitors against VEGF or VEGF receptors, including bevacizumab and cediranib.

study design

This phase II, randomised, double-blind, placebo-controlled study assessed the efficacy of first-line treatment with cediranib plus mFOLFOX6 compared with mFOLFOX6 alone. Patients were randomised 1 : 1 : 1 to receive once-daily cediranib (20 or 30 mg) or placebo, each in combination with 14-day treatment cycles of mFOLFOX6 (oxaliplatin 85 mg/m^2 IV, day 1; leucovorin 200 mg/m^2 IV, day 1; 5-FU 400 mg/m^2 IV bolus, day 1 and then 2400 mg/m^2 continuous IV infusion over 46 h). Patients were stratified at randomisation according to a two-level liver function covariate [based on baseline albumin and alkaline phosphatase (ALP) levels] and WHO PS (0 versus 1). Randomised treatment was continued until objective disease progression (as defined by RECIST) or until the occurrence of toxicity, death, withdrawal of patient consent or other discontinuation criteria. RECIST measurements were made using computed tomography or magnetic resonance imaging scans; clinical assessment of these scans was conducted by the study investigators.

The primary objective was to determine the efficacy of cediranib plus mFOLFOX6 compared with mFOLFOX6 alone by assessment of progression-free survival (PFS). Secondary objectives included comparison of OS, objective response rate (ORR: complete response + partial response), duration of response, change in tumour size and assessment of the safety

and tolerability of cediranib plus mFOLFOX6. An exploratory end point was to investigate the effect of treatment on soluble markers of angiogenesis (VEGF and sVEGFR-2). VEGF and sVEGFR-2 were measured by enzyme-linked immunosorbent assay of plasma samples from patients who provided separate informed consent.

PFS and ORR were determined from objective tumour assessments (RECIST) carried out at weeks 6, 12, 18, 24 and then every 12 weeks until disease progression or death. Adverse events (AEs) were recorded and graded according to Common Terminology Criteria for Adverse Events version 3.0. The study was approved by each centre's institutional review board and was carried out in accordance with the Declaration of Helsinki, the International Conference on Harmonisation/Good Clinical Practice, applicable regulatory requirements and the AstraZeneca policy on Bioethics.

statistical analysis

Assuming a median PFS of 9 months in the placebo group, an 18-month accrual period and a minimum 12-month follow-up, a total of 55 patients per group was required to have 80% power to detect a true PFS hazard ratio (HR) of 0.6 at two-sided significance level of $P < 0.2$ (one-sided $P < 0.1$), which was considered appropriate evidence of activity for a randomised phase II study [25]. The primary PFS analysis was conducted using a log-rank test stratified by WHO PS (0 or 1) and a two-level baseline liver function covariate (covariate 1 for baseline albumin < 3.5 g/l or ALP > 320 U/l; covariate 0 for all other values). PFS and OS were summarised by treatment group using the Kaplan–Meier method. The formal analysis was conducted when ~105 progression events had occurred across the three groups. No formal statistical analysis was carried out on safety data.

The results in the present study were relatively immature (65% of PFS events versus 81% in HORIZON II) and the HR was favourable compared with HORIZON II (HR = 0.84). Furthermore, there was a higher proportion of patients with a PS of zero. Therefore, further analysis of efficacy and safety outcomes was carried out when 81% of progression events had occurred.

results

patients

Between January 2008 and January 2009, 172 Japanese patients were randomised to treatment with cediranib 20 mg plus mFOLFOX6 ($n = 58$), cediranib 30 mg plus mFOLFOX6 ($n = 56$) or placebo plus mFOLFOX6 ($n = 58$) (Figure 1). Patient characteristics were representative of the patient population (Table 1). All patients were Japanese and 20%

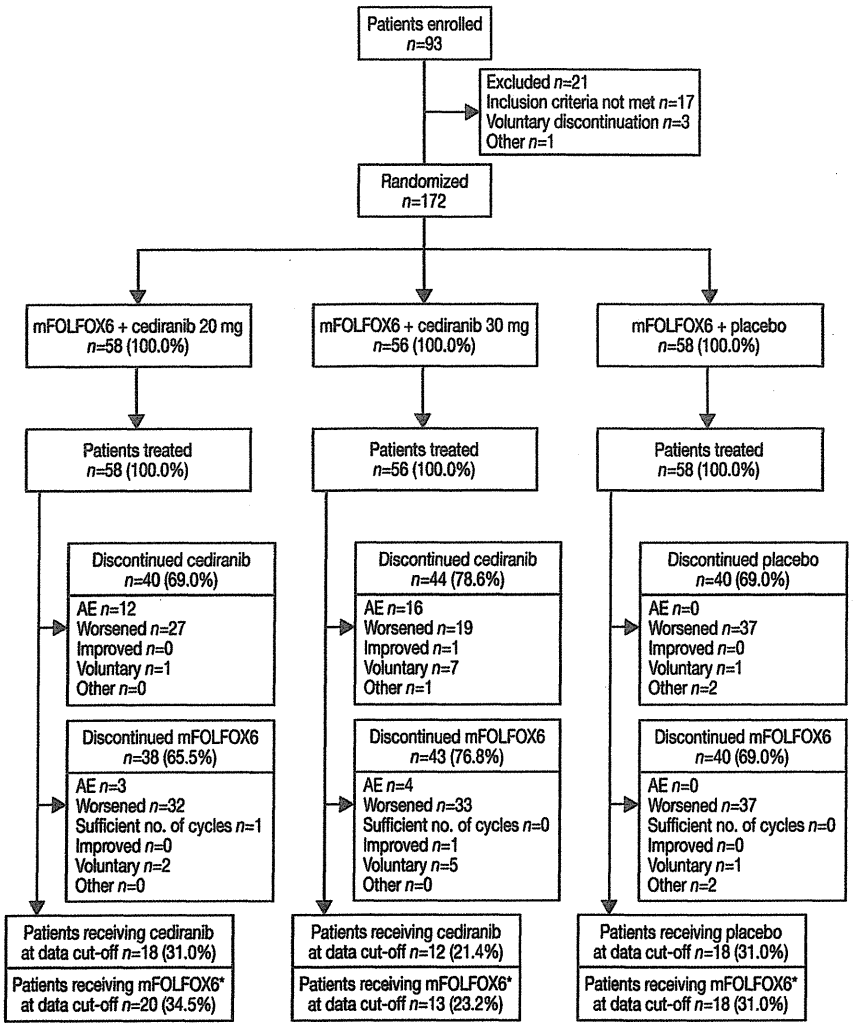

Figure 1. CONSORT diagram.

were receiving antihypertensive treatment at baseline. Baseline characteristics were generally well balanced across the groups, although there were more female patients in the cediranib 30 mg group. Imbalances were noted in metastases at baseline, time from initial diagnosis to randomisation, tumour grading, baseline ALP and baseline liver function (Table 1).

At the protocolled data cut-off (13 October 2009), 65% (112) of patients had progressed and 22% (38) had died. The most common reason for discontinuation of placebo/cediranib was worsened condition. At the second data cut-off (11 June 2010), 81% of patients had progressed and median OS follow-up was 19.0 months with 74 OS events.

efficacy

For the PFS comparison of cediranib 20 mg versus placebo, the HR was 0.70 [95% confidence interval (CI) 0.44–1.11], two-sided $P = 0.167$ (Figure 2A), which met the protocol-defined criterion for evidence of activity ($P < 0.2$). Median PFS was 10.2 and 8.3 months, respectively. For the PFS comparison of cediranib 30 mg versus placebo, the HR was 0.82 (95% CI 0.54–1.31), two-sided $P = 0.261$ (Figure 2B), which did not meet the predefined criterion. Median PFS was 8.9 months in the cediranib 30 mg arm. Predefined subgroup analysis of PFS for both dose groups did not identify a particular patient

Table 1. Patient demographics and baseline characteristics

Characteristic	Cediranib 20 mg + mFOLFOX6 ($n = 58$)	Cediranib 30 mg + mFOLFOX6 ($n = 56$)	Placebo + mFOLFOX6 ($n = 58$)
Median age (range), years	63.5 (33–79)	64.5 (40–82)	64.0 (36–80)
Sex, *n* (%)			
Male	38 (65.5)	30 (53.6)	39 (67.2)
Female	20 (34.5)	26 (46.4)	19 (32.8)
World Health Organisation performance status, *n* (%)			
0	44 (75.9)	43 (76.8)	47 (81.0)
1	14 (24.1)	13 (23.2)	11 (19.0)
Type of cancer, *n* (%)			
Colon	39 (67.2)	34 (60.7)	36 (62.1)
Rectal	19 (32.8)	22 (39.3)	22 (37.9)
Tumour grading, *n* (%)			
Well differentiated (G1)	11 (19.0)	14 (25.0)	16 (27.6)
Moderately differentiated (G2)	44 (75.9)	38 (67.9)	36 (62.1)
Poorly differentiated (G3)	2 (3.4)	3 (5.4)	4 (6.9)
Undifferentiated (G4)	1 (1.7)	1 (1.8)	1 (1.7)
Unassessable (GX)	0	0	1 (1.7)
Metastatic sites, *n* (%)			
1	32 (55.2)	29 (51.8)	28 (48.3)
>1	26 (44.8)	27 (48.2)	30 (51.7)
Metastases at baseline, *n* (%)			
Patients with liver only metastases at baseline	14 (24.1)	10 (17.9)	14 (24.1)
Patients with liver and other metastases at baseline	25 (43.1)	22 (39.3)	32 (55.2)
Patients with no liver involvement at baseline	19 (32.8)	24 (42.9)	12 (20.7)
Prior adjuvant therapy, *n* (%)			
Yes	13 (22.4)	9 (16.1)	8 (13.8)
No	45 (77.6)	47 (83.9)	50 (86.2)
Time from initial diagnosis to randomisation, *n* (%)			
<6 months	36 (62.1)	38 (67.9)	45 (77.6)
6 to <12 months	2 (3.4)	0	1 (1.7)
12 to <24 months	6 (10.3)	10 (17.9)	4 (6.9)
24 to <36 months	6 (10.3)	2 (3.6)	3 (5.2)
≥36 months	8 (13.8)	6 (10.7)	5 (8.6)
Baseline ALP, *n* (%)			
≤320 U/l	31 (53.4)	35 (62.5)	29 (50.0)
>320 U/l	27 (46.6)	21 (37.5)	29 (50.0)
Baseline liver function			
ALP > 320U/l or albumin < 35 g/l	29 (50.0)	22 (39.3)	30 (51.7)
Other	29 (50.0)	34 (60.7)	28 (48.3)
Baseline vascular endothelial growth factor			
n	36	37	38
Mean (standard deviation), pg/ml	146.5 (416.3)	74.3 (56.6)	96.9 (100.7)
Median (min, max), pg/ml	46.6 (31.2, 2520.5)	55.5 (31.2, 243.3)	54.6 (31.2, 508.1)

mFOLFOX6, modified FOLFOX6; ALP, alkaline phosphatase.

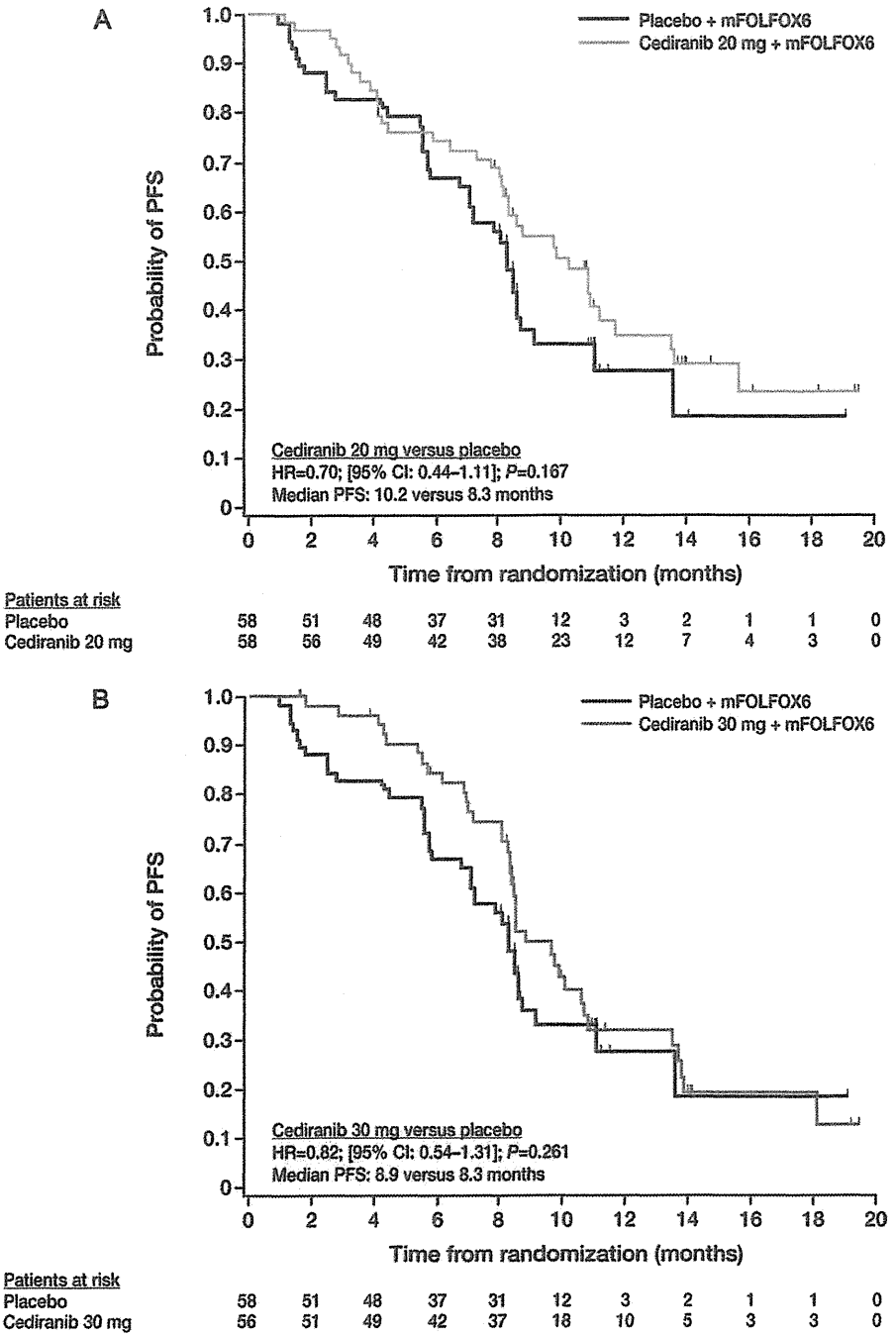

Figure 2. (A) Progression-free survival (PFS) for patients who received cediranib 20 mg + modified FOLFOX6 (mFOLFOX6) versus placebo + mFOLFOX6. (B) PFS for patients who received cediranib 30 mg + mFOLFOX6 versus placebo + mFOLFOX6.

population that derived a differential PFS benefit from cediranib versus placebo (supplemental Figure S1, available at *Annals of Oncology* online).

The ORR was 53.4%, 69.6% and 53.4% in the cediranib 20 mg, cediranib 30 mg and placebo arms, respectively; RECIST best response is summarised in Table 2. The median best percentage changes in tumour size were −37.3% (cediranib 20 mg), −43.4% (cediranib 30 mg) and −40.0% (placebo). The median duration of response was 9.2 (cediranib 20 mg), 6.7 (cediranib 30 mg) and 7.1 months (placebo) (Figure 3). At the primary analysis, there were insufficient deaths (total = 38; 15, 9 and 14 in the cediranib 20 mg, cediranib 30 mg and placebo arms, respectively) to draw conclusions on OS.

safety and tolerability

Overall, the most common AEs were diarrhoea and hypertension (Table 3); neither caused discontinuation of cediranib at the 20 mg dose. The incidence of AEs leading to discontinuation of cediranib/placebo was higher in the cediranib 30 mg group (27%) compared with the cediranib 20 mg (19%) or placebo (0%) groups; of these, only decreased

Table 2. Best RECIST response

Best response, *n* (%)	Cediranib 20 mg + mFOLFOX6 (*n* = 58)	Cediranib 30 mg + mFOLFOX6 (*n* = 56)	Placebo + mFOLFOX6 (*n* = 58)
CR	0	0	2 (3.4)
PR	31 (53.4)	39 (69.6)	29 (50.0)
Stable disease ≥6 weeks	24 (41.4)	14 (25.0)	20 (34.5)
Progressive disease	3 (5.2)	1 (1.8)	7 (12.1)
Non-evaluable	0	2 (3.6)	0

mFOLFOX6, modified FOLFOX6; CR, complete response; PR, partial response.

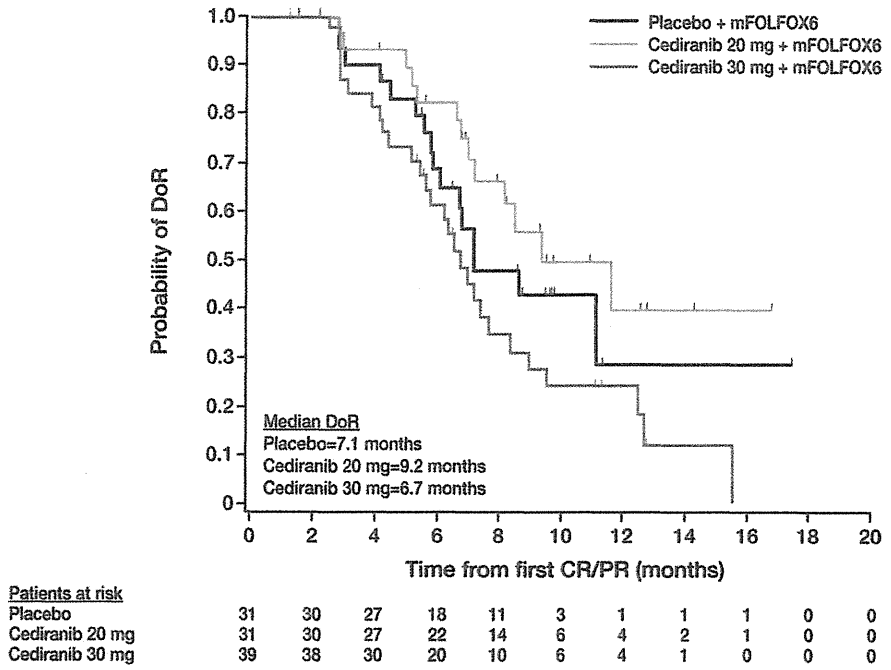

Figure 3. Duration of response for patients who received cediranib 20 mg, cediranib 30 mg or placebo, each in combination with modified FOLFOX6.

Table 3. AEs (frequency ≥30% in any group)

AE, *n* (%)	Cediranib 20 mg + mFOLFOX6 (*n* = 58)	Cediranib 30 mg + mFOLFOX6 (*n* = 56)	Placebo + mFOLFOX6 (*n* = 58)
Diarrhoea	53 (91.4)	49 (87.5)	22 (37.9)
Hypertension	47 (81.0)	48 (85.7)	18 (31.0)
Decreased appetite	43 (74.1)	43 (76.8)	39 (67.2)
Fatigue	39 (67.2)	40 (71.4)	36 (62.1)
Peripheral neuropathy	42 (72.4)	35 (62.5)	38 (65.5)
Nausea	39 (67.2)	37 (66.1)	37 (63.8)
PPES	31 (53.4)	34 (60.7)	8 (13.8)
Stomatitis	33 (56.9)	30 (53.6)	25 (43.1)
Vomiting	24 (41.4)	27 (48.2)	14 (24.1)
Dysphonia	24 (41.4)	16 (28.6)	2 (3.4)
Dysgeusia	18 (31.0)	17 (30.4)	18 (31.0)
Constipation	21 (36.2)	14 (25.0)	16 (27.6)
Alopecia	12 (20.7)	17 (30.4)	15 (25.9)
Epistaxis	15 (25.9)	19 (33.9)	9 (15.5)
Dysphonia	24 (41.4)	16 (28.6)	2 (3.4)

AE, adverse event; mFOLFOX6, modified FOLFOX6; PPES, palmar–plantar erythrodysaesthesia syndrome (hand–foot syndrome).