endothelial cells and myeloma cells. On the basis of these findings, Am80 could be a promising clinical agent against MM.

Acknowledgements

We thank Dr H Kagechika (University of Tokyo, Tokyo, Japan) for the kind gift of Am80. This work is supported in part by Grant-in-Aids for SI, MO, MK and RU from the Ministry of Education, Culture, Sports, Science and Technology, and for SI and RU from the Ministry of Health, Labor and Welfare. SI is supported by a Grant of the Princess Takamatsu Cancer Research Fund.

Supplementary Information

Supplementary Information accompanies the paper on the *Leukemia* website (http://www.nature.com/leu).

References

1 Iida S, Ueda R. Multistep tumorigenesis of multiple myeloma: its molecular delineation. *Int J Hematol* 2003; 77: 207–212.
2 Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. *Nat Rev Cancer* 2002; 2: 927–937.
3 Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM *et al.* Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromall cell interactions in multiple myeloma. *Blood* 2000; 95: 2630–2636.
4 Urashima M, Ogata A, Chauhan D, Vidriales MB, Teoh G, Hoshi Y *et al.* Interleukin-6 promotes multiple myeloma cell growth via phosphorylation of retinoblastoma protein. *Blood* 1996; 88: 2219–2227.
5 Vacca A, Ribatti D, Roncali L, Ranieri G, Serio G, Silvestris F *et al.* Bone marrow angiogenesis and progression in multiple myeloma. *Br J Haematol* 1994; 87: 503–508.
6 Ribatti D, Vacca A, Nico B, Quondamatteo F, Ria R, Minischetti M *et al.* Bone marrow angiogenesis and mast cell density increase simultaneously with progression of human multiple myeloma. *Br J Cancer* 1999; 79: 451–455.
7 Giuliani N, Colla S, Lazzaretti M, Sala R, Roti G, Mancini C *et al.* Proangiogenic properties of human myeloma cells: production of angiopoietin-1 and its potential relationship to myeloma-induced angiogenesis. *Blood* 2003; 102: 638–645.
8 Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L *et al.* Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997; 57: 4593–4599.
9 Bellamy WT, Richter L, Sirjani D, Roxas C, Glinsmann-Gibson B, Frutiger Y *et al.* Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. *Blood* 2001; 97: 1427–1434.
10 Lin B, Podar K, Gupta D, Tai YT, Li S, Weller E *et al.* The vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584 inhibits growth and migration of multiple myeloma cells in the bone marrow microenvironment. *Cancer Res* 2002; 62: 5019–5026.
11 Moehler TM, Ho AD, Goldschmidt H, Barlogie B. Angiogenesis in hematologic malignancies. *Crit Rev Oncol Hematol* 2003; 45: 227–244.
12 D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA* 1994; 91: 4082–4085.
13 Breitman TR, Selonick SE, Collins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci USA* 1980; 77: 2936–2940.
14 Ponzoni M, Bocca P, Chiesa V, Decensi A, Pistoia V, Raffaghello L *et al.* Differential effects of *N*-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: apoptosis *vs* differentiation. *Cancer Res* 1995; 55: 853–861.
15 Lu XP, Fanjul A, Picard N, Pfahl M, Rungta D, Nared-Hood K *et al.* Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells *in vivo*. *Nat Med* 1997; 3: 686–690.
16 Chen YH, Desai P, Shiao RT, Lavelle D, Haleem A, Chen J. Inhibition of myeloma cell growth by dexamethasone and all-trans retinoic acid: synergy through modulation of interleukin-6 autocrine loop at multiple sites. *Blood* 1996; 87: 314–323.
17 Taetle R, Dos Santos B, Akamatsu K, Koishihara Y, Ohsugi Y. Effects of all-*trans* retinoic acid and antireceptor antibodies on growth and programmed cell death of human myeloma cells. *Clin Cancer Res* 1996; 2: 253–259.
18 Ogata A, Nishimoto N, Shima Y, Yoshizaki K, Kishimoto T. Inhibitory effect of all-*trans* retinoic acid on the growth of freshly isolated myeloma cells via interference with interleukin-6 signal transduction. *Blood* 1994; 84: 3040–3046.
19 Delva L, Cornic M, Balitrand N, Guidez F, Miclea JM, Delmer A *et al.* Resistance to all-*trans* retinoic acid (ATRA) therapy in relapsing acute promyelocytic leukemia: study of *in vitro* ATRA sensitivity and cellular retinoic acid binding protein levels in leukemic cells. *Blood* 1993; 82: 2175–2181.
20 Takagi K, Suganuma M, Kagechika H, Shudo K, Ninomiya M, Muto Y *et al.* Inhibition of ornithine decarboxylase induction by retinobenzoic acids in relation to their binding affinities to cellular retinoid-binding proteins. *J Cancer Res Clin Oncol* 1988; 114: 221–224.
21 Hashimoto Y, Kagechika H, Shudo K. Expression of retinoic acid receptor genes and the ligand-binding selectivity of retinoic acid receptors (RAR's). *Biochem Biophys Res Commun* 1990; 166: 1300–1307.
22 Tobita T, Takeshita A, Kitamura K, Ohnishi K, Yanagi M, Hiraoka A *et al.* Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-*trans* retinoic acid. *Blood* 1997; 90: 967–973.
23 Kagechika H, Kawachi E, Fukasawa H, Saito G, Iwanami N, Umemiya H *et al.* Inhibition of IL-1-induced IL-6 production by synthetic retinoids. *Biochem Biophys Res Commun* 1997; 231: 243–248.
24 Oikawa T, Okayasu I, Ashino H, Morita I, Murota S, Shudo K. Three novel synthetic retinoids, Re 80, Am 580 and Am 80, all exhibit anti-angiogenic activity *in vivo*. *Eur J Pharmacol* 1993; 249: 113–116.
25 Sanda T, Iida S, Ogura H, Asamitsu K, Murata T, Bacon KB *et al.* Growth inhibition of multiple myeloma cells by a novel IkB kinase inhibitor. *Clin Cancer Res* 2005; 11: 1974–1982.
26 Hayami Y, Iida S, Nakazawa N, Hanamura I, Kato M, Komatsu H *et al.* Inactivation of the E3/LAPTm5 gene by chromosomal rearrangement and DNA methylation in human multiple myeloma. *Leukemia* 2003; 17: 1650–1657.
27 Kagechika H, Kawachi E, Hashimoto Y, Himi T, Shudo K. Retinobenzoic acids. 1. Structure-activity relationships of aromatic amides with retinoidal activity. *J Med Chem* 1988; 31: 2182–2192.
28 Fong TA, Shawver LK, Sun L, Tang C, App H, Powell TJ *et al.* SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 1999; 59: 99–106.
29 Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M *et al.* Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB J* 2004; 18: 300–310.
30 Itokawa T, Nokihara H, Nishioka Y, Sone S, Iwamoto Y, Yamada Y *et al.* Antiangiogenic effect by SU5416 is partly attributable to inhibition of Flt-1 receptor signaling. *Mol Cancer Ther* 2002; 1: 295–302.

31 Hirata A, Ogawa S, Kometani T, Kuwano T, Naito S, Kuwano M *et al*. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 2002; 62: 2554–2560.
32 Nefedova Y, Landowski TH, Dalton WS. Bone marrow stromal-derived soluble factors and direct cell contact contribute to *de novo* drug resistance of myeloma cells by distinct mechanisms. *Leukemia* 2003; 17: 1175–1182.
33 Koskela K, Pelliniemi TT, Pulkki K, Remes K. Treatment of multiple myeloma with all-*trans* retinoic acid alone and in combination with chemotherapy: a phase I/II trial. *Leuk Lymphoma* 2004; 45: 749–754.

厚生労働科学研究研究費補助金
第３次対がん総合戦略研究事業

新しい薬物療法の導入と その最適化に関する研究

平成１６年度～１８年度　総合研究報告書

主任研究者　西條　長宏

平成１９（２００７）年　４月

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakamura H, Takamori S, Fujii T, Ono M, Yamana H, Kuwano M, and Shirouzu K.	Cooperative cell-growth inhibition by combination treatment with ZD1839 (Iressa) and trastuzumab (Herceptin) in non-small-cell lung cancer.	Cancer Lett.	230	33-46	2005
Nakao S, Kuwano T, Tsutsumi-Miyahara C, Ueda S, Hamano S, Sonoda K, Saijo Y, Nukiwa T, Ishibashi T, Kuwano M, and Ono M.	Infiltration of COX2-expressing macrophage is a prerequisite for IL-1beta-induced neovascularization and tumor growth.	J. Clinic. Invest.	115	2979-2991	2005
Fujii T, Nakamura A M, Yokoyama G, Yamaguchi M, Tayama K,Miwa K, Toh U, Kawamura D, Shirouzu K, Yamana H, Kuwano M, and Tsuda H.	Antineoplaston induces G_1 arrest by PKCα and MAPK pathway in SKBR-3 Breast cancer cells.	Oncology Rep	14	489-494	2005
Sata R, Ohtani H, Tsujimoto M, Murakami H, Koyabu N, Nakamura T, Uchiumi T, Kuwano M, NagataH, Tsukimori K, Nakano H, and Sawada Y.	Functional analysis of organic cation transporter 3 expressed inhuman placenta.	J. Pharmacol. Expt. Therapeut.	315	888-895	2005
桑野信彦、藤井輝彦	がん分子標的治療開発に関する基盤研究ーベッドサイドへの貢献を目指して	久留米医学会雑誌	68(1・2)別冊	16-21	2005
桑野信彦	癌の分子標的治療の進歩	癌治療と宿主	17(3)	5	2005
桑野信彦	“イレッサ(ゲフィチニブ)”と“サリドマイド”と基礎医学への期待	臨床と研究	82(10)	19-20	2005
桑野信彦、小野眞弓	血管新生阻害剤	医学のあゆみ	215 (7)	607-612	2005
Fukuda T, Ashizuka M, Nakamura T, Shibahara K, Maeda K, Izumi H, Kohno K, Kuwano M, and Uchiumi T.	Characterization of 5'-untranslated region of YB-1 mRNA and autoregulation of translation by YB-1 protein.	Nucleic AcidsRes.	32	611-622	2004
Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M, and Ono M.	Cyclooxygenase 2 is a keyenzyme for inflammatory cytokinase-induced angiogenesis.	FASEB J.	18	300-310	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ono M., Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, Fujii T, and Kuwano M.	Sensitivity to gefitinib ('Iressa', ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation.	Molec. Cancer Therapeut.	3	465-472	2004
Shibahara K, Uchiumi T, Fukuda T, Kura S, Tominaga Y, Maehara Y, Kohno K, Nakabeppu Y, Tsuzuki T, and Kuwano, M.	Targeted disruption of one allèle of the Y-box binding-1 (YB-1) gene in mouse embryonic stem cells and increased sensitivity to cisplatin and mitomycin C.	Cancer Sci.	95	348-353	2004
Hisaeda K, Inokuchi A, Nakamura T, Kohno K, Iwamoto Y, Kuwano M, and Uchiumi T.	IL-1β represses multidrug resistance protein2 gene expression through inactivation of IRF3 in human hepatic cells.	Hepatology	39	1574-1582	2004
Hirata A, Uehara H, Izumi K, Naito S, Kuwano M, and Ono M.	Direct inhibition of EGF receptor activation in vascular endothelial cells by gefitinib ('Iressa', ZD1839).	Cancer Sci.	95	614-618	2004
Hirata K, Masuda K, Morikawa W, He J-W, Kuraoka A, Kuwano M, and Kawabuchi M.	N-myc downstream-regulated gene 1 expression in injured sciatic nerves.	GLIA	47	325-334	2004
Kuwano M, Oda Y, Izumi H, Yang S-J, Uchiumi T, Iwamoto Y, Toi M, Fujii T, Yamana H, Kinoshita H, Kamura T, Tsuneyoshi M, Yasumoto K, and Kohno K.	The role of nuclear YB-1 as a global marker in drug resistance.	Molec. Cancer Therapeut.	3	1458-1492	2004
桑野信彦、和田守正、和泉弘人、河野公俊	7.多剤耐性トランスポーター：抗がん剤の MDR　臨床薬理	臨床薬理	35(2)	89-96	2004
藤井輝彦、山名秀明、桑野信彦	8. その他の治療 (1)血管新生阻害薬	胃癌治療 2004 臨牀消化器内科	19(7)	980-987	2004
桑野信彦、藤井輝彦、小野眞弓、河野公俊	序文：分子標的治療の現状と将来への展望	日本臨牀	62(7)	1211-1215	2004
河野公俊、和泉弘人、吉田毅、五十嵐友紀、若杉哲郎、新名一郎、籾井泰朋、今泉拓也、桑野信彦	4. 抗癌剤の感受性を担う分子標的とオーダーメイド化学療法	実験医学 増刊	22 (14)	180(2078)-186(2084)	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yanase K, Tsukahara S, Mitsuhashi J, and Sugimoto Y.	Functional SNPs of the breast cancer resistance protein-therapeutic effects and inhibitor development.	Cancer Letters	234	73-80	2006
Mutoh K, Mitsuhashi J, Kimura Y, Tsukahara S, Ishikawa E, Sai K, Ozawa S, Sawada J, Ueda K, Katayama K, and Sugimoto Y.	A T3587G germ-line mutation of the MDR1 gene encodes a nonfunctional P-glycoprotein.	Mol. Cancer Ther.	5	877-884	2006
Mutoh K, Tsukahara S, Mitsuhashi J, Katayama K, and Sugimoto Y.	Estrogen-mediated post transcriptional down-regulation of P-glycoprotein in *MDR1*-transduced human breast cancer cells.	Cancer Sci.	97	1198-1204	2006
Imai Y, Ishikawa E, Asada S, and Sugimoto Y	Estrogen-mediated post transcriptional down-regulation of breast cancer resistance protein/ABCG2.	Cancer Res.	65	596-604	2005
Morita H, Koyama K, Sugimoto Y, and Kobayashi J	Antimitotic activity and reversal of breast cancer resistance protein-mediated drug resistance by stilbenoids from *Bletilla striata*.	Bioorg. Med. Chem. Lett.	15	1051-1054	2005
Sugimoto Y, Tsukahara S, Ishikawa E, and Mitsuhashi J	Breast cancer resistance protein: molecular target for anticancer drug resistance and pharmacokinetics/ pharmacodynamics.	Cancer Sci.	96	457-465	2005
Kage K, Fujita T, and Sugimoto Y	Role of Cys-603 in dimer/oligomer formation of the breast cancer resistance protein BCRP/ABCG2.	Cancer Sci.	96	866-872	2005
Imai Y, Tsukahara S, Asada S, and Sugimoto Y	Phytoestrogens/flavonoids reverse breast cancer resistance protein/ABCG2-mediated multidrug resistance.	Cancer Res.	64	4346-4352	2004
Yanase K, Tsukahara S, Asada S, Ishikawa E, Imai Y, and Sugimoto Y.	Gefitinib reverses breast cancer resistance protein-mediated drug resistance.	Mol. Cancer Ther.	3	1119-1125	2004

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wang X, Nitanda T, Shi M, Okamoto M, Furukawa T, Sugimoto Y, Akiyama S, and Baba M.	Induction of cellular resistance to nucleoside reverse transcriptase inhibitors by the wild-type breast cancer resistance protein.	Biochem. Pharmacol.	68	1363-1370	2004
Tokunaga E, Oki E, Kimura Y, Yamanaka T, Egashira A, Nishida K, Koga T, Morita M, Kakeji Y, Maehara Y.	Coexistence of the loss of heterozygosity at the PTEN locus and HER2 overexpression enhances the Akt activity thus leading to a negative progesterone receptor expression in breast carcinoma.	Breast Cancer Res Treat	101(3)	249-57	2007
Yamanaka T, Okamoto T, Ichinose Y, Oda S, Maehara Y.	Methodological aspects of current problems in target-based anticancer drug development.	Int J Clin Oncol	11(3)	167-75	2006
Tokunaga E, Oki E, Nishida K, Koga T, Yoshida R, Ikeda K, Kojima A, Egashira A, Morita M, Kakeji Y, Maehara Y.	Aberrant hypermethylation of the promoter region of the *CHFR* gene is rare in primary breast cancer.	Breast Cancer Res Treat	97(2)	199-203	2006
Tokunaga E, Oki E, Nishida K, Koga T, Egashira A, Morita M, Kakeji Y, Maehara Y.	Trastuzumab and breast cancer: developments and current status.	Int J Clin Oncol	11(3)	199-208	2006
Tokunaga E, Kimura Y, Oki E, Ueda N, Futatsugi M, Mashino K, Yamamoto M, Ikebe M, Kakeji Y, Baba H, Maehara Y.	Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients.	Int J Cancer	118(2)	284-9	2006
Tokunaga E, Kimura Y, Mashino K, Oki E, Kataoka A, Ohno S, Morita M, Kakeji Y, Baba H, Maehara Y.	Activation of PI3K/Akt signaling and hormone resistance in breast cancer.	Breast Cancer	13(2)	137-44	2006
Tokunaga E, Kataoka A, Kimura Y, Oki E, Mashino K, Nishida K, Koga T, Morita M, Kakeji Y, Baba H, Ohno S, Maehara Y.	The association between Akt activation and resistance to hormone therapy in metastatic breast cancer.	Eur J Cancer	42(5)	629-35	2006
Takahashi I, Emi Y, Kakeji Y, Tokunaga E, Ushiro S, Oki E, Watanabe M, Baba H, Maehara Y.	Phase I study of S-1 and biweekly docetaxel combination chemotherapy for advanced and recurrent gastric cancer.	Oncol Rep	15(4)	849-54	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Oki E, Kakeji Y, Yoshida R, Ikeda K, Nishida K, Koga T, Egashira A, Tokunaga E, Morita M, Baba H, Maehara Y.	[A randomized controlled trial to evaluate the effect of adjuvant oral fluoropyrimidine derivative therapy after curative resection for stage II/III rectal cancer-adjuvant chemotherapy trial of S-1 for rectal cancer (ACTS-RC)].	Gan To Kagaku Ryoho	33 Suppl 1	138-43	2006
Oki E, Kakeji Y, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, Yamamoto M, Ikebe M, Maehara Y.	Impact of loss of heterozygosity of encoding phosphate and tensin homolog on the prognosis of gastric cancer.	J Gastroenterol Hepatol	21(5)	814-8	2006
Mizokami K, Kakeji Y, Oda S, Maehara Y.	Relationship of hypoxia-inducible factor 1alpha and $p21^{WAF1/CIP1}$ expression to cell apoptosis and clinical outcome in patients with gastric cancer.	World J Surg Oncol	4	94	2006
Mizokami K, Kakeji Y, Oda S, Irie K, Yonemura T, Konishi F, Maehara Y.	Clinicopathologic significance of hypoxia-inducible factor 1alpha overexpression in gastric carcinomas.	J Surg Oncol	94(2)	149-54	2006
Aishima S, Basaki Y, Oda Y, Kuroda Y, Nishihara Y, Taguchi K, Taketomi A, Maehara Y, Hosoi F, Maruyama Y, Fotovati A, Oie S, Ono M,Ueno T, Sata M, Yano H, Kojiro M, Kuwano M, and Tsuneyoshi M.	High expression of insulin-like growth factor bindingprotein-3 is correlated with lower portal invasion and better prognosis in human hepatocellular carcinoma.	Cancer Sci.	97	1182-1190	2006
Maehara Y, Oki E, Tokunaga E, Maehara S, Tsujita E, Yamashita Y, Egashira A, Taketomi A, Yoshihiro K.	[Development of a molecular target therapy on the basis of global gene analyses of gastrointestinal carcinoma].	Fukuoka Igaku Zasshi	97(2)	30-6	2006
Yoshino I, Osoegawa A, Yohena T, Kameyama T, Oki E, Oda S, Maehara Y.	Loss of heterozygosity (LOH) in non-small cell lung cancer: difference between adenocarcinoma and squamous cell carcinoma.	Respir Med	99(3)	308-12	2005
Yamamoto M, Mashino K, Shibahara K, Oki E, Kakeji Y, Baba H, Maehara Y.	[Evaluation of positive cases with peritoneal lavage cytology in gastric cancer].	Gan To Kagaku Ryoho	32(10)	1389-92	2005
Yamamoto M, Mashino K, Shibahara K, Oki E, Kakeji Y, Baba H, Maehara Y.	[Evaluation of positive cases with peritoneal lavage cytology in gastric cancer].	Gan To Kagaku Ryoho	32(10)	1389-92	2005

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tanaka Y, Miyamoto S, Suzuki SO, Oki E, Yagi H, Sonoda K, Yamazaki A, Mizushima H, Maehara Y, Mekada E, Nakano H.	Clinical significance of heparin-binding epidermal growth factor-like growth factor and a disintegrin and metalloprotease 17 expression in human ovarian cancer.	Clin Cancer Res	11(13)	4783-92	2005
Oki E, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, Mashino K, Yamamoto M, Watanabe M, Ikebe M, Kakeji Y, Baba H, Maehara Y.	Genetic mutual relationship between PTEN and p53 in gastric cancer.	Cancer Lett	227(1)	33-8	2005
Oki E, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, Mashino K, Yamamoto M, Ikebe M, Kakeji Y, Maehara Y.	Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer.	Int J Cancer	117(3)	376-80	2005
Oda S, Zhao Y, Maehara Y.	Microsatellite instability in gastrointestinal tract cancers: a brief update.	Surg Today	35(12)	1005-15	2005
Oda S, Maehara Y, Ikeda Y, Oki E, Egashira A, Okamura Y, Takahashi I, Kakeji Y, Sumiyoshi Y, Miyashita K, Yamada Y, Zhao Y, Hattori H, Taguchi K, Ikeuchi T, Tsuzuki T, Sekiguchi M, Karran P, Yoshida MA.	Two modes of microsatellite instability in human cancer: differential connection of defective DNA mismatch repair to dinucleotide repeat instability.	Nucleic Acids Res	33(5)	1628-36	2005
Kimura Y, Oda S, Egashira A, Kakeji Y, Baba H, Nakabeppu Y, Maehara Y.	A variant form of *hMTH1*, a human homologue of the *E coli mutT* gene, correlates with somatic mutation in the *p53* tumour suppressor gene in gastric cancer patients.	J Med Genet	41(5)	e57	2004
Araki K, Wang B, Miyashita K, Cui Q, Ohno S, Baba H, Zhang RG, Sugimachi K, Maehara Y, Oda S.	Frequent loss of heterozygosity but rare microsatellite instability in oesophageal cancer in Japanese and Chinese patients.	Oncology	67(2)	151-8	2004
Koizumi F, Kitagawa M, Negishi T, Onda T, Matsumoto S, Hamaguchi T, and Matsumura Y.	Novel SN-38–incorporating polymeric micelles, NK012, eradicate vascular endothelial growth factor–secreting bulky tumors.	Cancer Res.	66	10048-10056	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Negishi T, Koizumi F, Uchino H, Kuroda J, Kawaguchi T, Naito S, and Matsumura Y.	NK105, a paclitaxel-incorporating micellar nanoparticle, is a more potent radiosensitising agent compared to free paclitaxel.	Br. J. Cancer	95	601-606	2006
Shimoyama T., Koizumi F, Fukumoto H, Kiura K, Tanimoto M, Saijo N, and Nishio K.	Effects of different combinations of gefitinib and irinotecan in lung cancer cell lines expressing wild or deletional EGFR.	Lung Cancer	53	13-21	2006
Shimoyama T, Hamano T, Natsume T, Koizumi F, Kiura K, Tanimoto M, and Nishio K.	Reference profiling of the genomic response induced by an anti-microtubule agent TZT-1027 (Soblidotin) *in vitro*.	Pharmacogenomics J.	6	388-396	2006
Arao T, Yanagihara K, Takigahira M, Takeda M, Koizumi F, Shiratori Y, and Nishio K.	ZD6474 inhibits tumor growth and intraperitoneal dissemination in a highly metastatic orthotopic gastric cancer model.	Int. J. Cancer	118	483-489	2006
Nishio K, and Arao T.	Progress in the field of molecular biology and application of biotechnology to medical oncology.	Acta Med Kinki Univ.	31	57-62	2006
Kawaishi M, Yokote H, Kimura H, Kasahara K, and Nishio K.	Development and characterization of an antibody specifically recognizing a mutant EGFR (L858R) protein expressed frequently in non-small cell lung cancer.	Acta Med Kinki Univ.	31	67-74	2006
Nishio M, Taguchi F, Ohyanagi F, Horiike A, Ishikawa Y, Satoh Y, Okumura S, Nakagawa K, and Nishio K.	Gefitinib efficacy associated with multiple expression of HER family in non-small cell lung cancer.	Anticancer Res.	26	3761-3765	2006
Kato T, and Nishio K.	Clinical aspects of epidermal growth factor receptor inhibitors: Benefit and risk.	Respirology	11	693-698	2006
Basaki Y, Hosoi F, Oda Y, Fotovati A, Maruyama Y, Oie S, Ono M, Izumi H, Kohno K, SakaiK, Shikmoyama T, Nishio K, and Kuwano M.	Akt-dependent nuclear localization of malignant characteristics by ovarian cancer cells.	Oncogene	Epub ahead of print		2006
Nishio K, Arao T, Kato T, and Yokote H.	*EGFR* mutation in various tissues.	Cancer Chemother Pharmacol.	58	39-41	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yanagihara K, Takigahira M, Takeshita F, Komatsu T, NishioK, Hasegawa F, and Ochiya T.	A photon counting technique for quantitatively evaluating progression of peritoneal tumor dissemination.	Cancer Res.	66	7532-7539	2006
Park S, Shimizu C, Shimoyama T, Takeda M, Kinoshita T, Kohno T, Katsumata N, Kang YK, and Nishio K, and Fujiwara Y.	Gene expression profiling of ATP-binding cassette (ABC) transporters as a predictor of the pathologic response to neoadjuvant chemotherapy in breast cancer patients.	Breast Cancer Res.	99	9-17	2006
Sekine I, Minna D, Nishio K, Tamura T, Saijo N.	A literature review of molecular markers predictive of clinical response to cytotoxic chemotherapy in patients with lung cancer.	J Thoracic Oncol.	1	31-37	2006
Yamanaka R, Arao T, Yajima N, Homma J, Genkai N, Sano M, Sekijima M, and Nishio K.	Identification of expressed genes characterizing long-term survival in malignant glioma patients.	Oncogene	25	5994-6002	2006
Nishio K, Arao T, Shimojima T, Fujiwara Y, Tamura T, and Saijo N.	Translational studies for target-based drugs.	Cancer Chemother Pharmacol	56	S90-S93	2005
Ando K, Ohmori T,Inoue F, Kadofuku T, Hosaka T, Ishida H, Shirai T, Okuda K, Hirose T, Horichi N, Nishio K, Saijo N, Adachi M, and Kuroki T.	Enhancement of sensitivity to tumor necrosis factor α in non-small cell lung cancer cells with acquired resistance to gefitinib.	Clin Cancer Res.	11	8872-8879	2005
Shimura M, Saito A, Matsuyama S, Sakuma T, Terui Y, Ueno K, Yumoto H, Yamauchi K, Yamamura K, Mimura H, Sano Y, Yabashi M, Tamasaku K, Nishio K, NishinoY, Endo K, Hatake K, Mori Y,Ishizaka Y, and Ishikawa T.	Element array by scanning X-ray fluorescence microscopy after cis-deamminedichloro-platium (II) treatment.	Cancer Res.	65	4998-5002	2005
Yanagihara K, Takigahira M, Tanaka H, KomatsuT, Fukumoto H, Koizumi F, NishioK, Ochiya T, Ino Y, and Hirohashi S.	Development and biological analysis ofperitoneal metastasis mouse models for humanscirrhous stomach cancer.	Cancer Sci.	96	323-332	2005
Nishio M, Ohyanagi F, Horiike A, Ishikawa Y, SatohY, Okumura S, Nakagawa K, Nishio K, and Horai T.	Gegitinib treatment affects androgen levels in non-small-cell lung cancer patients.	Br. J. Cancer	92	1887-1880	2005

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Park JK, Lee SH, Kang JH, Nishio K, Saijo N, and Kuh HJ.	Synergistic interaction between gefitinib (Iressa, ZD1839) and paclitaxelagainst human gastric carcinoma cells.	Anticancer Drugs	15	809-818	2004

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Cancer Letters 230 (2005) 33–46
CANCER Letters
www.elsevier.com/locate/canlet

Cooperative cell-growth inhibition by combination treatment with ZD1839 (Iressa) and trastuzumab (Herceptin) in non-small-cell lung cancer☆

Hisashi Nakamura[a,b], Shinzo Takamori[a,*], Teruhiko Fujii[b], Mayumi Ono[c], Hideaki Yamana[b], Michihiko Kuwano[b], Kazuo Shirouzu[a]

[a]*Department of Surgery, Kurume University School of Medicine, 67 Asahi-Machi, Kurume 830-0011, Japan*
[b]*Research Center for Innovative Cancer Therapy of the 21st Century COE Program for Medical Science, Kurume University School of Medicine, Kurume 830-0011, Japan*
[c]*Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University School of Medicine, Fukuoka 812-8582, Japan*

Received 21 September 2004; received in revised form 26 November 2004; accepted 15 December 2004

Abstract

An important recent advance in anticancer therapy was the development of molecular-targeting drugs, such as the epidermal growth-factor receptor (EGFR)-targeting drug ZD1839 (Iressa) and the HER2-trageting anti-HER2 monoclonal antibody trastuzumab (Herceptin). ZD1839 and trastuzumab are reported to improve the therapeutic efficacy of treatment for non-small-cell lung cancer (NSCLC) and breast cancer, respectively, although the effectiveness of either drug alone is not satisfactory. NSCLC cells often express both EGFR and HER2. We therefore investigated whether a combination of ZD1839 and trastuzumab had an additive or synergistic antitumor effect. In culture ZD1839 inhibited the growth of four NSCLC cell lines (A549, NCI-H23, NCI-H727, and NCI-H661) that expressed various levels of EGFR, HER2, HER3, and HER4. A significant cytotoxic effect was observed when ZD1839 was combined with trastuzumab in A549 cells. However, this combination had no apparent effect in NCI-H23 cells. Significant G_1-phase arrest, increased p27 expression and decreased cyclin E or D1 levels were detected in A549 cells treated with ZD1839 and trastuzumab. No significant effects were detected in NCI-H23 cells examined. The combination treatment significantly inhibited the phosphorylation of EGFR, HER2, retinoblastoma, extracellular signal-regulated kinase-1/2, and protein kinase B/Akt in A549 cells, but not in NCI-H23 cells. Our results indicated that increased levels of constitutive EGFR/HER2 heterodimers were formed in A549 cells in the presence of ZD1839, whereas no heterodimer formation was detected in NCI-H23 cells. We therefore suggest that combination treatment with ZD1839 and trastuzumab might have improved therapeutic efficacy against NSCLC cells expressing both EGFR and HER2.

Keywords: Heterodimer formation; Non-small-cell lung cancer; ZD1839; Trastuzumab

☆ Iressa is a trademark of the AstraZeneca group of companies.

* Corresponding author. Tel.: +81 942 31 7566; fax: +81 942 34 0709.
E-mail address: stakam@med.kurume-u.ac.jp (S. Takamori).

0304-3835/$ - see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2004.12.020

1. Introduction

Epidermal growth-factor receptor (EGFR) is the prototypic member of the EGFR family of receptors, which also contains HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) [1–4]. This family is involved in regulating signaling pathways that are implicated in the proliferation, invasion, migration, survival, adhesion, and differentiation of cancer cells [3,5]. EGFR and/or HER2 are expressed at high levels in many human malignancies of epithelial origin, including lung, breast, head and neck, and bladder cancer [6,7]. They exist as monomeric receptors spanning the plasma membrane and are activated by dimerization after binding to ligands, such as EGF/TGFα, heparin-binding EGF, epiregulin, betacellulin, and amphiregulin. The homodimerization and/or the heterodimerization of EGFR with other family members induces the ligand-specific activation of a number of intracellular signal-transduction cascades, including phospholipase Cγ, phosphatidylinositol-3-kinase (PI3K)-Akt, small G-proteins, Ras. Ras GTPase-activating protein, extracellular signal-regulated kinase (ERK)-1/2, Src family kinases, and signal transducer and activator of transcription (STAT) [8]. So far, no ligand has been identified for HER2, which seems to be the preferred heterodimeric partner for all other members of the EGFR family [9]. In fact, heterodimers containing HER2 have been reported to show greater affinity for ligands [10], to generate more prolonged signals and to enhance the biological effects of EGFR ligands, such as EGF [9,11]. High expression levels of EGFR and/or HER2 are associated with disease progression and poor prognosis in patients with various malignant cancers [4,5,12–14]. EGFR and HER2 proteins are therefore attractive targets for novel anticancer therapies [3,15].

The EGFR tyrosine-kinase inhibitor ZD1839 (Iressa) is a small-molecule anticancer agent and a synthetic anilinoquinazoline [16]. It is orally active and blocks signal-transduction pathways implicated in the proliferation and survival of cancer cells, as well as other host-dependent processes that promote cancer growth [3,17–19]. It shows antiproliferative activity in various different human cancer cell in vitro [20]. ZD1839-induced tumor-growth inhibition in vivo is potentiated by combination with a range of cytotoxic anticancer agents [20–23]. Clinically significant antitumor activity and symptom relief were reported in two phase II trials (known as IDEAL 1 and 2) of ZD1839 monotherapy in patients with advanced non-small-cell lung cancer (NSCLC), all of whom had previously received treatment with platinum-based chemotherapy [24–27]. ZD1839 has now been approved in several countries—including Japan, Australia, and United States—for use in patients with inoperable or recurrent NSCLC.

The humanized anti-HER2 monoclonal antibody trastuzumab (Herceptin) is an anticancer agent that was developed against HER2. Trastuzumab activity is dependent on HER2-expression levels [28–31]. Clinical trials have clearly demonstrated that trastuzumab has significant activity against HER2-positive metastatic breast cancer [32,33].

EGFR and HER2 are overexpressed in 40–80% and 25–30%, respectively, of NSCLC patients [26, 34]. A recent report suggested that they might act in concert to sustain the autonomous proliferation of breast cancer cells [35–38]. Moreover, breast tumors that co-express EGFR and HER2 have a relatively poor prognosis compared with tumors that express only one of these receptors [39–41]. Improved therapeutic effects might therefore be obtained against NSCLC cells expressing both EGFR and HER2 through combined treatment with ZD1839 and trastuzumab.

The present study aimed to determine whether a combination of ZD1839 and trastuzumab showed significant cytotoxicity against NSCLC cells. The effects of this combination treatment are discussed with respect to cell growth, cell signaling and the cell cycle.

2. Materials and methods

2.1. Materials

ZD1839 was provided by AstraZeneca (Macclesfield, UK), trastuzumab was purchased from Chuga Pharmaceutical Company (Tokyo, Japan).

2.2. Cell lines and cell culture

The human NSCLC cell lines A549, NCI-H23, NCI-H727, and NCI-H661, and the human vulval squamous carcinoma cell line A431, were purchased from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 were used as culture media, supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. A549 and A431 were cultured in DMEM, whilst NCI-H23, NCI-H727, and NCI-H661 were cultured in RPMI 1640. All cells were maintained under standard cell-culture conditions at 37 °C and 5% CO_2 in a humid environment.

2.3. Cell viability assay

For cell viability assays, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assays (Dojindo, Kumamoto, Japan) were used in this study. Briefly, cells were seeded at a density of $3–5 \times 10^3$ cell/well in 96-well plates. After 24 h, various concentrations of ZD1839 and/or trastuzumab were added, as indicated, and the cells were incubated for a further 96 h. Subsequently, the cells were washed with phosphate-buffered salin (PBS) and 10% WST-8 solution for 2 h at 37 °C. Absorbance was measured at 450 nm using a microplate reader model 550 (Bio-Rad Laboratories, Hercules, CA). Absorbance values were expressed as the percentage of untreated controls, and the concentration of ZD1839 and/or trastuzumab resulting in 50% growth inhibition (IC_{50}) was calculated.

2.4. Colony-formation assay

Cells survival were determined by plating 1×10^3 cells in 35-mm dishes. After 24 h, the medium was replaced with medium containing ZD1839 and/or trastuzumab at the concentrations indicated. The cells were incubated in a humidified chamber at 37 °C for 10 days. They were then washed with PBS and fixed with 100% methanol. Finally, the cells were stained with 10% crystal violet in water for at least 2 h at room temperature, and the number of colonies present was counted. Clonogenic survival was expressed as the percentage of colony-forming units present in the treated cultures relative to the untreated controls.

2.5. Cell-cycle analysis

Cells were seeded at a density of 1×10^5 cells/well in six-well plates. After 24 h, the medium was replaced with medium containing ZD 1839 and/or trastuzumab at concentrations of 1 and 1 μM, respectively, as indicated. Cells were incubated in a humidified chamber at 37 °C for 48 h, and then fixed with 70% ethanol, stained with 1 mg/ml propidium iodide (PI), and examined for changes in the cell-cycle distribution using flow cytometry (FACScan: Becton Dickinson).

2.6. Western blot analysis

Cells were cultured in six-well plates until subconfluence, and then incubated with ZD1839 and/or trastuzumab at the indicated concentrations for 3 h at 37 °C. Cells were washed with PBS and lysed in buffer containing 50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% β-mercaptoethanol, and then lysed by sonication. After the removal of cell debris by centrifugation, protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Cell lysates were boiled for 5 min at 100 °C, and equal amounts of protein (10 μg) were subjected to 7.5–15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked with PBS containing 5% milk and 0.1% Tween 20, and then incubated with the following primary antibodies: anti-EGFR (1:2500; BD Transduction Laboratories, Lexington, KY); anti-HER2 (1:50; Zymed Laboratories, San Francisco, CA); anti-HER3 and anti-HER4 (1:500; Neomarkers, Freemont, CA); anti-phospho EGFR (1:2000; Upstate Biotechnology, Lake Placid, NY); anti-phospho HER2, anti-ERK-1/2, anti-phospho ERK-1/2, anti-Akt, anti-phospho Akt, anti-retinoblastoma (Rb), and anti-phospho Rb (1:1000; Cell Signaling Technology, Beverly, MA); and anti-p27, anti-cyclin E and anti-cyclin D1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing three times with Tween 20-PBS (containing 0.1% Tween 20), membranes were

incubated with secondary antibody conjugated to anti-mouse or anti-rabbit horseradish peroxidase (HRP) (Bio-Rad Laboratories). The protein conducts were visualized using the enhanced chemiluminescence Western-blotting detection system (Amersham Pharmacia Biotech). The relative expression was calculated using the NIH Image version 1.62) program (rsb.info.nih.gov/nih-image/download.html).

2.7. Immunoprecipitation and immunoblot analysis

Cells were cultured in 100-mm dishes until subconfluence, and then incubated with ZD1839 at the concentrations indicated for 3 h at 37 °C. Cells were washed with PBS and lysed in NP-40 buffer (50 mM Tris, 1 mM EDTA. 80 mM NaCl, 0.3% NP-40, and 10% glycerol containing 1 mM PMSF,

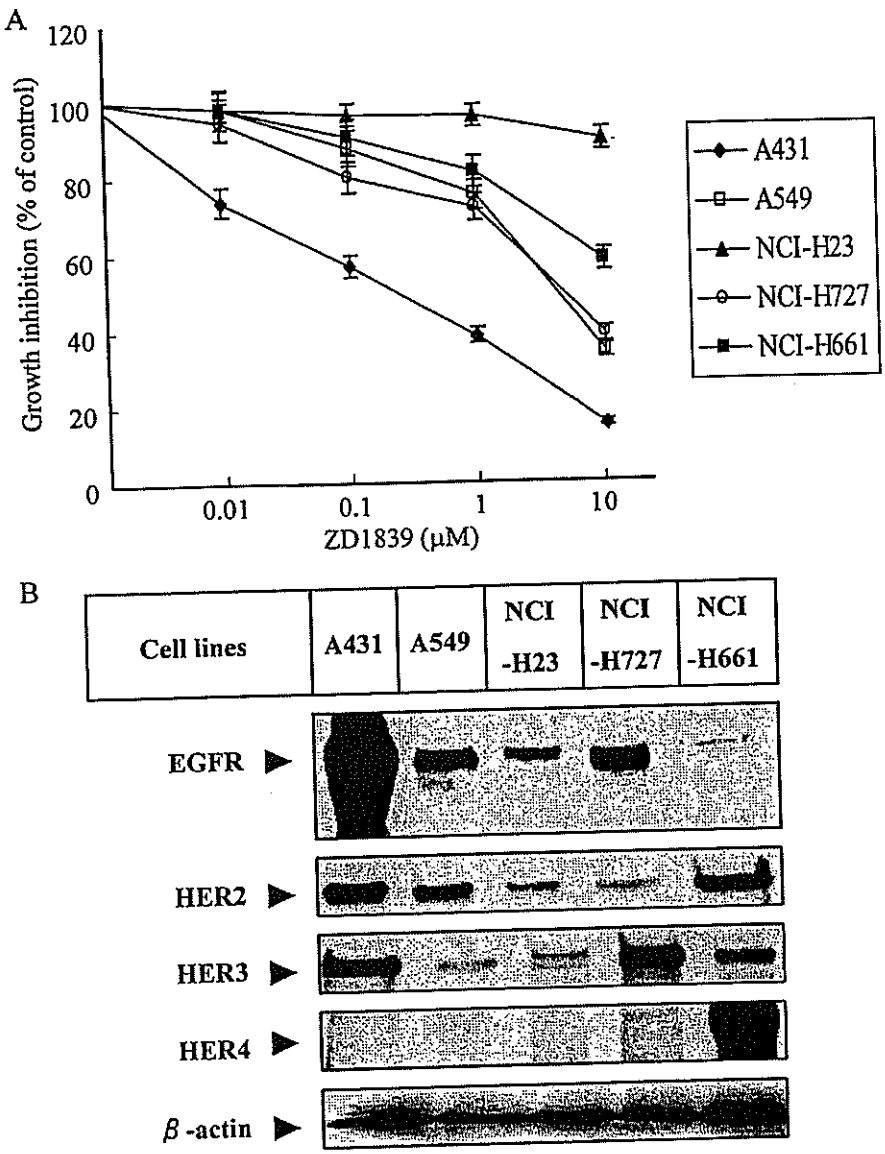

Fig. 1. Effects of ZD1839 on the growth of NSCLC cells and variation in the expression of EGFR family proteins. (A) Cell viability assays wer performed as described in Section 2, with various concentrations of ZD1839. Cells ($3–5 \times 10^3$/well) were seeded into 96-well plates. After 24 h the cells were incubated with ZD1839 for 96 h. The number of viable cells in each well was estimated using WST-8 assays. Each data poi represents the mean ± SD of triplicate experiments, expressed as a percentage of the cell growth relative to untreated controls. (B) Th expression levels of EGFR family proteins were measured using Western blot analysis. Cells were cultured in six-well plates unt subconfluence, and then cells were harvested. Equal amounts of protein were loaded onto a 7.5% SDS-PAGE gel and immunoblotted with th antibodies indicated, as detailed in Section 2. EGFR, HER2, HER3, and HER4 protein levels were determined using anti-EGFR, anti-HER anti-HER3, and anti-HER4 antibodies. Similar results were obtained in repeated experiments.

10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate). For immunoprecipitation, 2 mg of total protein from cell lysates were incubated with appropriate amounts of primary antibody at 4 °C for 3 h. Protein A/G PLUS agarose (15 μl, Santa Cruz Biotechnology) was then added for a further 2 h at 4 °C with rocking. Immnoprecipitates were pelleted by centrifugation and washed three times with lysis buffer. Captured proteins were then eluted by boiling the beads in SDS sample buffer for 5 min at 100 °C. Samples were subjected to 7.5% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. Immunoblot analysis was performed as described above.

2.8. Statistical analysis

Statistical analysis was performed using the Student's *t*-test. A probability (*P*) level of <0.05 was considered statistically significant.

3. Results

3.1. Differential effects of ZD1839 on NSCLC cell growth and EGFR family protein expression

We first examined the effects of ZD1839 on cell proliferation in four human NSCLC cell lines—A549, NCI-H23, NCI-H727, and NCI-H661—compared with the human vulval squamous carcinoma cell line A431, which is known to be highly susceptible to growth inhibition by ZD1839 [42]. ZD1839 induced dose-dependent growth inhibition in the NSCLC cell lines, and significantly inhibited the proliferation of A431 cells (Fig. 1(A)). Among the NSCLC cell lines, A549 was the most sensitive to ZD1839, whereas NCI-H23 showed the least effects.

The four NSCLC cell lines examined expressed varying amounts of EGFR, HER2, HER3, and HER4 proteins as summarized in Table 1. Consistent with previous findings [42], A431 cells expressed high levels of EGFR (Fig. 1(B)). A549, NCI-H23, and NCI-H727 cells expressed comparable levels of EGFR, whereas little, if any, EGFR was expressed in NCI-H661 cells. Comparable levels of HER2 expression were detected in A549 and NCI-H661 cells, but the levels were much lower in NCI-H23 and NCI-H727 cells. NCI-H727 and NCI-H661cells showed greater levels of HER3 expression than A549 and NCI-H23 cells. By contrast, only NCI-H661 cells expressed high levels of HER4.

3.2. Effects of combination treatment with ZD1839 and trastuzumab on NSCLC cell growth

In the next experiment, we evaluated the effects of various doses of ZD1839 on the growth of NSCLC cells in the presence or absence of trastuzumab at doses of 0.01, 0.1, and 1 μM. A combination of ZD1839 and trastuzumab induced significant growth inhibition in A549 cells (Fig. 2(A)). This combination appeared to have a slight additive effects, or no effects at all, on the growth of NCI-H23, NCI-H727, and NCI-H661 cells (Fig. 2(B), and data not shown). NCI-H23 cells in particular showed no significant growth inhibition.

Table 1
Comparison of protein expression levels of EGFR family and ZD1839-induced growth inhibition

Cell lines	EGFR family protein levels (%)[a]				Growth inhibition[b]
	EGFR	HER2	HER3	HER4	IC_{50} (μM)
A431	270	108	89	<10	0.7
A549	92	69	<10	<10	5.4
NCI-H23	41	15	<10	<10	>10
NCI-H727	100	<10	100	<10	7.6
NCI-H661	<10	100	40	100	>10

[a] Protein expression levels were examined by Western blotting. Further, the indensity of specific staining for each cell lines were quantitatived using a NIH Image, according to the manufacture's instructions. Relative EGFR family levels of five cell lines are presented when normalized by EGFR level in NCI-H727, HER2 level in NCI-H661, HER3 level in NCI-H727, HER4 level in NCI-H661.

[b] ZD1839 concentrations (μM) responsible for 50% growth inhibition in WST-8 assay at 96 h, calculated with data of triplicate experiments.

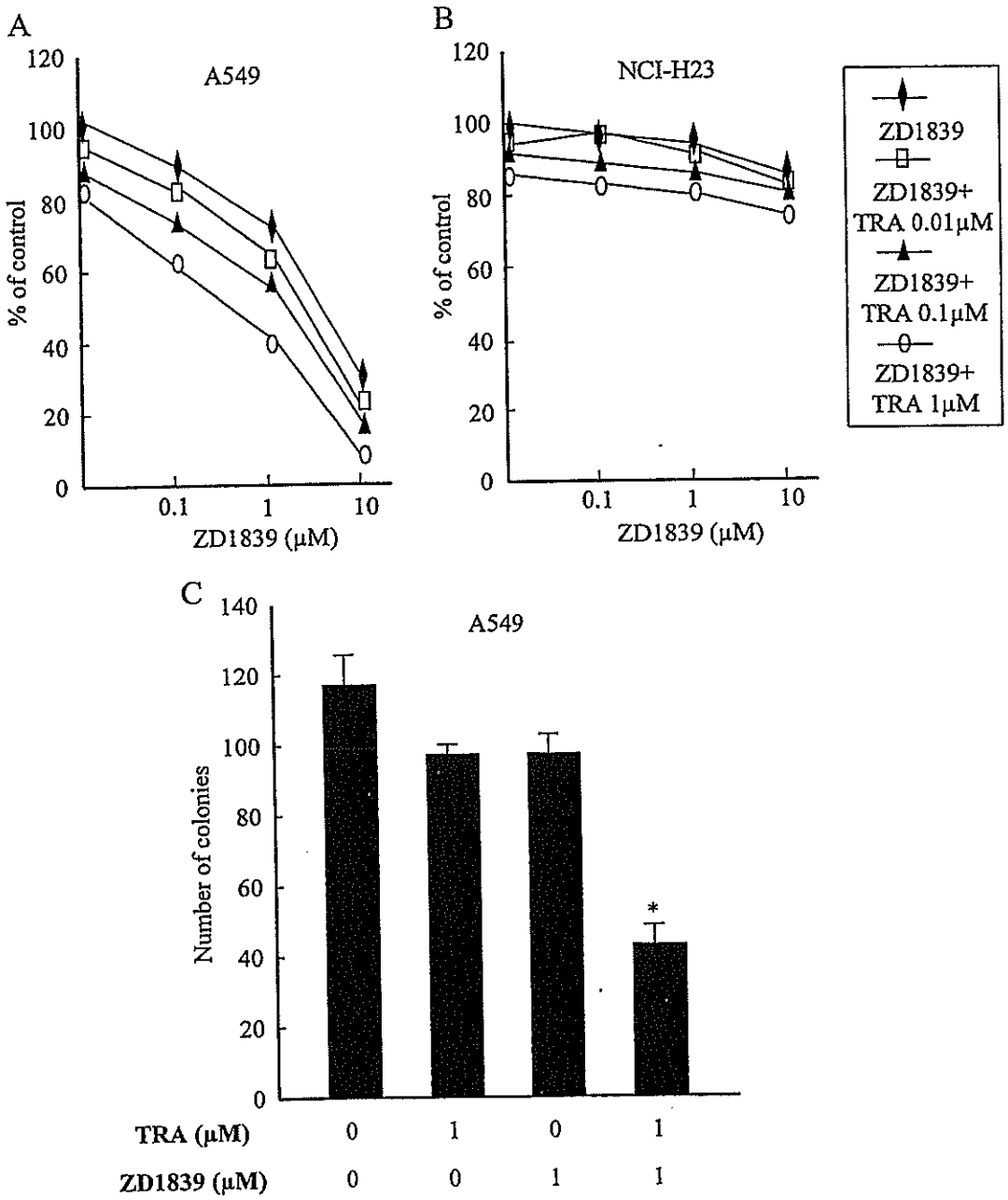

Fig. 2. Effects of combination treatment with ZD1839 and trastuzumab (TRA) on NSCLC cell proliferation and cell-colony survival. (A) A54 cells; (B) NCI-H23 cells. Cells ($3–5\times10^3$/well) were seeded into 96-well plates and incubated the following day with the concentrations c ZD1839 and/or TRA indicated. After 96 h of treatment, the number of viable cells in each well was estimated using WST-8 assays, as describe in Section 2. Similar results were obtained in triplicate experiments. The coefficient of variation was always below 10%. For reasons of clarity is not shown. (C) A549 cells (1×10^3/dish) were seeded in colony-formation assays with the concentrations of ZD1839 and/or TRA indicated, a described in Section 2. Each column represents the mean number of colonies with a diameter >50 μm in three dishes. The bars show th mean ± SD of triplicate experiments. An asterisk denotes a statistically significant difference ($P<0.05$) compared with the untreated controls c cells treated with either drug alone.

We further evaluated the effects in A549 cells using a colony-formation assay. Combination treatment with ZD1839 at 1 μM and trastuzumab at 1 μM induced significant growth inhibition in A549 cells compared with untreated controls or cells treated with either drug on its own (Fig. 2(C)), These data clearl show that a combination of ZD1839 and trastuzuma produces significant growth inhibition in A549 cell These effects were not observed in the other cell line examined (data not shown).

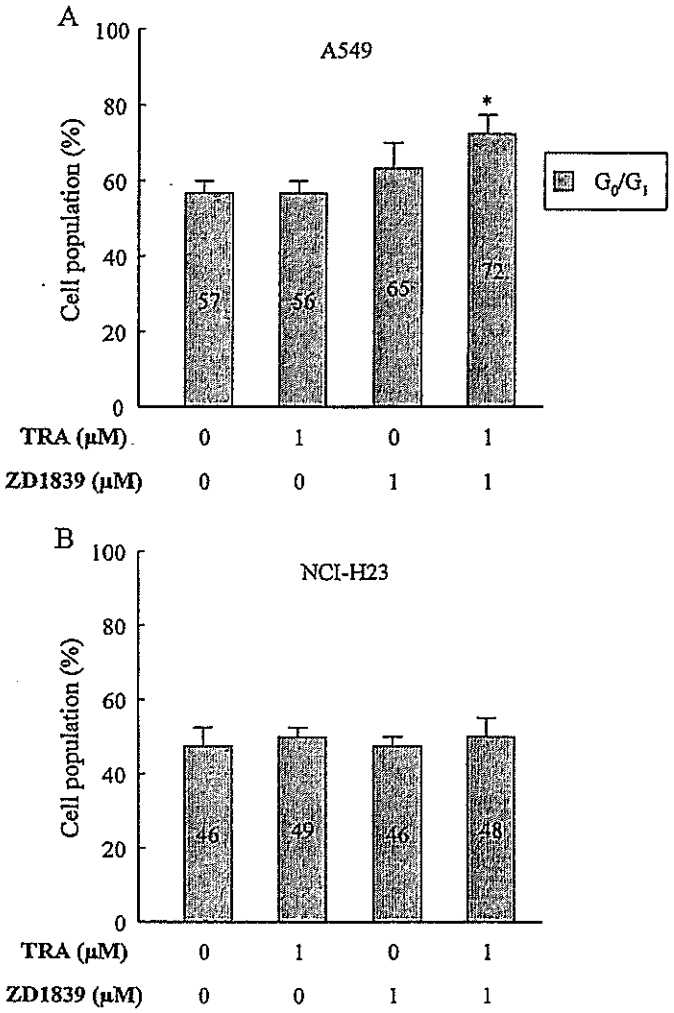

Fig. 3. Effects of combination treatment with ZD1839 and trastuzumab (TRA) on NSCLC cell-cycle progression. (A) A549 cells; (B) NCI-H23 cells. Cells were treated with ZD1839 at 1 μM and/or TRA at 1μM for 48 h. They were then fixed with 70% ethanol, stained with PI, and changes in the cell-cycle distribution were examined using flow-cytometric analysis, as described in Section 2. The numbers of cells percentage in the G_0/G_1 phase are shown in the bar graph. The data represent the mean values and the bars indicated the SD of triplicate experiments. An asterisk denotes a statistically significant difference ($P<0.05$) compared with the untreated controls or cells treated with either drug alone. Similar results were obtained in repeated experiments.

3.3. Effects of combination treatment with ZD1839 and trastuzumab on cell-cycle progression and expression of cell-cycle-related proteins

We then examined the cell growth inhibitory effects of a combination of ZD1839 and trastuzumab on cell-cycle progression using FACScan. In A549 cells, a combination of ZD1839 at 1 μM and trastuzumab at 1 μM produced a significant increase in the numbers of cells in the G_0/G_1 phase compared with untreated control cells or those treated with either drug on its own (Fig. 3(A)). This was accompanied by a corresponding decrease in the number of A549 cells in the S and G_2/M phases. Combination treatment therefore induced G_1 arrest in A549 cells. By contrast, no significant changes were observed in cell-cycle distribution in NCI-H23, NCI-H727, and NCI-H661 cells after combination treatment (Fig. 3(B), and data not shown). We next examined the expression of cell-cycle regulators, such as p27 and cyclin E or D1, which are essential for G_1/S phase progression, in NSCLC cells. Combination treatment increased the expression of cyclin-dependent kinase inhibitor p27, but decreased the expression of cyclin E or D1 (Fig. 4). The phosphorylation of Rb (Ser-780) protein was also inhibited compared with the untreated controls or cells treated with either drug alone. By contrast, the combination treatment had no significant effects on the expression of p27, cyclin E or D1, and Rb phosphorylation in NCI-H23 cells.

3.4. EGFR and HER2-induced cell-growth signaling in NSCLC cells after combination treatment with ZD1839 and trastuzumab

HER2 as well as EGFR activates a number of cytoplasmic signal transduction pathways such as the PI3K-Akt and Ras-MAP kinase. We then evaluated the effects of combination of ZD1839 and trastuzumab on EGFR, HER2, ERK-1/2, and Akt (ser-473) phosphorylation in A549 and NCI-H23 cells under basal growth conditions in the presence of 10% serum. Combination treatment with ZD1839 at 1 μM and trastuzumab at 1 μM produced significant inhibition of phosphorylation of EGFR and HER2 in A549 cells, compared with treatment with either drug alone (Fig. 5). Moreover, the combination treatment also significantly inhibited the phosphorylation of ERK-1/2 and Akt in A549 cells. No effects were detected in NCI-H23 cells.

3.5. Formation of constitutive EGFR/HER2 heterodimers in NSCLC cells

A combination of ZD1839 and trastuzumab was found to block EGFR and HER2 signaling in A549

Cell lines	A549				NCI-H23			
TRA (1 μM)	-	+	-	+	-	+	-	+
ZD1839 (1 μM)	-	-	+	+	-	-	+	+

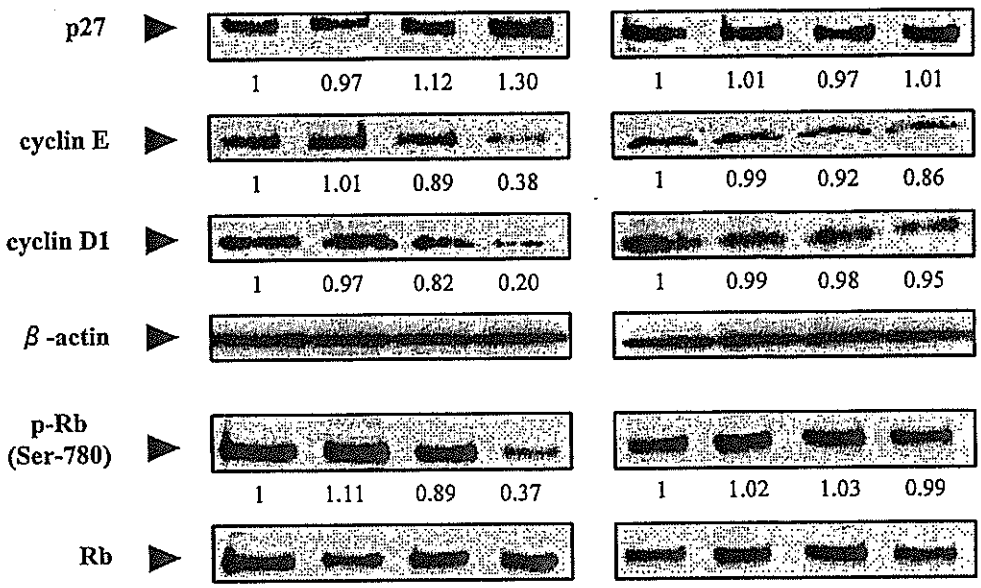

Fig. 4. Effects of combination treatment with ZD1839 and trastuzumab (TRA) on the expression of cell-cycle related proteins. The expression levels of p27, cyclin E or D1, and Rb were measured in drug-sensitive A549 and drug-resistant NCI-H23) cells. Cells were treated for 48 h with ZD1839 at 1 μM and/or TRA at 1 μM. Equal amounts of protein were separated by SDS-PAGE and subjected to immunoblot analysis with the antibodies indicated, as detailed in Section 2. p27 and cyclin E or D1 levels were determined using anti-p27 and anti-cyclin E or D1 antibodies, respectively. Rb activity was determined using an anti-phospho Rb antibody, and Rb protein levels were evaluated using anti-Rb antibody. Similar results were obtained in repeated experiments. Values indicate the density of the bands.

cells but not in NCI-H23 cells. The EGFR/HER2 heterodimer is frequently detected in cancer cell lines in culture [43,44] and is thought to have an important role in cell signaling. We therefore compared the formation of EGFR/HER2 heterodimers in A549 and NCI-H23 cells. Immunoprecipitation and immunoblot analysis revealed the formation of constitutive EGFR/HER2 heterodimers in A549 cells (Fig. 6), which showed a twofold increase in the presence of ZD1839. By contrast, NCI-H23) cells showed little, if any, formation of constitutive EGFR/HER2 heterodimers.

4. Discussion

EGFR and HER2 are important molecular targets for anticancer drugs. ZD1839 (Iressa), which selectively inhibits EGFR tyrosine kinase, is effective in patients with advanced NSCLC. The humanized anti-HER2 antibody trastuzumab (Herceptin) is effective against metastatic breast cancer [32,33]. Ye et al. [45] reported that a combination of trastuzumab and the anti-EGFR monoclonal antibody C225 had an additive antiproliferative effect in ovarian cancer cells. Moreover, several studies have demonstrated synergistic growth inhibition by trastuzumab and ZD1839 in breast cancer cells [35–38]. In the present study, we evaluated the potential cooperative antiproliferative effects of ZD1839 and trastuzumab on NSCLC cells in culture. These drugs showed significant cytotoxic effects on the proliferation of ZD1839-sensitive NSCLC cells in both WST-8 and colony-formation assays. By contrast, the combination treatment had either a slight additive effects or