96. Narisawa, T.; Fukaura, Y.; Hasebe, M.; Ito, M.; Aizawa, R.; Murakoshi, M.; Uemura, S.; Khachik, F.; Nishino, H. Inhibitory effects of natural carotenoids, α-carotene, β-carotene, lycopene and lutein, on colonic aberrant crypt foci formation in rats. *Cancer Lett.* **1996**, *107*, 137–142.
97. Faure, H.; Fayol, V.; Galabert, C.; Grolier, P.; Le Moel, G.; Steghens, J.P.; van Kappel, A.; Nabet, F. Carotenoids: 1. Metabolism and physiology. *Ann. Biol. Clin. (Paris)* **1999**, *57*, 169–183.
98. Tanaka, T.; Sugiura, H.; Inaba, R.; Nishikawa, A.; Murakami, A.; Koshimizu, K.; Ohigashi, H. Immunomodulatory action of citrus auraptene on macrophage functions and cytokine production of lymphocytes in female BALB/c mice. *Carcinogenesis* **1999**, *20*, 1471–1476.
99. Narisawa, T.; Fukaura, Y.; Oshima, S.; Inakuma, T.; Yano, M.; Nishino, H. Chemoprevention by the oxygenated carotenoid β-cryptoxanthin of *N*-methylnitrosourea-induced colon carcinogenesis in F344 rats. *Jpn. J. Cancer Res.* **1999**, *90*, 1061–1065.
100. Kohno, H.; Taima, M.; Sumida, T.; Azuma, Y.; Ogawa, H.; Tanaka, T. Inhibitory effect of mandarin juice rich in β-cryptoxanthin and hesperidin on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary tumorigenesis in mice. *Cancer Lett.* **2001**, *174*, 141–150.
101. Tanaka, T.; Makita, H.; Kawabata, K.; Mori, H.; Kakumoto, M.; Satoh, K.; Hara, A.; Sumida, T.; Fukutani, K.; Ogawa, H. Modulation of *N*-methyl-*N*-amylnitrosamine-induced rat oesophageal tumourigenesis by dietary feeding of diosmin and hesperidin, both alone and in combination. *Carcinogenesis* **1997**, *18*, 761–769.
102. Tanaka, T.; Makita, H.; Kawabata, K.; Mori, H.; Kakumoto, M.; Satoh, K.; Hara, A.; Sumida, T.; Ogawa, H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* **1997**, *18*, 957–965.
103. Tanaka, T.; Makita, H.; Ohnishi, M.; Hirose, Y.; Wang, A.; Mori, H.; Satoh, K.; Hara, A.; Ogawa, H. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by dietary curcumin and hesperidin: Comparison with the protective effect of β-carotene. *Cancer Res.* **1994**, *54*, 4653–4659.
104. Tanaka, T.; Makita, H.; Ohnishi, M.; Mori, H.; Satoh, K.; Hara, A.; Sumida, T.; Fukutani, K.; Ogawa, H. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats by flavonoids diosmin and hesperidin, each alone and in combination. *Cancer Res.* **1997**, *57*, 246–252.
105. Yang, M.; Tanaka, T.; Hirose, Y.; Deguchi, T.; Mori, H.; Kawada, Y. Chemopreventive effects of diosmin and hesperidin on *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary-bladder carcinogenesis in male ICR mice. *Int. J. Cancer* **1997**, *73*, 719–724.
106. Kohno, H.; Maeda, M.; Honjo, S.; Murakami, M.; Shimada, R.; Masuda, S.; Sumida, T.; Azuma, Y.; Ogawa, H.; Tanaka, T. Prevention of colonic preneoplastic lesions by the β-cryptoxanthin and hesperidin rich powder prepared from *Citrus unshiu* Marc. Juice in male F344 rats. *J. Toxicol. Pathol.* **1999**, *12*, 209–215.
107. Tanaka, T.; Tanaka, T.; Tanaka, M.; Kuno, T. Cancer chemoprevention by citrus pulp and juices containing high amounts of β-cryptoxanthin and hesperidin. *J. Biomed. Biotechnol.* **2012**, doi:10.1155/2012/516981.
108. Suzuki, R.; Kohno, H.; Yasui, Y.; Hata, K.; Sugie, S.; Miyamoto, S.; Sugawara, K.; Sumida, T.; Hirose, Y.; Tanaka, T. Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male db/db mice. *Int. J. Cancer* **2007**, *120*, 252–258.

109. Tanaka, T.; Yasui, Y.; Ishigamori-Suzuki, R.; Oyama, T. Citrus compounds inhibit inflammation- and obesity-related colon carcinogenesis in mice. *Nutr. Cancer* **2008**, *60*, S70–S80.
110. Narisawa, T.; Fukaura, Y.; Hasebe, M.; Nomura, S.; Oshima, S.; Sakamoto, H.; Inakuma, T.; Ishiguro, Y.; Takayasu, J.; Nishino, H. Prevention of *N*-methylnitrosourea-induced colon carcinogenesis in F344 rats by lycopene and tomato juice rich in lycopene. *Jpn. J. Cancer Res.* **1998**, *89*, 1003–1008.
111. Sharoni, Y.; Giron, E.; Rise, M.; Levy, J. Effects of lycopene-enriched tomato oleoresin on 7,12-dimethyl-benz[a]anthracene-induced rat mammary tumors. *Cancer Detect. Prev.* **1997**, *21*, 118–123.
112. Nagasawa, H.; Mitamura, T.; Sakamoto, S.; Yamamoto, K. Effects of lycopene on spontaneous mammary tumour development in SHN virgin mice. *Anticancer Res.* **1995**, *15*, 1173–1178.
113. Kim, D.J.; Takasuka, N.; Kim, J.M.; Sekine, K.; Ota, T.; Asamoto, M.; Murakoshi, M.; Nishino, H.; Nir, Z.; Tsuda, H. Chemoprevention by lycopene of mouse lung neoplasia after combined initiation treatment with DEN, MNU and DMH. *Cancer Lett.* **1997**, *120*, 15–22.
114. Astorg, P.; Gradelet, S.; Berges, R.; Suschetet, M. Dietary lycopene decreases the initiation of liver preneoplastic foci by diethylnitrosamine in the rat. *Nutr. Cancer* **1997**, *29*, 60–68.
115. Okajima, E.; Tsutsumi, M.; Ozono, S.; Akai, H.; Denda, A.; Nishino, H.; Oshima, S.; Sakamoto, H.; Konishi, Y. Inhibitory effect of tomato juice on rat urinary bladder carcinogenesis after *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine initiation. *Jpn. J. Cancer Res.* **1998**, *89*, 22–26.
116. Cohen, L.A.; Zhao, Z.; Pittman, B.; Khachik, F. Effect of dietary lycopene on *N*-methylnitrosourea-induced mammary tumorigenesis. *Nutr. Cancer* **1999**, *34*, 153–159.
117. Hu, K.Q.; Liu, C.; Ernst, H.; Krinsky, N.I.; Russell, R.M.; Wang, X.D. The biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids *in vitro* and *in vivo*. *J. Biol. Chem.* **2006**, *281*, 19327–19338.
118. Lian, F.; Smith, D.E.; Ernst, H.; Russell, R.M.; Wang, X.D. Apo-10'-lycopenoic acid inhibits lung cancer cell growth *in vitro*, and suppresses lung tumorigenesis in the A/J mouse model *in vivo*. *Carcinogenesis* **2007**, *28*, 1567–1574.
119. Lian, F.; Wang, X.D. Enzymatic metabolites of lycopene induce Nrf2-mediated expression of phase II detoxifying/antioxidant enzymes in human bronchial epithelial cells. *Int. J. Cancer* **2008**, *123*, 1262–1268.
120. Yang, C.M.; Huang, S.M.; Liu, C.L.; Hu, M.L. Apo-8'-lycopenal induces expression of HO-1 and NQO-1 via the ERK/p38-Nrf2-ARE pathway in human HepG2 cells. *J. Agric. Food Chem.* **2012**, *60*, 1576–7585.
121. Hsing, A.W.; Comstock, G.W.; Abbey, H.; Polk, B.F. Serologic precursors of cancer. Retinol, carotenoids, and tocopherol and risk of prostate cancer. *J. Natl. Cancer Inst.* **1990**, *82*, 941–946.
122. Schuurman, A.G.; Goldbohm, R.A.; Brants, H.A.; van den Brandt, P.A. A prospective cohort study on intake of retinol, vitamins C and E, and carotenoids and prostate cancer risk (Netherlands). *Cancer Causes Control* **2002**, *13*, 573–582.
123. Giovannucci, E.; Ascherio, A.; Rimm, E.B.; Stampfer, M.J.; Colditz, G.A.; Willett, W.C. Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl. Cancer Inst.* **1995**, *87*, 1767–1776.

124. Giovannucci, E.; Rimm, E.B.; Liu, Y.; Stampfer, M.J.; Willett, W.C. A prospective study of tomato products, lycopene, and prostate cancer risk. *J. Natl. Cancer Inst.* **2002**, *94*, 391–398.
125. Wu, K.; Erdman, J.W., Jr.; Schwartz, S.J.; Platz, E.A.; Leitzmann, M.; Clinton, S.K.; DeGroff, V.; Willett, W.C.; Giovannucci, E. Plasma and dietary carotenoids, and the risk of prostate cancer: A nested case-control study. *Cancer Epidemiol. Biomark. Prev.* **2004**, *13*, 260–269.
126. Kucuk, O.; Sarkar, F.H.; Sakr, W.; Djuric, Z.; Pollak, M.N.; Khachik, F.; Li, Y.W.; Banerjee, M.; Grignon, D.; Bertram, J.S.; *et al.* Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy. *Cancer Epidemiol. Biomark. Prev.* **2001**, *10*, 861–868.
127. Bowen, P.; Chen, L.; Stacewicz-Sapuntzakis, M.; Duncan, C.; Sharifi, R.; Ghosh, L.; Kim, H.S.; Christov-Tzelkov, K.; van Breemen, R. Tomato sauce supplementation and prostate cancer: Lycopene accumulation and modulation of biomarkers of carcinogenesis. *Exp. Biol. Med. (Maywood)* **2002**, *227*, 886–893.
128. Cohen, L.A. A review of animal model studies of tomato carotenoids, lycopene, and cancer chemoprevention. *Exp. Biol. Med. (Maywood)* **2002**, *227*, 864–868.
129. Michaud, D.S.; Feskanich, D.; Rimm, E.B.; Colditz, G.A.; Speizer, F.E.; Willett, W.C.; Giovannucci, E. Intake of specific carotenoids and risk of lung cancer in 2 prospective US cohorts. *Am. J. Clin. Nutr.* **2000**, *72*, 990–997.
130. Le Marchand, L.; Hankin, J.H.; Bach, F.; Kolonel, L.N.; Wilkens, L.R.; Stacewicz-Sapuntzakis, M.; Bowen, P.E.; Beecher, G.R.; Laudon, F.; Baque, P.; *et al.* An ecological study of diet and lung cancer in the South Pacific. *Int. J. Cancer* **1995**, *63*, 18–23.
131. Chew, B.P.; Park, J.S. Carotenoid action on the immune response. *J. Nutr.* **2004**, *134*, 257S–261S.
132. Kawashima, T. A marine carotenoid, fucoxanthin, induces regulatory T cells and inhibits Th17 cell differentiation *in vitro*. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 2066–2069.
133. Iannone, A.; Rota, C.; Bergamini, S.; Tomasi, A.; Canfield, L.M. Antioxidant activity of carotenoids: An electron-spin resonance study on β-carotene and lutein interaction with free radicals generated in a chemical system. *J. Biochem. Mol. Toxicol.* **1998**, *12*, 299–304.
134. Sujak, A.; Gabrielska, J.; Grudzinski, W.; Borc, R.; Mazurek, P.; Gruszecki, W.I. Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: The structural aspects. *Arch. Biochem. Biophys.* **1999**, *371*, 301–307.
135. Haegele, A.D.; Gillette, C.; O'Neill, C.; Wolfe, P.; Heimendinger, J.; Sedlacek, S.; Thompson, H.J. Plasma xanthophyll carotenoids correlate inversely with indices of oxidative DNA damage and lipid peroxidation. *Cancer Epidemiol. Biomark. Prev.* **2000**, *9*, 421–425.
136. Stahl, W.; Junghans, A.; de Boer, B.; Driomina, E.S.; Briviba, K.; Sies, H. Carotenoid mixtures protect multilamellar liposomes against oxidative damage: Synergistic effects of lycopene and lutein. *FEBS Lett.* **1998**, *427*, 305–308.
137. Gonzalez de Mejia, E.; Loarca-Pina, G.; Ramos-Gomez, M. Antimutagenicity of xanthophylls present in Aztec Marigold (*Tagetes erecta*) against 1-nitropyrene. *Mutat. Res.* **1997**, *389*, 219–226.
138. Gonzalez de Mejia, E.; Ramos-Gomez, M.; Loarca-Pina, G. Antimutagenic activity of natural xanthophylls against aflatoxin B1 in Salmonella typhimurium. *Environ. Mol. Mutagen.* **1997**, *30*, 346–353.

139. Park, J.S.; Chew, B.P.; Wong, T.S.; Zhang, J.X.; Magnuson, N.S. Dietary lutein but not astaxanthin or β-carotene increases pim-1 gene expression in murine lymphocytes. *Nutr. Cancer* **1999**, *33*, 206–212.
140. Tsubono, Y.; Tsugane, S.; Gey, K.F. Plasma antioxidant vitamins and carotenoids in five Japanese populations with varied mortality from gastric cancer. *Nutr. Cancer* **1999**, *34*, 56–61.
141. Slattery, M.L.; Benson, J.; Curtin, K.; Ma, K.N.; Schaeffer, D.; Potter, J.D. Carotenoids and colon cancer. *Am. J. Clin. Nutr.* **2000**, *71*, 575–582.
142. Wingerath, T.; Sies, H.; Stahl, W. Xanthophyll esters in human skin. *Arch. Biochem. Biophys.* **1998**, *355*, 271–274.
143. Stahl, W.; Heinrich, U.; Jungmann, H.; Sies, H.; Tronnier, H. Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in humans. *Am. J. Clin. Nutr.* **2000**, *71*, 795–798.
144. Hunter, D.J.; Manson, J.E.; Colditz, G.A.; Stampfer, M.J.; Rosner, B.; Hennekens, C.H.; Speizer, F.E.; Willett, W.C. A prospective study of the intake of vitamins C, E, and A and the risk of breast cancer. *N. Engl. J. Med.* **1993**, *329*, 234–240.
145. Potischman, N.; McCulloch, C.E.; Byers, T.; Nemoto, T.; Stubbe, N.; Milch, R.; Parker, R.; Rasmussen, K.M.; Root, M.; Graham, S.; *et al.* Breast cancer and dietary and plasma concentrations of carotenoids and vitamin A. *Am. J. Clin. Nutr.* **1990**, *52*, 909–915.
146. Rohan, T.E.; McMichael, A.J.; Baghurst, P.A. A population-based case-control study of diet and breast cancer in Australia. *Am. J. Epidemiol.* **1988**, *128*, 478–489.
147. Sumantran, V.N.; Zhang, R.; Lee, D.S.; Wicha, M.S. Differential regulation of apoptosis in normal *versus* transformed mammary epithelium by lutein and retinoic acid. *Cancer Epidemiol. Biomark. Prev.* **2000**, *9*, 257–263.
148. Freudenheim, J.L.; Marshall, J.R.; Vena, J.E.; Laughlin, R.; Brasure, J.R.; Swanson, M.K.; Nemoto, T.; Graham, S. Premenopausal breast cancer risk and intake of vegetables, fruits, and related nutrients. *J. Natl. Cancer Inst.* **1996**, *88*, 340–348.
149. Dorgan, J.F.; Sowell, A.; Swanson, C.A.; Potischman, N.; Miller, R.; Schussler, N.; Stephenson, H.E., Jr. Relationships of serum carotenoids, retinol, α-tocopherol, and selenium with breast cancer risk: Results from a prospective study in Columbia, Missouri (United States). *Cancer Causes Control* **1998**, *9*, 89–97.
150. Zhang, S.; Tang, G.; Russell, R.M.; Mayzel, K.A.; Stampfer, M.J.; Willett, W.C.; Hunter, D.J. Measurement of retinoids and carotenoids in breast adipose tissue and a comparison of concentrations in breast cancer cases and control subjects. *Am. J. Clin. Nutr.* **1997**, *66*, 626–632.
151. Toniolo, P.; van Kappel, A.L.; Akhmedkhanov, A.; Ferrari, P.; Kato, I.; Shore, R.E.; Riboli, E. Serum carotenoids and breast cancer. *Am. J. Epidemiol.* **2001**, *153*, 1142–1147.
152. Yeum, K.J.; Ahn, S.H.; Rupp de Paiva, S.A.; Lee-Kim, Y.C.; Krinsky, N.I.; Russell, R.M. Correlation between carotenoid concentrations in serum and normal breast adipose tissue of women with benign breast tumor or breast cancer. *J. Nutr.* **1998**, *128*, 1920–1926.
153. Anonymous. Eskimo diets and diseases. *Lancet* **1983**, *1*, 1139–1141.
154. Bates, C.; van Dam, C.; Horrobin, D.F.; Morse, N.; Huang, Y.S.; Manku, M.S. Plasma essential fatty acids in pure and mixed race American Indians on and off a diet exceptionally rich in salmon. *Prostaglandins Leukot. Med.* **1985**, *17*, 77–84.

155. Tanaka, T.; Morishita, Y.; Suzui, M.; Kojima, T.; Okumura, A.; Mori, H. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. *Carcinogenesis* **1994**, *15*, 15–19.
156. Tanaka, T.; Makita, H.; Ohnishi, M.; Mori, H.; Satoh, K.; Hara, A. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. *Cancer Res.* **1995**, *55*, 4059–4064.
157. Tanaka, T.; Kawamori, T.; Ohnishi, M.; Makita, H.; Mori, H.; Satoh, K.; Hara, A. Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis* **1995**, *16*, 2957–2963.
158. Yasui, Y.; Hosokawa, M.; Mikami, N.; Miyashita, K.; Tanaka, T. Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice via modulation of the inflammatory cytokines. *Chem. Biol. Interact.* **2011**, *193*, 79–87.
159. McCarty, M.F. Minimizing the cancer-promotional activity of cox-2 as a central strategy in cancer prevention. *Med. Hypotheses* **2011**, *78*, 45–57.
160. Nagendraprabhu, P.; Sudhandiran, G. Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFkB and COX-2. *Invest. New Drugs* **2011**, *29*, 207–224.
161. Gradelet, S.; Le Bon, A.M.; Berges, R.; Suschetet, M.; Astorg, P. Dietary carotenoids inhibit aflatoxin B1-induced liver preneoplastic foci and DNA damage in the rat: Role of the modulation of aflatoxin B1 metabolism. *Carcinogenesis* **1998**, *19*, 403–411.
162. Hix, L.M.; Frey, D.A.; McLaws, M.D.; Østerlie, M.; Lockwood, S.F.; Bertram, J.S. Inhibition of chemically-induced neoplastic transformation by a novel tetrasodium diphosphate astaxanthin derivative. *Carcinogenesis* **2005**, *26*, 1634–1641.
163. Black, H.S.; Mathews-Roth, M.M. Protective role of butylated hydroxytoluene and certain carotenoids in photocarcinogenesis. *Photochem. Photobiol.* **1991**, *53*, 707–716.
164. Santamaria, L.; Bianchi, A.; Arnaboldi, A.; Ravetto, C.; Bianchi, L.; Pizzala, R.; Andreoni, L.; Santagati, G.; Bermond, P. Chemoprevention of indirect and direct chemical carcinogenesis by carotenoids as oxygen radical quenchers. *Ann. N.Y. Acad. Sci.* **1988**, *534*, 584–596.
165. Grubbs, C.J.; Eto, I.; Juliana, M.M.; Whitaker, L.M. Effect of canthaxanthin on chemically induced mammary carcinogenesis. *Oncology* **1991**, *48*, 239–245.
166. Onogi, N.; Okuno, M.; Matsushima-Nishiwaki, R.; Fukutomi, Y.; Moriwaki, H.; Muto, Y.; Kojima, S. Antiproliferative effect of carotenoids on human colon cancer cells without conversion to retinoic acid. *Nutr. Cancer* **1998**, *32*, 20–24.
167. Bertram, J.S.; Pung, A.; Churley, M.; Kappock, T.J., IV; Wilkins, L.R.; Cooney, R.V. Diverse carotenoids protect against chemically induced neoplastic transformation. *Carcinogenesis* **1991**, *12*, 671–678.
168. Mathews-Roth, M.M.; Krinsky, N.I. Carotenoid dose level and protection against UV-B induced skin tumors. *Photochem. Photobiol.* **1985**, *42*, 35–38.

169. Hanusch, M.; Stahl, W.; Schulz, W.A.; Sies, H. Induction of gap junctional communication by 4-oxoretinoic acid generated from its precursor canthaxanthin. *Arch. Biochem. Biophys.* **1995**, *317*, 423–428.
170. Gradelet, S.; Astorg, P.; Leclerc, J.; Chevalier, J.; Vernevaut, M.F.; Siess, M.H. Effects of canthaxanthin, astaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat. *Xenobiotica* **1996**, *26*, 49–63.
171. Jewell, C.; O'Brien, N.M. Effect of dietary supplementation with carotenoids on xenobiotic metabolizing enzymes in the liver, lung, kidney and small intestine of the rat. *Br. J. Nutr.* **1999**, *81*, 235–242.
172. Palozza, P.; Maggiano, N.; Calviello, G.; Lanza, P.; Piccioni, E.; Ranelletti, F.O.; Bartoli, G.M. Canthaxanthin induces apoptosis in human cancer cell lines. *Carcinogenesis* **1998**, *19*, 373–376.
173. Goralczyk, R.; Buser, S.; Bausch, J.; Bee, W.; Zuhlke, U.; Barker, F.M. Occurrence of birefringent retinal inclusions in cynomolgus monkeys after high doses of canthaxanthin. *Invest. Ophthalmol. Vis. Sci.* **1997**, *38*, 741–752.
174. Leyon, H.; Ros, A.M.; Nyberg, S.; Algvere, P. Reversibility of canthaxanthin deposits within the retina. *Acta Ophthalmol. (Copenh.)* **1990**, *68*, 607–611.
175. Das, S.K.; Hashimoto, T.; Kanazawa, K. Growth inhibition of human hepatic carcinoma HepG2 cells by fucoxanthin is associated with down-regulation of cyclin D. *Biochim. Biophys. Acta* **2008**, *1780*, 743–749.
176. Hosokawa, M.; Kudo, M.; Maeda, H.; Kohno, H.; Tanaka, T.; Miyashita, K. Fucoxanthin induces apoptosis and enhances the antiproliferative effect of the PPARgamma ligand, troglitazone, on colon cancer cells. *Biochim. Biophys. Acta* **2004**, *1675*, 113–119.
177. Zhang, Z.; Zhang, P.; Hamada, M.; Takahashi, S.; Xing, G.; Liu, J.; Sugiura, N. Potential chemoprevention effect of dietary fucoxanthin on urinary bladder cancer EJ-1 cell line. *Oncol. Rep.* **2008**, *20*, 1099–1103.
178. Yonekura, L.; Kobayashi, M.; Terasaki, M.; Nagao, A. Keto-carotenoids are the major metabolites of dietary lutein and fucoxanthin in mouse tissues. *J. Nutr.* **2010**, *140*, 1824–1831.
179. Kim, J.M.; Araki, S.; Kim, D.J.; Park, C.B.; Takasuka, N.; Baba-Toriyama, H.; Ota, T.; Nir, Z.; Khachik, F.; Shimidzu, N.; *et al.* Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis* **1998**, *19*, 81–85.
180. Nishino, H.; Murakoshi, M.; Tokuda, H.; Satomi, Y. Cancer prevention by carotenoids. *Arch. Biochem. Biophys.* **2009**, *483*, 165–168.
181. Okuzumi, J.; Takahashi, T.; Yamane, T.; Kitao, Y.; Inagake, M.; Ohya, K.; Nishino, H.; Tanaka, Y. Inhibitory effects of fucoxanthin, a natural carotenoid, on *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine-induced mouse duodenal carcinogenesis. *Cancer Lett.* **1993**, *68*, 159–168.
182. Yoshiko, S.; Hoyoku, N. Fucoxanthin, a natural carotenoid, induces G1 arrest and GADD45 gene expression in human cancer cells. *In Vivo* **2007**, *21*, 305–309.
183. Miyashita, K.; Nishikawa, S.; Beppu, F.; Tsukui, T.; Abe, M.; Hosokawa, M. The allenic carotenoid fucoxanthin, a novel marine nutraceutical from brown seaweeds. *J. Sci. Food Agric.* **2011**, *91*, 1166–1174.

184. Liu, C.L.; Huang, Y.S.; Hosokawa, M.; Miyashita, K.; Hu, M.L. Inhibition of proliferation of a hepatoma cell line by fucoxanthin in relation to cell cycle arrest and enhanced gap junctional intercellular communication. *Chem. Biol. Interact.* **2009**, *182*, 165–172.
185. Liu, C.L.; Chiu, Y.T.; Hu, M.L. Fucoxanthin enhances HO-1 and NQO1 expression in murine hepatic BNL CL.2 cells through activation of the Nrf2/ARE system partially by its pro-oxidant activity. *J. Agric. Food Chem.* **2011**, *59*, 11344–11351.
186. Murakami, C.; Takemura, M.; Sugiyama, Y.; Kamisuki, S.; Asahara, H.; Kawasaki, M.; Ishidoh, T.; Linn, S.; Yoshida, S.; Sugawara, F.; *et al.* Vitamin A-related compounds, all-trans retinal and retinoic acids, selectively inhibit activities of mammalian replicative DNA polymerases. *Biochim. Biophys. Acta* **2002**, *1574*, 85–92.
187. Zhang, L.X.; Cooney, R.V.; Bertram, J.S. Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. *Carcinogenesis* **1991**, *12*, 2109–2114.
188. LeRoith, D.; Roberts, C.T., Jr. The insulin-like growth factor system and cancer. *Cancer Lett.* **2003**, *195*, 127–137.
189. Voskuil, D.W.; Vrieling, A.; van't Veer, L.J.; Kampman, E.; Rookus, M.A. The insulin-like growth factor system in cancer prevention: Potential of dietary intervention strategies. *Cancer Epidemiol. Biomark. Prev.* **2005**, *14*, 195–203.
190. Mantzoros, C.S.; Tzonou, A.; Signorello, L.B.; Stampfer, M.; Trichopoulos, D.; Adami, H.O. Insulin-like growth factor 1 in relation to prostate cancer and benign prostatic hyperplasia. *Br. J. Cancer* **1997**, *76*, 1115–1118.
191. Hankinson, S.E.; Willett, W.C.; Colditz, G.A.; Hunter, D.J.; Michaud, D.S.; Deroo, B.; Rosner, B.; Speizer, F.E.; Pollak, M. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* **1998**, *351*, 1393–1396.
192. Ma, J.; Pollak, M.N.; Giovannucci, E.; Chan, J.M.; Tao, Y.; Hennekens, C.H.; Stampfer, M.J. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J. Natl. Cancer Inst.* **1999**, *91*, 620–625.
193. Yu, H.; Spitz, M.R.; Mistry, J.; Gu, J.; Hong, W.K.; Wu, X. Plasma levels of insulin-like growth factor-I and lung cancer risk: A case-control analysis. *J. Natl. Cancer Inst.* **1999**, *91*, 151–156.
194. Hata, K.; Kubota, M.; Shimizu, M.; Moriwaki, H.; Toshiya, K.; Tanaka, T.; Hara, A.; Hirose, Y. C57BL/KsJ-*db/db-Apc*$^{Min/+}$ mice exhibit an increased incidence of intestinal neoplasms. *Int. J. Mol. Sci.* **2011**, *12*, 8133–8145.
195. Karas, M.; Amir, H.; Fishman, D.; Danilenko, M.; Segal, S.; Nahum, A.; Koifmann, A.; Giat, Y.; Levy, J.; Sharoni, Y. Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. *Nutr. Cancer* **2000**, *36*, 101–111.
196. Karas, M.; Danilenko, M.; Fishman, D.; LeRoith, D.; Levy, J.; Sharoni, Y. Membrane-associated insulin-like growth factor-binding protein-3 inhibits insulin-like growth factor-I-induced insulin-like growth factor-I receptor signaling in ishikawa endometrial cancer cells. *J. Biol. Chem.* **1997**, *272*, 16514–16520.
197. Amir, H.; Karas, M.; Giat, J.; Danilenko, M.; Levy, R.; Yermiahu, T.; Levy, J.; Sharoni, Y. Lycopene and 1,25-dihydroxyvitamin D3 cooperate in the inhibition of cell cycle progression and induction of differentiation in HL-60 leukemic cells. *Nutr. Cancer* **1999**, *33*, 105–112.

198. Murakoshi, M.; Takayasu, J.; Kimura, O.; Kohmura, E.; Nishino, H.; Iwashima, A.; Okuzumi, J.; Sakai, T.; Sugimoto, T.; Imanishi, J.; *et al.* Inhibitory effects of α-carotene on proliferation of the human neuroblastoma cell line GOTO. *J. Natl. Cancer Inst.* **1989**, *81*, 1649–1652.
199. Stivala, L.A.; Savio, M.; Quarta, S.; Scotti, C.; Cazzalini, O.; Rossi, L.; Scovassi, I.A.; Pizzala, R.; Melli, R.; Bianchi, L.; *et al.* The antiproliferative effect of β-carotene requires p21waf1/cip1 in normal human fibroblasts. *Eur. J. Biochem.* **2000**, *267*, 2290–2296.
200. Satomi, Y.; Nishino, H. Implication of mitogen-activated protein kinase in the induction of G1 cell cycle arrest and gadd45 expression by the carotenoid fucoxanthin in human cancer cells. *Biochim. Biophys. Acta* **2009**, *1790*, 260–266.
201. Ford, N.A.; Elsen, A.C.; Zuniga, K.; Lindshield, B.L.; Erdman, J.W., Jr. Lycopene and apo-12'-lycopenal reduce cell proliferation and alter cell cycle progression in human prostate cancer cells. *Nutr. Cancer* **2011**, *63*, 256–263.
202. Nahum, A.; Hirsch, K.; Danilenko, M.; Watts, C.K.; Prall, O.W.; Levy, J.; Sharoni, Y. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27(Kip1) in the cyclin E-cdk2 complexes. *Oncogene* **2001**, *20*, 3428–3436.
203. Gross, M.D.; Bishop, T.D.; Belcher, J.D.; Jacobs, D.R., Jr. Induction of HL-60 cell differentiation by carotenoids. *Nutr. Cancer* **1997**, *27*, 169–173.
204. Tarantilis, P.A.; Morjani, H.; Polissiou, M.; Manfait, M. Inhibition of growth and induction of differentiation of promyelocytic leukemia (HL-60) by carotenoids from *Crocus sativus* L. *Anticancer Res.* **1994**, *14*, 1913–1918.
205. Danilenko, M.; Wang, X.; Studzinski, G.P. Carnosic acid and promotion of monocytic differentiation of HL60-G cells initiated by other agents. *J. Natl. Cancer Inst.* **2001**, *93*, 1224–1233.
206. Steiner, M.; Priel, I.; Giat, J.; Levy, J.; Sharoni, Y.; Danilenko, M. Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Nutr. Cancer* **2001**, *41*, 135–144.
207. Stahl, W.; von Laar, J.; Martin, H.D.; Emmerich, T.; Sies, H. Stimulation of gap junctional communication: Comparison of acyclo-retinoic acid and lycopene. *Arch. Biochem. Biophys.* **2000**, *373*, 271–274.
208. Ben-Dor, A.; Nahum, A.; Danilenko, M.; Giat, Y.; Stahl, W.; Martin, H.D.; Emmerich, T.; Noy, N.; Levy, J.; Sharoni, Y. Effects of acyclo-retinoic acid and lycopene on activation of the retinoic acid receptor and proliferation of mammary cancer cells. *Arch. Biochem. Biophys.* **2001**, *391*, 295–302.
209. Muto, Y.; Moriwaki, H.; Omori, M. *In vitro* binding affinity of novel synthetic polyprenoids (polyprenoic acids) to cellular retinoid-binding proteins. *Gann* **1981**, *72*, 974–977.
210. Araki, H.; Shidoji, Y.; Yamada, Y.; Moriwaki, H.; Muto, Y. Retinoid agonist activities of synthetic geranyl geranoic acid derivatives. *Biochem. Biophys. Res. Commun.* **1995**, *209*, 66–72.
211. Butler, R.; Mitchell, S.H.; Tindall, D.J.; Young, C.Y. Nonapoptotic cell death associated with S-phase arrest of prostate cancer cells via the peroxisome proliferator-activated receptor gamma ligand, 15-deoxy-delta12,14-prostaglandin J2. *Cell Growth Differ.* **2000**, *11*, 49–61.

212. Kubota, T.; Koshizuka, K.; Williamson, E.A.; Asou, H.; Said, J.W.; Holden, S.; Miyoshi, I.; Koeffler, H.P. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both *in vitro* and *in vivo*. *Cancer Res.* **1998**, *58*, 3344–3352.
213. Mueller, E.; Smith, M.; Sarraf, P.; Kroll, T.; Aiyer, A.; Kaufman, D.S.; Oh, W.; Demetri, G.; Figg, W.D.; Zhou, X.P.; *et al.* Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10990–10995.
214. Yasui, Y.; Kim, M.; Tanaka, T. PPAR Ligands for Cancer Chemoprevention. *PPAR Res.* **2008**, *2008*, 548919.
215. Jaiswal, A.K. Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free Radic. Biol. Med.* **2000**, *29*, 254–262.
216. Venugopal, R.; Jaiswal, A.K. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 14960–14965.
217. Wang, X.D.; Liu, C.; Bronson, R.T.; Smith, D.E.; Krinsky, N.I.; Russell, M. Retinoid signaling and activator protein-1 expression in ferrets given β-carotene supplements and exposed to tobacco smoke. *J. Natl. Cancer Inst.* **1999**, *91*, 60–66.
218. Xie, W.; Evans, R.M. Orphan nuclear receptors: The exotics of xenobiotics. *J. Biol. Chem.* **2001**, *276*, 37739–37742.
219. Altucci, L.; Gronemeyer, H. The promise of retinoids to fight against cancer. *Nat. Rev. Cancer* **2001**, *1*, 181–193.
220. Pavan, B.; Biondi, C.; Dalpiaz, A. Nuclear retinoic acid receptor β as a tool in chemoprevention trials. *Curr. Med. Chem.* **2006**, *13*, 3553–3563.
221. Shimizu, M.; Sakai, H.; Shirakami, Y.; Iwasa, J.; Yasuda, Y.; Kubota, M.; Takai, K.; Tsurumi, H.; Tanaka, T.; Moriwaki, H. Acyclic retinoid inhibits diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BLKS/J- $+Lepr^{db}/+Lepr^{db}$ mice. *Cancer Prev. Res. (Phila.)* **2011**, *4*, 128–136.
222. Moriwaki, H.; Shimizu, M.; Okuno, M.; Nishiwaki-Matsushima, R. Chemoprevention of liver carcinogenesis with retinoids: Basic and clinical aspects. *Hepatol. Res.* **2007**, *37*, S299–S302.
223. Fabricius, E.M.; Kruse-Boitschenko, U.; Schneeweiss, U.; Wildner, G.P.; Hoffmeister, B.; Raguse, J.D. Model examination of chemoprevention with retinoids in squamous cell carcinomas of the head and neck region and suitable biomarkers for chemoprevention. *Int. J. Oncol.* **2011**, *39*, 1083–1097.
224. Prakash, P.; Liu, C.; Hu, K.Q.; Krinsky, N.I.; Russell, R.M.; Wang, X.D. β-Carotene and β-apo-14'-carotenoic acid prevent the reduction of retinoic acid receptor β in benzo[a]pyrene-treated normal human bronchial epithelial cells. *J. Nutr.* **2004**, *134*, 667–673.
225. Reuter, S.; Gupta, S.C.; Chaturvedi, M.M.; Aggarwal, B.B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* **2010**, *49*, 1603–1616.
226. Schmidt, M.V.; Brune, B.; von Knethen, A. The nuclear hormone receptor PPARgamma as a therapeutic target in major diseases. *ScientificWorldJournal* **2010**, *10*, 2181–2197.
227. Youssef, J.; Badr, M. Peroxisome proliferator-activated receptors and cancer: Challenges and opportunities. *Br. J. Pharmacol.* **2011**, *164*, 68–82.

228. Sarraf, P.; Mueller, E.; Smith, W.M.; Wright, H.M.; Kum, J.B.; Aaltonen, L.A.; de la Chapelle, A.; Spiegelman, B.M.; Eng, C. Loss-of-function mutations in PPAR gamma associated with human colon cancer. *Mol. Cell* **1999**, *3*, 799–804.
229. Simone, R.E.; Russo, M.; Catalano, A.; Monego, G.; Froehlich, K.; Boehm, V.; Palozza, P. Lycopene inhibits NF-kB-mediated IL-8 expression and changes redox and PPARgamma signalling in cigarette smoke-stimulated macrophages. *PLoS One* **2011**, *6*, e19652.
230. Yang, C.M.; Lu, I.H.; Chen, H.Y.; Hu, M.L. Lycopene inhibits the proliferation of androgen-dependent human prostate tumor cells through activation of PPARγ-LXRα-ABCA1 pathway. *J. Nutr. Biochem.* **2012**, *23*, 8–17.
231. Xie, W.; Barwick, J.L.; Simon, C.M.; Pierce, A.M.; Safe, S.; Blumberg, B.; Guzelian, P.S.; Evans, R.M. Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev.* **2000**, *14*, 3014–3023.
232. Moore, L.B.; Goodwin, B.; Jones, S.A.; Wisely, G.B.; Serabjit-Singh, C.J.; Willson, T.M.; Collins, J.L.; Kliewer, S.A. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7500–7502.
233. Ramos-Gomez, M.; Kwak, M.K.; Dolan, P.M.; Itoh, K.; Yamamoto, M.; Talalay, P.; Kensler, T.W. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3410–3415.
234. Kim, Y.C.; Masutani, H.; Yamaguchi, Y.; Itoh, K.; Yamamoto, M.; Yodoi, J. Hemin-induced activation of the thioredoxin gene by Nrf2. A differential regulation of the antioxidant responsive element by a switch of its binding factors. *J. Biol. Chem.* **2001**, *276*, 18399–18406.
235. Dhakshinamoorthy, S.; Jaiswal, A.K. Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* **2001**, *20*, 3906–3917.
236. Hayes, J.D.; McMahon, M. Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett.* **2001**, *174*, 103–113.
237. Kwak, M.K.; Egner, P.A.; Dolan, P.M.; Ramos-Gomez, M.; Groopman, J.D.; Itoh, K.; Yamamoto, M.; Kensler, T.W. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat. Res.* **2001**, *480–481*, 305–315.
238. Kong, A.N.; Owuor, E.; Yu, R.; Hebbar, V.; Chen, C.; Hu, R.; Mandlekar, S. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). *Drug Metab. Rev.* **2001**, *33*, 255–271.
239. Xu, C.; Li, C.Y.; Kong, A.N. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* **2005**, *28*, 249–268.
240. Morimitsu, Y.; Nakagawa, Y.; Hayashi, K.; Fujii, H.; Kumagai, T.; Nakamura, Y.; Osawa, T.; Horio, F.; Itoh, K.; Iida, K.; *et al.* A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J. Biol. Chem.* **2002**, *277*, 3456–3463.
241. Angel, P.; Karin, M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* **1991**, *1072*, 129–157.
242. Albanese, C.; Johnson, J.; Watanabe, G.; Eklund, N.; Vu, D.; Arnold, A.; Pestell, R.G. Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. *J. Biol. Chem.* **1995**, *270*, 23589–23597.

243. Palozza, P.; Parrone, N.; Catalano, A.; Simone, R. Tomato lycopene and inflammatory cascade: Basic interactions and clinical implications. *Curr. Med. Chem.* **2010**, *17*, 2547–2563.
244. Polakis, P. Wnt signaling and cancer. *Genes Dev.* **2000**, *14*, 1837–1851.
245. Schulenburg, A.; Cech, P.; Herbacek, I.; Marian, B.; Wrba, F.; Valent, P.; Ulrich-Pur, H. CD44-positive colorectal adenoma cells express the potential stem cell markers musashi antigen (msi1) and ephrin B2 receptor (EphB2). *J. Pathol.* **2007**, *213*, 152–160.
246. Cohen, P.; Frame, S. The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 769–776.
247. Korkaya, H.; Paulson, A.; Charafe-Jauffret, E.; Ginestier, C.; Brown, M.; Dutcher, J.; Clouthier, S.G.; Wicha, M.S. Regulation of mammary stem/progenitor cells by PTEN/Akt/β-catenin signaling. *PLoS Biol.* **2009**, *7*, e1000121.
248. Tang, F.Y.; Shih, C.J.; Cheng, L.H.; Ho, H.J.; Chen, H.J. Lycopene inhibits growth of human colon cancer cells via suppression of the Akt signaling pathway. *Mol. Nutr. Food Res.* **2008**, *52*, 646–654.
249. Lin, M.C.; Wang, F.Y.; Kuo, Y.H.; Tang, F.Y. Cancer chemopreventive effects of lycopene: Suppression of MMP-7 expression and cell invasion in human colon cancer cells. *J. Agric. Food Chem.* **2011**, *59*, 11304–11318.
250. Tanaka, T.; Suzuki, R. Inflammation and cancer. In *Cancer: Disease Progression and Chemoprevention*; Tanaka, T., Ed.; Research Signpost: Kerala, India, 2007; pp. 27–44.
251. Tanaka, T.; Kohno, H.; Suzuki, R.; Yamada, Y.; Sugie, S.; Mori, H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.* **2003**, *94*, 965–973.
252. Kim, H. Inhibitory mechanism of lycopene on cytokine expression in experimental pancreatitis. *Ann. N.Y. Acad. Sci.* **2011**, *1229*, 99–102.
253. Erkan, M.; Reiser-Erkan, C.; Michalski, C.W.; Kleeff, J. Tumor microenvironment and progression of pancreatic cancer. *Exp. Oncol.* **2010**, *32*, 128–131.
254. Nitsche, C.; Simon, P.; Weiss, F.U.; Fluhr, G.; Weber, E.; Gartner, S.; Behn, C.O.; Kraft, M.; Ringel, J.; Aghdassi, A.; *et al.* Environmental risk factors for chronic pancreatitis and pancreatic cancer. *Dig. Dis.* **2011**, *29*, 235–242.
255. Gallicchio, L.; Boyd, K.; Matanoski, G.; Tao, X.G.; Chen, L.; Lam, T.K.; Shiels, M.; Hammond, E.; Robinson, K.A.; Caulfield, L.E.; *et al.* Carotenoids and the risk of developing lung cancer: A systematic review. *Am. J. Clin Nutr.* **2008**, *88*, 372–383.

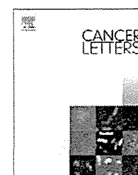
© 2012 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).

Cancer Letters 323 (2012) 215–222

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet



Synergistic growth inhibition of human hepatocellular carcinoma cells by acyclic retinoid and GW4064, a farnesoid X receptor ligand

Tomohiko Ohno, Yohei Shirakami, Masahito Shimizu *, Masaya Kubota, Hiroyasu Sakai, Yoichi Yasuda, Takahiro Kochi, Hisashi Tsurumi, Hisataka Moriwaki

Department of Medicine, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

ARTICLE INFO

Article history:
Received 4 November 2011
Received in revised form 29 March 2012
Accepted 20 April 2012

Keywords:
Acyclic retinoid
GW4064
Hepatocellular carcinoma
RXRα
FXR
Synergism

ABSTRACT

Abnormalities in the expression and function of retinoid X receptor (RXR), a master regulator of the nuclear receptor superfamily, are associated with the development of hepatocellular carcinoma (HCC). Dysfunction of farnesoid X receptor (FXR), one of the nuclear receptors that forms a heterodimer with RXR, also plays a role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), a synthetic retinoid targeting RXRα, plus GW4064, a ligand for FXR, on the growth of human HCC cells. We found that ACR and GW4064 preferentially inhibited the growth of HLE, HLF, and Huh7 human HCC cells in comparison with Hc normal hepatocytes. The combination of 1 μM ACR plus 1 μM GW4064 synergistically inhibited the growth of HLE cells by inducing apoptosis. The combined treatment with these agents acted cooperatively to induce cell cycle arrest in the G_0/G_1 phase and inhibit the phosphorylation of RXRα, which is regarded as a critical factor for liver carcinogenesis, through inhibition of ERK and Stat3 phosphorylation. This combination also increased the expression levels of $p21^{CIP1}$ and SHP mRNA, while decreasing the levels of c-*myc* and cyclin D1 mRNA in HLE cells. In addition, a reporter assay indicated that the FXRE promoter activity was significantly increased by treatment with ACR plus GW4064. Our results suggest that ACR and GW4064 cooperatively inhibit RXRα phosphorylation, modulate the expression of FXR-regulated genes, thus resulting in the induction of apoptosis and the inhibition of growth in HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Nuclear receptors are ligand-dependent transcription factors that are involved in various physiological processes. Retinoid X receptors (RXRs) are regarded as master regulators of nuclear receptors because they play an essential role in controlling normal cell proliferation and metabolism by forming a heterodimer with other nuclear receptors [1,2]. Therefore, abnormalities in the expression and function of RXRs are closely associated with the development of various disorders, including cancer, whereas using a retinoid might be an effective strategy for the prevention and treatment of human malignancies [3]. A malfunction of RXRα, one of the subtypes of RXR, due to phosphorylation by the Ras/MAPK signaling pathway is profoundly associated with liver carcinogenesis [4–8]. On the other hand, administration of acyclic retinoid (ACR), a synthetic retinoid which targets RXRα, reduced the incidence of post-therapeutic recurrence of hepatocellular carcinoma (HCC) and improved the survival rate of patients with this malignancy [9,10]. ACR also inhibits the growth of HCC-derived cells by inducing apoptosis and cell cycle arrest in the G_0/G_1 phase [11,12]. These findings suggest that nuclear receptors, especially RXRα, are critical targets for the prevention and treatment of HCC.

Farnesoid X receptor (FXR), which has been characterized as a bile acid receptor, is also a member of the nuclear receptor superfamily of ligand-dependent transcription factors that form heterodimers with RXR [13]. FXR has been shown to be essential in controlling bile acid, lipid, and glucose homeostasis [13]. It also plays a critical role in normal liver regeneration and promotes liver repair after injury by mediating its related signaling pathways [14].

Abbreviations: ACR, acyclic retinoid; CI, combination index; DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FXR, farnesoid X receptor; FXRE, farnesoid X receptor response element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; IFN, interferon; MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RTK, receptor tyrosine kinase; RT-PCR, reverse transcription PCR; RXR, retinoid X receptor; SHP, small heterodimer partner; Stat3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

* Corresponding author. Address: Department of Internal Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Tel.: +81 58 230 6313; fax: +81 58 230 6310.
E-mail address: shimim-gif@umin.ac.jp (M. Shimizu).

0304-3835/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2012.04.015

In addition, recent studies have revealed that aberrations in FXR are involved in liver carcinogenesis. FXR deficiency in mice leads to the development of neoplasms in the liver, including hepatic adenoma, HCC, and hepatocholangiocellular carcinoma [15,16]. A significant decrease in FXR expression and activity is also observed in human HCC samples [17]. Therefore, targeting FXR and improving its function might be a promising strategy for the prevention and treatment of HCC.

Recently, combination therapy and prevention have garnered much interest in the cancer field because they can synergistically inhibit growth and induce apoptosis in cancer cells. In human HCC-derived cells, ACR acts synergistically with other agents, such as interferon (IFN)-β, OSI-461, vitamin K_2, valproic acid, and trastuzumab, in suppressing growth and inducing apoptosis [11,18–21]. The agents that inhibit RXRα phosphorylation are among the most promising agents to use in combination with ACR [11,20,21]. In addition, the induction of nuclear receptors that dimerize with RXR, such as retinoic acid receptor (RAR)-β, and activation of these receptors by their ligands may also lead to synergistic growth inhibition in HCC cells when combined with ACR [11,19]. GW4064, a synthetic ligand for FXR, is known to induce the expression of genes involved in the transport of bile acids in the liver and intestines [22,23]. GW4064 also inhibits the growth of breast and prostate cancer cell lines [24–26], whereas the anticancer effects of this agent on HCC cells have not been evaluated. In the present study, we examined the effects of GW4064 on the growth of human HCC cells. We also investigated whether the combination of ACR plus GW4064 exerts synergistic growth inhibitory effects on HCC cells and examined the possible mechanisms responsible for such synergy.

2. Materials and methods

2.1. Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical Co. Ltd., (Tokyo, Japan). GW4064 was purchased from Sigma–Aldrich (St. Louis, MO, USA). The anti-RXRα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies for ERK, phosphorylated ERK (p-ERK), Stat3, phosphorylated Stat3 (p-Stat3), PARP, and GAPDH were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell lines and cell culture conditions

HLE, HLF, and Huh7 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HLE and HLF cells were maintained in DMEM and Huh7 cells were in RPMI1640 media, respectively. All media were supplemented with 10% FCS and 1% Penicillin/Streptomycin. Hc human normal hepatocyte cell line was purchased from Cell Systems (Kirkland, WA, USA) and maintained in a CS-S complete medium (Cell Systems). These cells were cultured in an incubator with humidified air with 5% CO_2 at 37 °C.

2.3. Cell proliferation assays

One thousand of HCC (HLE, HLF, and Huh7) or Hc cells were seeded on 96-well plates. The following day, the medium was changed to serum free medium and the cells were treated with the indicated concentrations of ACR or GW4064 for 48 h. Cell proliferation assays were performed using a MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. To determine whether the combined effects of ACR plus GW4064 were synergistic, HCC cells were treated with combinations of the indicated concentrations of ACR and GW4064 for 48 h and the combination index (CI)-isobologram was calculated. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were used to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate), as described previously [11,19,27].

2.4. Apoptosis assays

TUNEL, caspase-3 activity, and Annexin V assays are conducted to evaluate apoptosis. For TUNEL assay, HLE cells (1×10^6) were treated with l μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 48 h on glass bottom culture dishes. The cells were then fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with both 4',6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) methods using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) [11].

Caspase-3 activity and Annexin V assays were performed using HLE cells that were treated with the same concentration of test drugs for 72 h. The cell lysates were prepared and the caspase-3 activity assay was done using the Apoalert Caspase Fluorescent Assay Kit (Clontech Laboratories, Mountain View, CA, USA). The Annexin V-binding capacity of treated cells was investigated by flow cytometry using the Annexin V-FITC apoptosis detection kit I (BD, Franklin Lakes, NJ, USA). Cultured cells were washed with cold phosphate-buffered saline before incubation with Annexin V-FITC in a buffer containing propidium iodine (PI). Stained cells were analyzed by flow cytometry using the FACScan (BD). Annexin V-FITC-positive and PI negative cells were considered to be populations undergoing apoptosis.

2.5. Cell cycle assays

HLE cells were treated with l μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 72 h in DMEM medium with 1% FCS. The harvested cells were stained with PI using Cell Cycle Phase Determination Kit (Cayman, Ann Arbor, MI, USA), and the samples were then analyzed for DNA histograms and cell cycle phase distribution using a FACScan flow cytometer. The data were analyzed by using the CellQuest computer program (BD) [11].

2.6. Protein extraction and Western blot analysis

Equivalent amounts of extracted protein were examined by a Western blot analysis using specific antibodies [21]. To detect the expression level of phosphorylated RXRα (p-RXRα) protein, total phosphoprotein was affinity-purified from the total cell extracts using a PhosphoProtein Purification Column (QIAGEN, Valencia, CA, USA) and then was subjected to the Western blot analyses using an anti-RXRα antibody. GAPDH expression served as a loading control. The intensities of protein bands were quantified using NIH image software version 1.45.

2.7. RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from the HLE cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) [28]. Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify the *c-myc*, small heterodimer partner (SHP), $p21^{CIP1}$, cyclin D1, and β-actin genes. The specific primer sets for $p21^{CIP1}$, cyclin D1, and β-actin were used as described elsewhere [12,29]. The sequences for *c-myc*- and SHP-specific primers were as follows: FMYC (5'-CCC TGA GCG ATT CAG ATG AT-3') and RMYC (5'-GCT CCA GGA TGT TGT GGT TT-3'), and FSHP (5'-GCT GTC TGG AGT CCT TCT GG-3') and RSHP (5'-ACC TGA GCA AAA GCA TGT CC-3'), respectively.

2.8. FXRE reporter assays

HLE cells were transfected with FXR response element (FXRE) reporter plasmids (100 ng/well in 96-well dish), which were kindly provided by Dr. T. Nishimaki-Mogami (National Institute of Health Sciences, Tokyo, Japan), along with p*RL*-CMV (*Renilla* luciferase, 10 ng/well in 96-well dish; Promega) as an internal standard to normalize the transfection efficiency. Transfections were done using Lipofectamine LTX Reagent (Invitrogen). After exposure of the cells to the transfection mixture for 24 h, the cells were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 24 h. The cell lysates were then prepared, and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega) [11].

2.9. Statistical analysis

The data are expressed as the means ± SD. Statistical significance of the differences in the mean values was assessed with a one-way ANOVA, followed by Tukey–Kramer's multiple comparison tests. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. ACR and GW4064 cause preferential inhibition of the growth of human HCC cells in comparison with Hc normal hepatocytes

In our initial study, we examined the growth inhibitory effect of ACR and GW4064 on HLE, HLF, and Huh7 human HCC cells and on Hc hepatocytes. ACR inhibited the growth of HCC cells with an IC_{50} value of less than 4 μM. The HLF cells were most susceptible to ACR

because the IC_{50} value with this agent was 2 μM (Fig. 1A). GW4064 also inhibited the growth of this series of HCC cells with an IC_{50} value of about 1.4 μM (Fig. 1B). On the other hand, Hc cells were resistant to these agents up to 5 μM (Fig. 1). These results suggest that ACR and GW4064 preferentially inhibit the growth of HCC cells compared with that of normal hepatocytes.

3.2. ACR plus GW4064 cause synergistic inhibition of the growth of HCC cells

Next, the effects of combined treatment were examined with a range of concentrations of ACR plus GW4064 to determine whether they synergistically inhibited the growth of HLE (Fig. 2A), HLF (Fig. 2B), and Huh7 (Fig. 2C) HCC cells. We found that the CI indices for less than 1 μM ACR (0.5 or 1 μM) plus less than 0.5 μM GW4064 (0.1 or 0.5 μM) were 1+(slight synergism), 2+(moderate synergism), or 3+(synergism), respectively, in this series of HCC cells (Fig. 2D and Table 1). These findings suggest that ACR plus GW4064 might be an effective combination for the inhibition of HCC cell growth due to their synergistic activity. The combination of 1 μM ACR (about IC_{25} value) and 1 μM GW4064 (about IC_{30} value) in HLE cells (Fig. 2A and D, and Table 1) was used for the following experiments because a CI-isobologram analysis gave this combination a CI index of 1+(0.88), indicating slight synergism.

3.3. ACR plus GW4064 cooperatively induce apoptosis in HLE cells

We next examined whether the synergistic growth inhibition in HLE cells induced by treatment with ACR plus GW4064 might be associated with the induction of apoptosis. In TUNEL assays, the treatment of HLE cells with either 1 μM ACR or 1 μM GW4064 alone induced TUNEL-positive cells in approximately 19.3% or 11.9% of the total viable cells, respectively. However, the combination of these agents markedly enhanced the induction of apoptosis, with 51.6% of the total viable cells being TUNEL-positive (Fig. 3A). Similar results were also observed in the Western blot analysis for PARP expression; the combination of ACR plus GW4064 markedly enhanced PARP cleavage, indicating the induction of apoptosis (Fig. 3B). We also found an increase in the levels of caspase-3 activity in ACR alone- and GW4064 alone-treated cells, and this was significantly enhanced when the cells were treated with a combination of these agents (Fig. 3C). In addition, the percentage of Annexin V-positive cells, which was increased by treatment with GW4064 alone, was substantially increased by the combined treatment with ACR plus GW4064 (Fig. 3D). These findings suggest that the combination with ACR plus GW4064 synergistically inhibited growth of HLE human HCC cells, mainly, through the induction of apoptosis.

3.4. ACR plus GW4064 cooperatively induce G_0/G_1 cell cycle arrest in HLE cells

A cell cycle analysis was performed using DNA flow cytometry to determine whether the synergistic effects on growth inhibition caused by combined treatment with ACR plus GW4064 were associated with specific changes in cell cycle distribution. As shown in Fig. 4, the combined treatment with 1 μM ACR plus 1 μM GW4064 significantly increased the percentage of cells in the G_0/G_1 phase in comparison to that of untreated cells (76.1 ± 4.3% vs. 57.3 ± 5.8%, $P < 0.05$), whereas the population of cells in this phase was not significantly increased by treatment with ACR alone (63.6 ± 3.0%) or GW4064 alone (65.3 ± 4.5%). These findings suggest that the combination of ACR plus GW4064 cooperatively induced G_0/G_1 phase cell cycle arrest in HLE human HCC cells.

3.5. ACR plus GW4064 additively suppress the phosphorylation of RXRα, ERK, and Stat3 proteins in HLE cells

RXRα phosphorylation plays a critical role in the development of HCC and might be a promising target for HCC chemoprevention [4–8]. Therefore, the effects of the combination of ACR plus GW4064 on the phosphorylation of this nuclear receptor and related signaling molecules were investigated in HLE cells. As shown in Fig. 5, when the cells were treated with 1 μM ACR, there was a marked decrease in the expression levels of p-RXRα and p-Stat3 proteins. Treatment with 1 μM GW4064 alone also decreased the expression levels of p-ERK and p-Stat3 protein. Moreover, the expression levels of p-RXRα, p-ERK and p-Stat3 proteins were markedly decreased when the cells were treated with the combination of these agents.

3.6. ACR plus GW4064 cooperatively affect the expression levels of $p21^{CIP1}$, c-myc, cyclin D1, and SHP mRNA in HLE cells

We next examined the combined effects of ACR plus GW4064 on the expression levels of $p21^{CIP1}$, c-*myc*, and cyclin D1 mRNA in HLE cells because these genes control cell proliferation and cell cycle progression. The quantitative RT-PCR analyses revealed that treatment with neither 1 μM ACR nor 1 μM GW4064 alone had any apparent effect on the expression levels of $p21^{CIP1}$, c-*myc*, and cyclin D1 mRNA. However, when the cells were treated with the combination of these agents, there was a significant increase

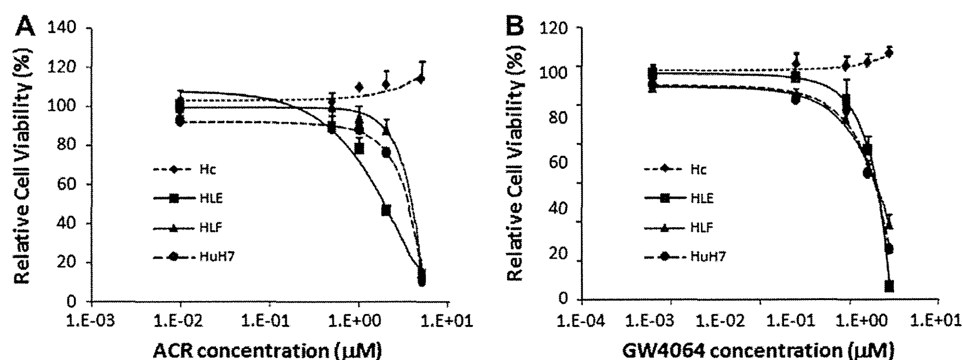


Fig. 1. Inhibition of cell growth by ACR and GW4064 in HLE, HLF, and Huh7 human HCC cells and Hc normal hepatocytes. HLE, HLF, Huh7, and Hc cells were treated with the indicated concentrations of ACR (A) or GW4064 (B) for 48 h. Cell viability was determined by MTS assay and was expressed as a percentage of the control value. *Error Bars*, SD of triplicate assays.

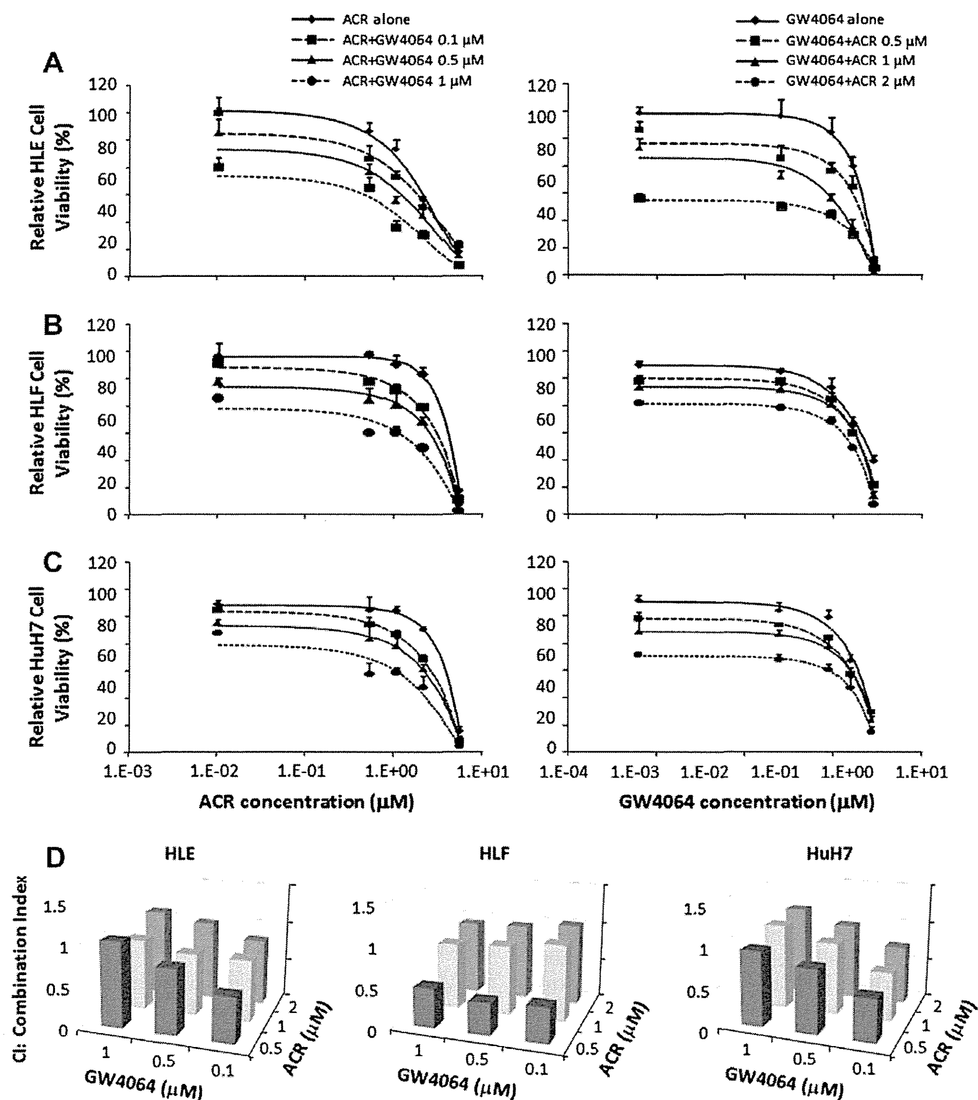


Fig. 2. Inhibition of cell growth by ACR alone, GW4064 alone, and various combinations of these agents in HCC cells. HLE (A), HLF (B), and Huh7 (C) cells were treated with the indicated concentrations of ACR alone, GW4064 alone, and various combinations of these agents for 48 h. Cell viability was determined by MTS assay and expressed as a percentage of the control value. *Error Bars*, SD of triplicate assays. (D) The data obtained in (A), (B), and (C) was used to calculate the combination index.

Table 1
Combined effects of ACR and GW4064 on HCC cells.

	HLE ACR concentration (μM)			HLF ACR concentration (μM)			HuH7 ACR concentration (μM)		
GW4064 concentration (μM)	0.5	1	2	0.5	1	2	0.5	1	2
0.1	+++	++	+	+++	+	±	+++	++	++
0.5	++	++	±	+++	++	±	++	+	±
1	±	+	±	+++	+	±	±	±	±

Note: "±", CI 0.9–1.1 additive effect; " + ", CI 0.8–0.9 slight synergism; "++", CI 0.6–0.8 moderate synergism; "+++", CI 0.4–0.6 synergism;
Abbreviations: CI, combination index; ACR, acyclic retinoid.

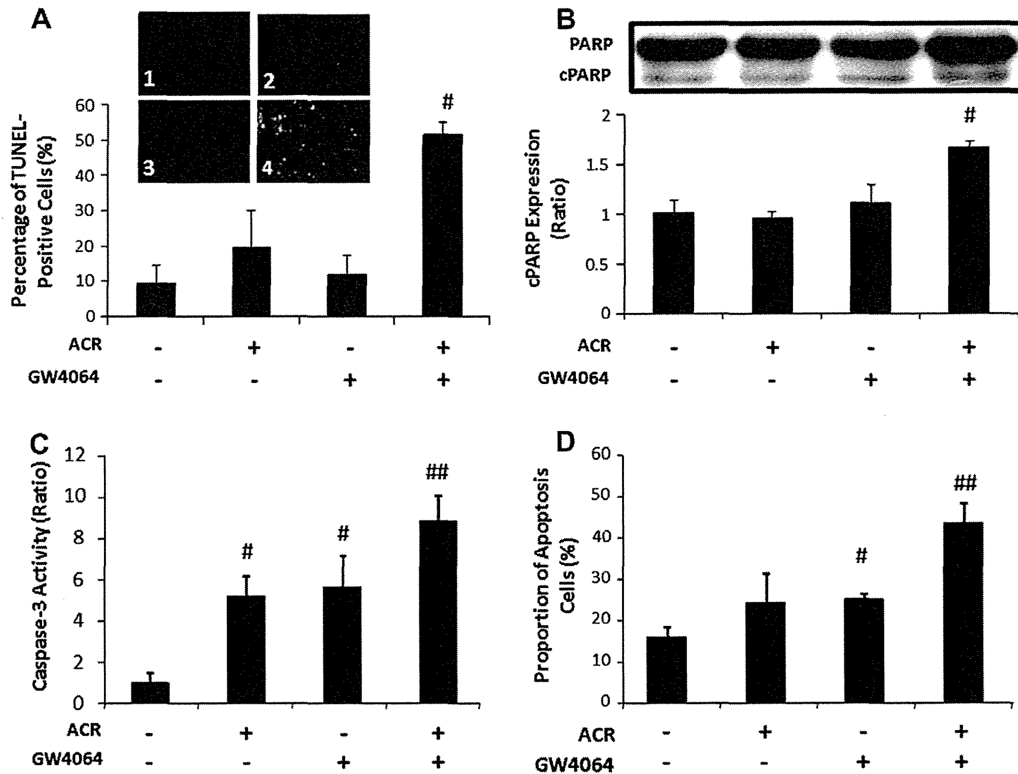


Fig. 3. Effects of the combination of ACR plus GW4064 on induction of apoptosis in HLE cells. The cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 48 h or 72 h. TUNEL assays (A) and Western blot analysis using a PARP-specific antibody (B, upper panel) were performed using cells treated with test drugs for 48 h. Caspase-3 activity assays (C) and Annexin V assays (D) were performed using samples treated for 72 h. (A) TUNEL-positive cells were counted and examined as the percentage of the DAPI-positive cell number (500 cells were counted in each flask). (B) The intensities of the cleaved PARP (c-PARP) blots were quantified using densitometry. Columns and lines indicate mean and SD of triplicate assays (lower panel). (C) Caspase-3 activity was performed with a fluorometric system. (D) Cultured cells were incubated with Annexin V-FITC in a buffer containing propidium iodine (PI). Stained cells were then analyzed by flow cytometry. Annexin V-FITC-positive and PI-negative cells were counted as apoptotic cells. #: $P < 0.05$, compared with vehicle treated cells. ##: $P < 0.05$, compared with vehicle, ACR alone, or GW4064 alone treated cells.

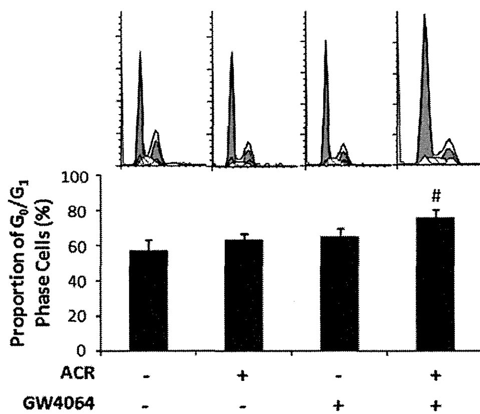


Fig. 4. Effects of the combination of ACR plus GW4064 on induction of the G_0/G_1 cell cycle arrest in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 72 h. The cells were then stained with propidium iodide to analyze their cell cycle progression. The distributions of cells in the G_0/G_1 of the cell cycle were calculated using a FACScan. *Error Bars*, SE of triplicate assays. #: $P < 0.05$, compared with vehicle treated cells.

in the levels p21^{CIP1}, and a decrease in the levels of c-*myc* and cyclin D1 mRNA expression (Fig. 6A–C). In addition, the expression level of SHP mRNA, which is one of the target genes of FXR [17,25,30,31], was also significantly increased by the combination treatment with ACR plus GW4064 (Fig. 6D).

3.7. ACR enhances the induction of FXRE promoter activities by GW4064

FXR and RXRs modulate the expression of target genes by interacting with FXRE elements located in the promoter regions of such genes [13]. Therefore, we next examined whether ACR might enhance the transcriptional activity of the FXRE promoter induced by GW4064 using transient transfection luciferase reporter assays. As shown in Fig. 6E, 1 μM GW4064 significantly increased the FXRE reporter activity in comparison with control HLE cells which were not treated with either ACR or GW4064. Moreover, when the cells were treated with a combination of 1 μM GW4064 plus 1 μM ACR, there was a significant increase in the transcriptional activity of the FXRE reporter, thus suggesting that treatment with these agents might cooperatively enhance the FXRE reporter activity.

4. Discussion

The prognosis for patients with HCC is poor and more effective strategies for the chemoprevention and chemotherapy of this malignancy are urgently required. The present study provides the

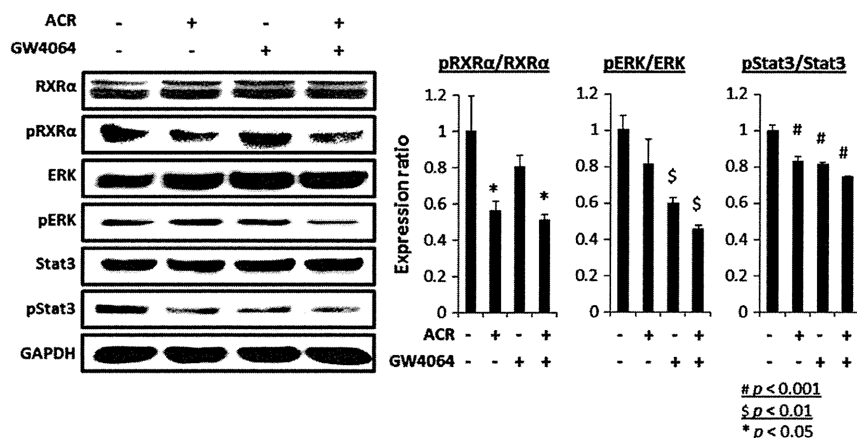


Fig. 5. Effects of the combination of ACR plus GW4064 on the phosphorylation of RXRα, ERK, and Stat3 proteins in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 12 h. The extracted proteins were examined by a Western blot analysis using the respective antibodies (left panels). The intensities of the blots were quantified using densitometry. Columns and lines indicate means and SD of triplicate assays (right panels). Repeat Western blots gave similar results. *: $P < 0.05$, $: $P < 0.01$, #: $P < 0.001$, compared with vehicle treated cells.

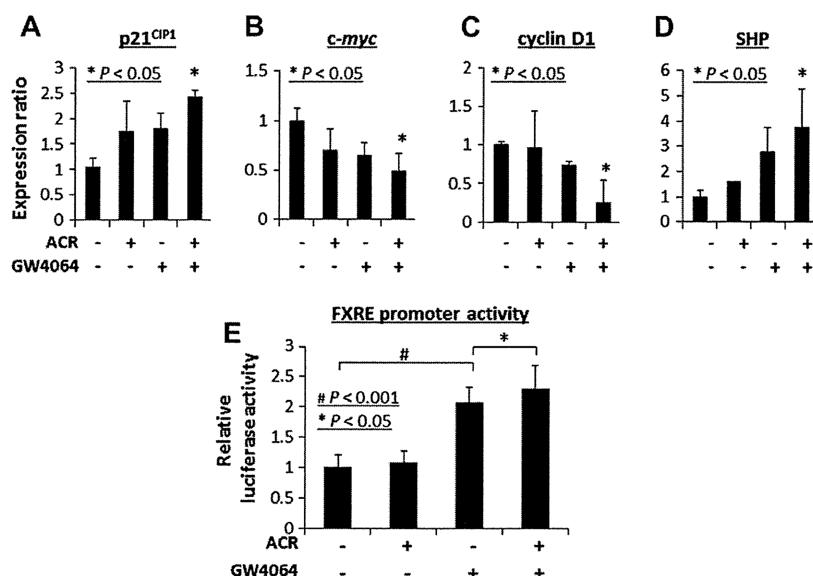


Fig. 6. Effects of the combination of ACR plus GW4064 on the expression of $p21^{CIP1}$, *c-myc*, cyclin D1, and SHP mRNA and on the transcriptional activity of the FXRE promoter in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 24 h. The extracted mRNAs were examined by a quantitative real-time RT-PCR analysis using the $p21^{CIP1}$ (A), *c-myc* (B), cyclin D1 (C), and SHP (D) specific primers. The expression levels of each mRNA were normalized to the level of β-actin mRNA. Values represent the means ± SD of triplicate analyses. *: $P < 0.05$. (E) A transient transfection reporter assay was performed with the FXRE luciferase reporter in the presence of vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064. Relative luciferase activity was determined after 24 h. Columns and lines indicate the means and SD of triplicate assays. #: $P < 0.001$, **: $P < 0.05$.

first evidence that GW4064, a synthetic ligand for FXR, preferentially inhibits the growth of HCC cells compared with Hc normal hepatocytes. This study also clearly indicates that the combination of GW4064 plus ACR, which is expected as a HCC chemopreventive agent [8–10], cause a synergistic inhibition of growth in HLE human HCC cells and that this is associated with the induction of apoptosis. This combination also acted cooperatively to induce the arrest of the cell cycle in the G_0/G_1 phase and the expression of $p21^{CIP1}$ and SHP mRNA, but suppress the expression levels of c-*myc* and cyclin D1 mRNA.

As illustrated in Fig. 7, we presume that the synergism generated by the combination of ACR plus GW4064 is mainly associated with the enhancement of the FXRE reporter activity. FXR regulates the expression of target genes by binding either as a monomer or as a heterodimer with RXR to the FXRE [13]. Therefore, in the present study, ACR and GW4064 cooperatively enhanced the binding of FXR to the FXRE promoter, thereby enhancing the expression of its target genes (Fig. 7, as indicated by A). Among the FXR target genes, SHP is considered to play a role in the inhibition of cell growth because it is a pivotal cell death receptor that targets the mitochondria, leading to the induction of apoptosis and inhibition of tumor growth [32]. GW4064 suppresses the growth of cancer cells through the activation of FXR targeted genes, including SHP [17,25,30,31]. SHP has also been shown to be a direct negative

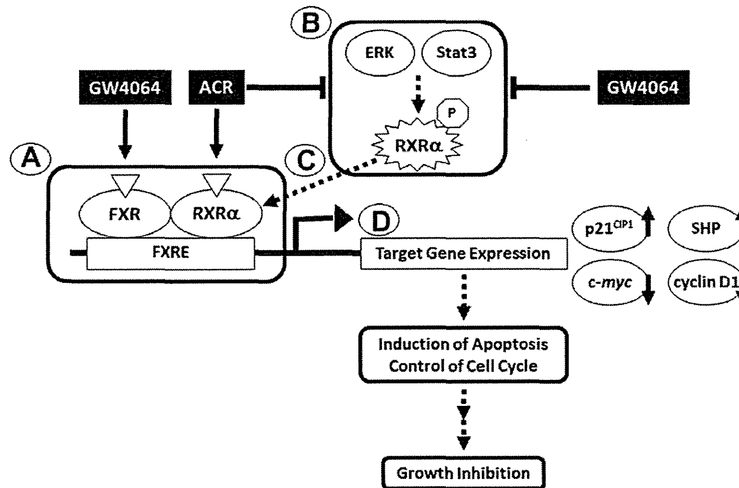


Fig. 7. A hypothetical schematic representation of the effects of the combination of ACR plus GW4064 on growth inhibition in HCC cells. ACR and GW4064 can bind to their receptors, RXRα and FXR, as ligands, and subsequently activate the FXRE promoter activity (A). ACR and GW4064 also inhibit RXRα phosphorylation, which is involved in liver carcinogenesis, by inhibiting ERK and Stat3 phosphorylation (B). The inhibition of RXRα phosphorylation by these agents might restore the function of this nuclear receptor as a heterodimeric partner for FXR (C), thus resulting in the activation of the FXRE promoter activity. Cooperative activation of this promoter activity by ACR and GW4064 regulates the expression of target genes, such as $p21^{CIP1}$, *c-myc*, cyclin D1, and SHP, which play a critical role in the induction of apoptosis, control of cell cycle progression, and inhibition of cancer cell growth (D). For additional details see Section 4.

regulator of cyclin D1 gene transcription [30]. Treatment with 1 μM GW4064 alone did not significantly increase the expression levels of SHP mRNA in the present study, whereas its expression was clearly increased by combined treatment with ACR plus GW4064. These findings may indicate that the concentration of 1 μM is insufficient to increase the levels of SHP mRNA in HCC cells and, therefore, an appropriate partner, such as ACR, is required for GW4064 to exert a synergistic effect on growth inhibition in HCC cells (Fig. 7).

In addition, recent studies have revealed that activating FXR suppresses the expression of cyclin D1 and *c-myc*, but induces the expression of $p21^{CIP1}$, by targeting the Wnt/β-catenin signaling pathway [17,31]. These findings seem to be significant because the Wnt/β-catenin pathway plays a critical role in liver carcinogenesis, and thus may be a promising target for the treatment of HCC [33]. In FXR knockout mice, sustained activation of this pathway was shown to be involved in the development of HCC [17]. On the other hand, ACR has been shown to exert growth inhibitory effects in HCC cells by targeting the Wnt/β-catenin pathway [12]. ACR also induces apoptosis and cell cycle arrest in the G_0/G_1 phase in HCC cells by regulating the expression of $p21^{CIP1}$, cyclin D1, and *c-myc* [12,19,34,35]. Therefore, the activation of FXR by GW4064 may act cooperatively with ACR to inhibit the activation of the Wnt/β-catenin pathway, subsequently decreasing the expression of cyclin D1 and *c-myc*, but increasing the expression of $p21^{CIP1}$, as was demonstrated in the present study.

In addition to chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis virus, increased evidence has indicated that a malfunction of RXRα due to phosphorylation is profoundly involved in the development of HCC [4–8]. In HCC cells, the Ras/MAPK signaling pathway is highly activated, leading to phosphorylation of RXRα, which indicates that the Ras/MAPK pathway and p-RXRα are potential targets for inhibiting the growth of HCC cells [4–8]. Indeed, ACR dephosphorylates RXRα, ERK, and Stat3 proteins, and restores the function of RXRα, thus inhibiting the growth of HCC cells and suppressing liver tumorigenesis in obese mice [4,11,20,36]. The combinations of ACR plus valproic acid or vitamin K_2 also synergistically suppressed the growth of HCC cells by inhibiting RXRα phosphorylation [11,20]. Similar to these previous studies [11,20], in the present study, inhibition of RXRα phosphorylation by the combination of ACR plus GW4064 may also have restored the function of RXRα as a master regulator of nuclear receptors, thus contributing to synergistic growth inhibition in HCC cells (Fig. 7, as indicated by B). Dephosphorylation of RXRα by this combination treatment may play a role in the observed enhancement of the FXRE promoter activity because phosphorylation of RXRα abolishes its ability to form heterodimers with other nuclear receptors, but inhibition of this phosphorylation can restore its heterodimeric activity [7]. The combination of ACR plus GW4064 may also promote RXRs homodimerization and thus enhance the promoter activity of retinoid X response element, which is associated with the anticancer mechanisms of ACR [11,37].

One of the major questions that arose was how the combination of ACR plus GW4064 could inhibit the phosphorylation of ERK and Stat3 proteins. One of the mechanisms which might explain this phenomenon is that the effects of ACR and GW4064 inhibit the activation of specific receptor tyrosine kinases (RTKs). ACR has been shown to reduce HCC development and inhibit cancer growth by targeting growth factors and their corresponding RTKs, such as the epidermal growth factor (EGF) receptor (EGFR), and downstream signaling pathways, including the Ras/MAPK and Jak/Stat3 pathways [29,38]. The activation of FXR by its ligand also reduces the expression of HER2, a member of the EGFR family of RTKs, and inhibits EGF-mediated HER2 and ERK phosphorylation in human breast cancer cells [24]. Therefore, GW4064 may increase the inhibitory effects of ACR on certain types of RTKs by activating FXR, which results in the inhibition of ERK and Stat3 phosphorylation and subsequent RXRα phosphorylation. Future studies are required to clarify whether both ACR and GW4064 synergistically exert inhibitory effects on the activation of specific RTKs.

Finally, it should be noted that, in a clinical trial showing the chemopreventive effects of ACR on the recurrence of secondary HCC [9,10], the plasma concentration of this agent (which ranged from 1 to 5 μM) was approximately the same as the concentration used in the present study (1 μM). In phase II clinical trials, a FXR

ligand also ameliorated the increase in the alkaline phosphatase levels in patients with primary biliary cirrhosis and improved the insulin sensitivity in patients with diabetes and liver steatosis, although some unfavorable events that might be associated with FXRE reporter overactivity were observed [39]. The combination of ACR plus GW4064 may resolve such problems because this combination permits the administration of lower doses of both agents for treatment. Future pharmacokinetic studies are required to determine whether the dose of GW4064 used in this study is clinically relevant and pilot studies confirming are thus called for to clarify the safety of this agent.

In conclusion, the observation that a combination of appropriate concentrations of ACR plus GW4064 can inhibit the growth of human HCC cells without affecting the growth of normal hepatocytes should encourage further clinical studies using these agents to investigate their potential for HCC chemoprevention and chemotherapy. The results of our present study suggest that combining ACR with GW4064 might hold promise as a clinical modality for the prevention and treatment of HCC, due to their synergistic effects.

References

[1] D.J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, R.M. Evans, The nuclear receptor superfamily: the second decade, Cell 83 (1995) 835–839.

[2] P. Chambon, A decade of molecular biology of retinoic acid receptors, Faseb. J. 10 (1996) 940–954.

[3] L. Altucci, M.D. Leibowitz, K.M. Ogilvie, A.R. de Lera, H. Gronemeyer, RAR and RXR modulation in cancer and metabolic disease, Nat. Rev. Drug Discov. 6 (2007) 793–810.

[4] R. Matsushima-Nishiwaki, M. Okuno, Y. Takano, S. Kojima, S.L. Friedman, H. Moriwaki, Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid, Carcinogenesis 24 (2003) 1353–1359.

[5] R. Matsushima-Nishiwaki, M. Okuno, S. Adachi, T. Sano, K. Akita, H. Moriwaki, S.L. Friedman, S. Kojima, Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma, Cancer Res. 61 (2001) 7675–7682.

[6] S. Adachi, M. Okuno, R. Matsushima-Nishiwaki, Y. Takano, S. Kojima, S.L. Friedman, H. Moriwaki, Y. Okano, Phosphorylation of retinoid X receptor suppresses its ubiquitination in human hepatocellular carcinoma, Hepatology 35 (2002) 332–340.

[7] K. Yoshimura, Y. Muto, M. Shimizu, R. Matsushima-Nishiwaki, M. Okuno, Y. Takano, H. Tsurumi, S. Kojima, Y. Okano, H. Moriwaki, Phosphorylated retinoid X receptor alpha loses its heterodimeric activity with retinoic acid receptor beta, Cancer Sci. 98 (2007) 1868–1874.

[8] M. Shimizu, K. Takai, H. Moriwaki, Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention, Cancer Sci. 100 (2009) 369–374.

[9] Y. Muto, H. Moriwaki, M. Ninomiya, S. Adachi, A. Saito, K.T. Takasaki, T. Tanaka, K. Tsurumi, M. Okuno, E. Tomita, T. Nakamura, T. Kojima, Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma prevention study group, New Engl. J. Med. 334 (1996) 1561–1567.

[10] Y. Muto, H. Moriwaki, A. Saito, Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma, New Engl. J. Med. 340 (1999) 1046–1047.

[11] H. Tatebe, M. Shimizu, Y. Shirakami, H. Sakai, Y. Yasuda, H. Tsurumi, H. Moriwaki, Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells, Cancer Lett. 285 (2009) 210–217.

[12] M. Suzui, M. Masuda, J.T. Lim, C. Albanese, R.G. Pestell, I.B. Weinstein, Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1, Cancer Res. 62 (2002) 3997–4006.

[13] Y.D. Wang, W.D. Chen, D.D. Moore, W. Huang, FXR: a metabolic regulator and cell protector, Cell Res. 18 (2008) 1087–1095.

[14] W. Huang, K. Ma, J. Zhang, M. Qatanani, J. Cuvillier, J. Liu, B. Dong, X. Huang, D.D. Moore, Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration, Science 312 (2006) 233–236.

[15] F. Yang, X. Huang, T. Yi, Y. Yen, D.D. Moore, W. Huang, Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor, Cancer Res. 67 (2007) 863–867.

[16] I. Kim, K. Morimura, Y. Shah, Q. Yang, J.M. Ward, F.J. Gonzalez, Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice, Carcinogenesis 28 (2007) 940–946.

[17] A. Wolfe, A. Thomas, G. Edwards, R. Jaseja, G.L. Guo, U. Apte, Increased activation of the Wnt/{beta}-catenin pathway in spontaneous hepatocellular carcinoma observed in farnesoid x receptor knockout mice, J. Pharmacol. Exp. Ther. 338 (2011) 12–21.

[18] A. Obora, Y. Shiratori, M. Okuno, S. Adachi, Y. Takano, R. Matsushima-Nishiwaki, I. Yasuda, Y. Yamada, K. Akita, T. Sano, J. Shimada, S. Kojima, Y. Okano, S.L. Friedman, H. Moriwaki, Synergistic induction of apoptosis by acyclic retinoid and interferon-beta in human hepatocellular carcinoma cells, Hepatology 36 (2002) 1115–1124.

[19] M. Shimizu, M. Suzui, A. Deguchi, J.T. Lim, D. Xiao, J.H. Hayes, K.P. Papadopoulos, I.B. Weinstein, Synergistic effects of acyclic retinoid and OSI-461 on growth inhibition and gene expression in human hepatoma cells, Clin. Cancer Res. 10 (2004) 6710–6721.

[20] T. Kanamori, M. Shimizu, M. Okuno, R. Matsushima-Nishiwaki, H. Tsurumi, S. Kojima, H. Moriwaki, Synergistic growth inhibition by acyclic retinoid and vitamin K_2 in human hepatocellular carcinoma cells, Cancer Sci. 98 (2007) 431–437.

[21] H. Tatebe, M. Shimizu, Y. Shirakami, H. Tsurumi, H. Moriwaki, Synergistic growth inhibition by 9-cis-retinoic acid plus trastuzumab in human hepatocellular carcinoma cells, Clin. Cancer Res. 14 (2008) 2806–2812.

[22] Y. Liu, Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis, J. Clin. Invest. 112 (2003) 1678–1687.

[23] I. Kim, S.H. Ahn, T. Inagaki, M. Choi, S. Ito, G.L. Guo, S.A. Kliewer, F.J. Gonzalez, Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine, J. Lipid Res. 48 (2007) 2664–2672.

[24] C. Giordano, S. Catalano, S. Panza, D. Vizza, I. Barone, D. Bonofiglio, L. Gelsomino, P. Rizza, S.A. Fuqua, S. Ando, Farnesoid X receptor inhibits tamoxifen-resistant MCF-7 breast cancer cell growth through downregulation of HER2 expression, Oncogene (2011).

[25] K.E. Swales, M. Korbonits, R. Carpenter, D.T. Walsh, T.D. Warner, D. Bishop-Bailey, The farnesoid X receptor is expressed in breast cancer and regulates apoptosis and aromatase expression, Cancer Res. 66 (2006) 10120–10126.

[26] J. Kaeding, E. Bouchaert, J. Belanger, P. Caron, S. Chouinard, M. Verreault, O. Larouche, G. Pelletier, B. Staels, A. Belanger, O. Barbier, Activators of the farnesoid X receptor negatively regulate androgen glucuronidation in human prostate cancer LNCAP cells, Biochem. J. 410 (2008) 245–253.

[27] L. Zhao, M.G. Wientjes, J.L. Au, Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram, and combination index analyses, Clin. Cancer Res. 10 (2004) 7994–8004.

[28] M. Shimizu, Y. Yasuda, H. Sakai, M. Kubota, D. Terakura, A. Baba, T. Ohno, T. Kochi, H. Tsurumi, T. Tanaka, H. Moriwaki, Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-db/db obese mice, BMC Cancer 11 (2011) 281.

[29] M. Shimizu, M. Suzui, A. Deguchi, J.T. Lim, I.B. Weinstein, Effects of acyclic retinoid on growth, cell cycle control, epidermal growth factor receptor signaling, and gene expression in human squamous cell carcinoma cells, Clin. Cancer Res. 10 (2004) 1130–1140.

[30] Y. Zhang, P. Xu, K. Park, Y. Choi, D.D. Moore, L. Wang, Orphan receptor small heterodimer partner suppresses tumorigenesis by modulating cyclin D1 expression and cellular proliferation, Hepatology 48 (2008) 289–298.

[31] R.R. Maran, A. Thomas, M. Roth, Z. Sheng, N. Esterly, D. Pinson, X. Gao, Y. Zhang, V. Ganapathy, F.J. Gonzalez, G.L. Guo, Farnesoid X receptor deficiency in mice leads to increased intestinal epithelial cell proliferation and tumor development, J. Pharmacol. Exp. Ther. 328 (2009) 469–477.

[32] Y. Zhang, J. Soto, K. Park, G. Viswanath, S. Kuwada, E.D. Abel, L. Wang, Nuclear receptor SHP, a death receptor that targets mitochondria, induces apoptosis and inhibits tumor growth, Mol. Cell Biol. 30 (2010) 1341–1356.

[33] D.J. Mulholland, S. Dedhar, G.A. Coetzee, C.C. Nelson, Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know?, Endocr Rev. 26 (2005) 898–915.

[34] Y. Yamada, Y. Shidoji, Y. Fukutomi, T. Ishikawa, T. Kaneko, H. Nakagama, M. Imawari, H. Moriwaki, Y. Muto, Positive and negative regulations of albumin gene expression by retinoids in human hepatoma cell lines, Mol. Carcinog. 10 (1994) 151–158.

[35] M. Suzui, M. Shimizu, M. Masuda, J.T. Lim, N. Yoshimi, I.B. Weinstein, Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells, Mol. Cancer Ther. 3 (2004) 309–316.

[36] M. Shimizu, H. Sakai, Y. Shirakami, J. Iwasa, Y. Yasuda, M. Kubota, K. Takai, H. Tsurumi, T. Tanaka, H. Moriwaki, Acyclic retinoid inhibits diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BLKS/J- +(db)/+Lepr(db) mice, Cancer Prev. Res. 4 (2011) 128–136.

[37] H. Araki, Y. Shidoji, Y. Yamada, H. Moriwaki, Y. Muto, Retinoid agonist activities of synthetic geranyl geranoic acid derivatives, Biochem. Biophys. Res. Commun. 209 (1995) 66–72.

[38] T. Sano, M. Kagawa, M. Okuno, N. Ishibashi, M. Hashimoto, M. Yamamoto, R. Suzuki, H. Kohno, R. Matsushima-Nishiwaki, Y. Takano, H. Tsurumi, S. Kojima, S.L. Friedman, H. Moriwaki, T. Tanaka, Prevention of rat hepatocarcinogenesis by acyclic retinoid is accompanied by reduction in emergence of both TGF-alpha-expressing oval-like cells and activated hepatic stellate cells, Nutr. Cancer 51 (2005) 197–206.

[39] S. Fiorucci, S. Cipriani, A. Mencarelli, F. Baldelli, G. Bifulco, A. Zampella, Farnesoid X Receptor Agonist for the Treatment of Liver and Metabolic Disorders: Focus on 6-ethyl-CDCA, Mini Rev. Med. Chem. 11 (2011) 753–762.

Cancer Science
The official journal of the Japanese Cancer Association

Suppression of azoxymethane-induced colonic preneoplastic lesions in rats by 1-methyltryptophan, an inhibitor of indoleamine 2,3-dioxygenase

Kengo Ogawa,[1] Takeshi Hara,[1] Masahito Shimizu,[1] Soranobu Ninomiya,[1] Junji Nagano,[1] Hiroyasu Sakai,[1] Masato Hoshi,[2] Hiroyasu Ito,[2] Hisashi Tsurumi,[1,5] Kuniaki Saito,[3] Mitsuru Seishima,[2] Takuji Tanaka[4] and Hisataka Moriwaki[1]

[1]First Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu; [2]Department of Informative Clinical Medicine, Gifu University Graduate School of Medicine, Gifu; [3]Human Health Sciences, Graduate School of Medicine and Faculty of Medicine, Kyoto University, Kyoto; [4]The Tohkai Cytopathology Institute, Cancer Research and Prevention (TCI-CaRP), Gifu, Japan

(Received November 29, 2011/Revised February 3, 2012/Accepted February 4, 2012/Accepted manuscript online February 9, 2012/Article first published online March 6, 2012)

The escape of preneoplastic cells from the immune system, which is caused by immune tolerance, occurs during the development of several types of tumors. Indoleamine 2,3-dioxygenase (IDO) plays a critical role in the induction of immune tolerance. In the present study we investigated the effects of 1-methyltryptophan (1-MT), an IDO inhibitor, and (–)-epigallocatechin gallate (EGCG), the major catechin in green tea, on the development of azoxymethane (AOM)-induced colonic preneoplastic lesions by focusing on the inhibition of IDO. To induce colonic premalignant lesions, male F344 rats were injected with AOM (20 mg/kg body weight, s.c.) once a week for 2 weeks. They also received 0.2% 1-MT or 0.1% EGCG in their drinking water for 4 weeks, starting 1 week before the first dose of AOM. Both 1-MT and EGCG significantly decreased the total number of aberrant crypt foci and β-catenin-accumulated crypts, which overexpressed IDO protein. Treatment with EGCG decreased IDO mRNA expression in both the colonic epithelium and stroma of rats induced by AOM. The AOM-induced increase in *cyclooxygenase-2* mRNA expression in the colonic stroma was significantly decreased by EGCG. Furthermore, AOM-induced increases in IDO activity in the serum and stroma were significantly inhibited by 1-MT and EGCG. Inhibition of IDO activity by 1-MT and EGCG was also observed in cell-free assays. These findings suggest that upregulation of IDO activity is observed in the early stages of colon carcinogenesis and that the use of IDO inhibitors, such as 1-MT and EGCG, which suppress the occurrence of colonic preneoplastic lesions, could be a novel strategy for the chemoprevention of colon cancer. (*Cancer Sci* 2012; 103: 951–958)

The immune system recognizes preneoplastic cells and, in most cases, eliminates these cells before they expand into clinically detectable tumors. Therefore, the escape of precancerous cells from the immune system, which is closely associated with immune tolerance, is involved in the development of several types of tumors.[1] Recent studies have suggested that indoleamine 2,3-dioxygenase (IDO) plays a crucial role in the induction of immune tolerance.[2] Indoleamine 2,3-dioxygenase is an intracellular enzyme that catalyses the first and rate-limiting steps in the catabolism of the essential amino acid tryptophan along the kynurenine pathway.[3] In the tumor microenvironment, increased IDO activity inhibits the proliferation of T and natural killer cells and induces apoptosis through tryptophan depletion and the production of toxic tryptophan catabolites.[4] Overexpression of IDO has been shown to be correlated with poor clinical outcome in patients with ovarian carcinoma, endometrial carcinoma, and colorectal carcinoma.[5–7] We have recently reported that, in diffuse large B-cell lymphoma, IDO expression in tumor cells and serum concentrations of L-kynurenine, which reflect IDO activity, are useful indicators of a poor prognosis.[8,9] Several preclinical studies using rodent cancer models have demonstrated that IDO inhibitors, such as 1-methyltryptophan (1-MT), are therapeutically beneficial, especially when combined with different types of cytotoxic chemotherapeutic agents.[10,11] These reports suggest that targeting IDO, and therefore regulating tryptophan catabolism, may be an effective strategy for the treatment of certain types of human malignancies.[12] However, the possibility of cancer chemoprevention by inhibiting IDO expression and/or activity has not been considered.

(−)-Epigallocatechin gallate (EGCG), one of the major catechins in green tea, is the most biologically active component of green tea. It has been shown to exert its cancer chemopreventive and anti-carcinogenic effects in various organs, including the colon.[13,14] Previously, we demonstrated that EGCG can inhibit the growth of and induce apoptosis in human colorectal cancer cells.[15–17] The inhibitory effects of EGCG on both inflammation- and obesity-related colon carcinogenesis have also been demonstrated.[18,19] In addition, green tea polyphenols in the drinking water have been shown to inhibit the development of putative preneoplastic lesions called aberrant crypt foci (ACF) in rats treated with azoxymethane (AOM), which induces ACF.[20–22]

Recently, it was been reported that EGCG administration suppresses the expression of IDO in interferon (IFN)-γ-stimulated murine dendritic cells[23] and human oral cancer cell lines.[24] Although the mechanisms underlying the role of IDO in carcinogenesis have not yet been clarified, we hypothesized that the inhibitory effect of EGCG on IDO expression may contribute to the anti-carcinogenic properties of EGCG. To confirm our hypothesis, we examined the effects of 1-MT and EGCG on the development in the colon of AOM-induced preneoplastic lesions, namely ACF[21] and β-catenin-accumulated crypts (BCAC),[25] in male F344 rats by focusing on the inhibition of IDO expression and activity.

Materials and Methods

Animals, chemicals, and diets. Male F344 rats, aged 4 weeks (Charles River Japan, Tokyo, Japan), were maintained at the Gifu University Animal Facility according to Institutional Animal Care Guidelines. All rats were housed in plastic cages with free access to drinking water and a pelleted basal diet

[5]To whom correspondence should be addressed.
E-mail: htsuru@gifu-u.ac.jp

doi: 10.1111/j.1349-7006.2012.02237.x
© 2012 Japanese Cancer Association