

Figure 1 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on the growth of Huh7 xenografts. BALB/c nude mice were injected s.c. with 5×10^6 Huh7 cells. One week after the injection, the mice were divided into four groups and treated as follows for 5 weeks: group 1, untreated control group (Con); group 2, 0.03% ACR-treated group; group 3, 3.0% BCAA-treated group; group 4, 0.03% ACR and 3.0% BCAA-treated group. The tumor volumes in each group at the termination of experiment are represented. Bars, standard deviation. * $P < 0.01$, significant differences obtained by comparisons to groups 1, 2, and 3.

of HCC in clinical trials.^{3,15} On the other hand, the simultaneous treatment of the mice with these concentrations of ACR plus BCAA produced a significant decrease in the growth of HCC xenografts; the tumor volume was inhibited by 73% in the combination treatment group in comparison to the control group ($P < 0.01$).

BCAA inhibits the phosphorylation of Akt and IGF-1R, and enhances the suppression of the RXR- α and ERK phosphorylation by ACR in HCC xenografts

Retinoid X receptor- α phosphorylation by Ras/mitogen-activated protein kinase is closely associated with the development of HCC, and thus might be a critical target for chemoprevention of HCC.^{2,9} BCAA inhibits the activation of IGF-1R and Akt and this is associated with the cancer chemopreventive effects of this agent.^{16,21} Therefore, the combined effects of ACR plus BCAA on the phosphorylation of RXR- α , ERK, Akt and IGF-1R proteins were investigated in Huh7 xenografts. The expression levels of p-RXR- α and p-ERK proteins, which decreased in the ACR alone-treated group in comparison to the control group (Fig. 2a, column 2), decreased

to a greater extent when the mice were treated with the combination of ACR plus BCAA (Fig. 2a, column 4). The expression levels of p-Akt and p-IGF-1R proteins, which were decreased in the BCAA alone-treated group (Fig. 2a, column 3), were also further reduced by combined treatment with ACR plus BCAA (Fig. 2a, column 4).

Combined treatment of ACR plus BCAA induces the RAR- β and p21^{CIP1} mRNA in HCC xenografts

The combined effect of ACR plus BCAA on the induction of the RAR- β and p21^{CIP1} mRNA was next examined because, in addition to the inhibition of RXR- α phosphorylation, ACR is known to inhibit the growth of HCC cells by enhancing the expression of these molecules.^{4,5,7} Semiquantitative RT-PCR analyses showed that treatment with both ACR alone and BCAA alone tended to increase the levels of RAR- β mRNA, but the differences were not significant (Fig. 2b, columns 2 and 3). On the other hand, when ACR was combined with BCAA, the expression levels of this mRNA were significantly enhanced in comparison to the control group (Fig. 2b, column 4). In addition, treatment with ACR alone and the combination of ACR plus BCAA significantly increased the expression of p21^{CIP1} mRNA (Fig. 2c, columns 2 and 4), a negative modulator of cell cycle progression,²² in comparison to the control group.

DISCUSSION

COMBINATION CHEMOPREVENTION IS often advantageous because it provides the potential for additive or, in some instances, synergistic effects between specific agents. The present study clearly indicated that the combination of ACR plus BCAA, both of which exert chemopreventive properties on HCC development,^{3,15} causes potent inhibition of growth in human HCC xenografts. The hypotheses that explain this beneficial effect are summarized in Figure 3.

Initially, it should be emphasized that the phosphorylation of RXR- α and ERK proteins was strongly inhibited by the combination of ACR plus BCAA. This study and prior ones^{5,8,14,23} show that ACR alone inhibits the phosphorylation of these proteins, thus indicating that BCAA could enhance the effect of ACR in HCC xenografts. These findings seem to be significant because restoration of the function of RXR- α as a master regulator of nuclear receptors by targeting its phosphorylation might be an effective strategy for the prevention and treatment of HCC.² BCAA may support the effect of

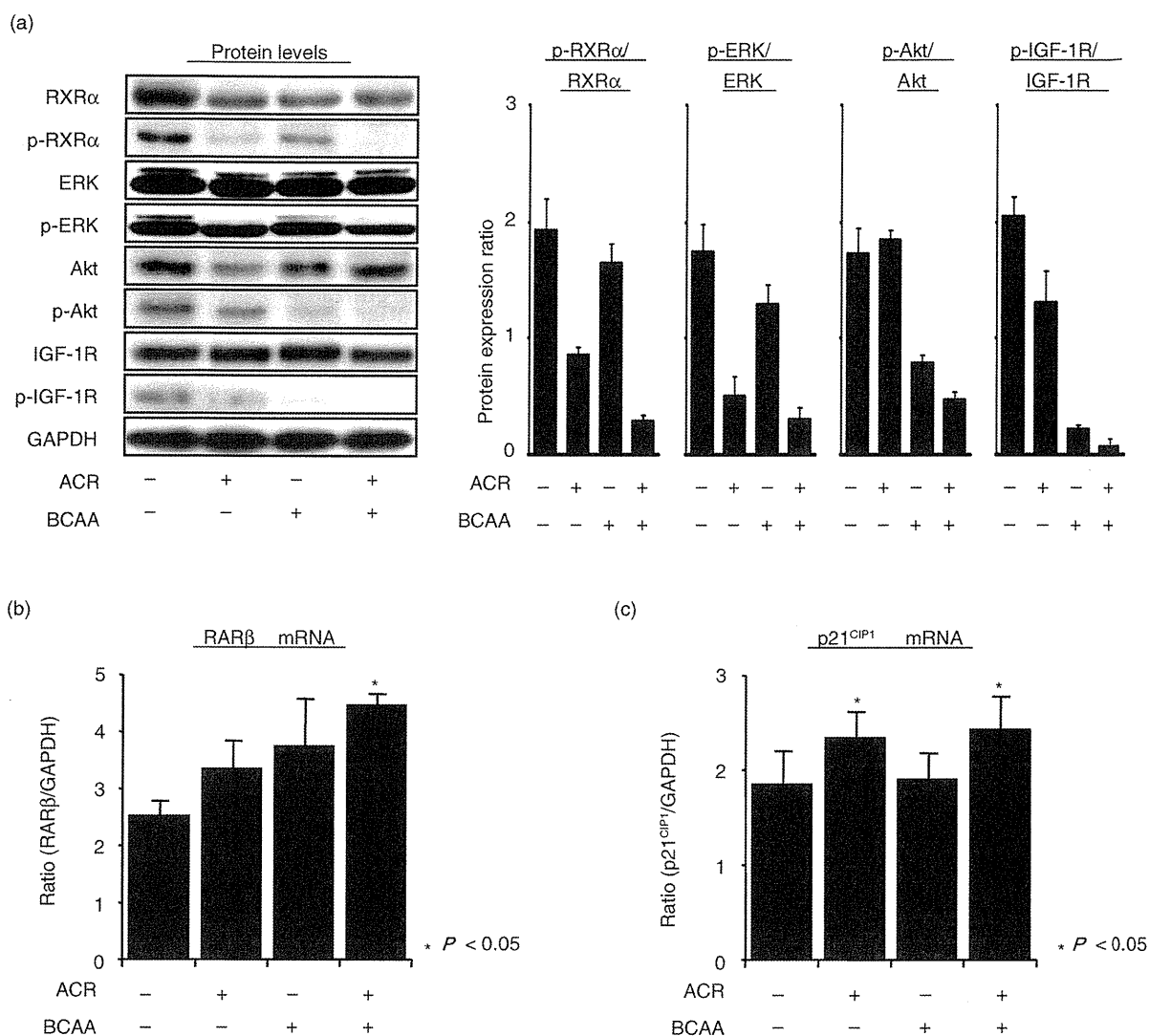


Figure 2 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on phosphorylation of retinoid X receptor- α (RXR- α), extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins and expression levels of retinoic acid receptor- β (RAR- β) and p21^{CIP1} mRNA in Huh7 xenografts. The xenografts were excised from each animal at the termination of the experiment and tumor extracts were examined by a western blot analysis using the respective antibodies (a) or were examined by a quantitative real-time reverse transcription polymerase chain reaction analysis using RAR- β (b) and p21^{CIP1} (c) specific primers. (a) Western blot analysis for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using a single membrane and equal protein loading was verified by the detection of this protein. Repeat western blots yielded similar results. Lanes, protein samples from each group (left). The intensities of blots were quantitated by densitometry (right). (b,c) The expression levels of RAR- β (b) and p21^{CIP1} (c) genes were normalized to GAPDH expression. Bars, standard deviations of triplicate assays. * $P < 0.05$, significant differences obtained by comparison to the control group (group 1). p-, phosphorylated.

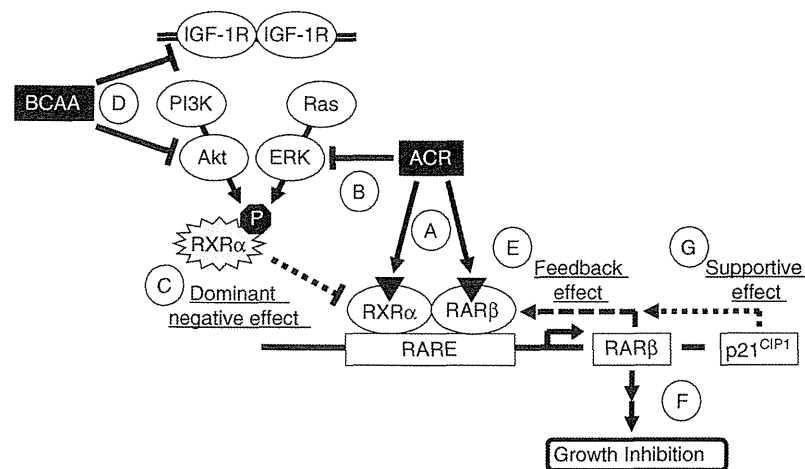


Figure 3 Hypothetical schematic representation of the effect of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on growth inhibition in hepatocellular carcinoma (HCC) xenografts. ACR can bind to both retinoic acid receptor (RAR) and retinoid X receptor (RXR) as a ligand (a), and activate the retinoic acid responsive element (RARE) promoter activity, thus increasing the levels of both RAR- β and p21^{CIP1} because the promoter region of these molecules contains RARE. In parallel, ACR inactivates the Ras/mitogen-activated protein kinase signaling pathways (b). This signaling pathway phosphorylates RXR- α , and thus impairs the function of this receptor in a dominant-negative manner (c). On the other hand, BCAA inhibits the activation of IGF-1R and its downstream Akt, which is also involved in RXR- α phosphorylation (d). Cooperative inhibition of RXR- α phosphorylation by ACR plus BCAA might restore the function of this receptor and subsequently increase RAR- β expression. This induction of RAR- β and its activation by the ligand ACR might produce a positive feedback effect on the expression of RAR- β itself (e), thus enhancing inhibition of growth in HCC cells (f). Induction of p21^{CIP1} might support this positive feedback effect (g). For additional details see the "Discussion" section. ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor.

ACR, at least in part, by inhibiting the activation of IGF-1R and its downstream Akt, because some types of receptor tyrosine kinases (RTK), including IGF-1R, might phosphorylate RXR- α through the phosphorylation of ERK and Akt. ACR and BCAA reduce the development of HCC and suppress the growth of cancer cells by inhibiting the activation of specific RTK, including IGF-1R and epidermal growth factor receptor (EGFR).^{6,16,24} BCAA also suppresses insulin-induced hepatic tumor cell proliferation by inhibiting ERK and Akt phosphorylation.¹⁸ The previous reports showing that there is a cross-talk between EGFR and IGF-1R, and that the simultaneous targeting of these RTK induces a synergistic inhibition of growth in HCC cells, might give this hypothesis credibility.^{25,26}

The reduction in the dominant negative effect of RXR- α phosphorylation by combining ACR plus BCAA might activate the transcriptional activity of retinoic acid responsive element (RARE).^{5,10} This is associated with the increased expression of RAR- β and p21^{CIP1} mRNA because the promoter region of these genes contains RARE.^{27,28} RAR- β , which is also a receptor for ACR, can

exert tumor-suppressive effects in cancer cells.²⁹ Therefore, the induction of RAR- β by the treatment with ACR plus BCAA might have played a critical role in inhibiting the growth of HCC xenografts in the present study. In addition, this induction might be, at least in part, associated with p21^{CIP1} upregulation by ACR plus BCAA because introduction of the p21^{CIP1} gene into cells induces RAR- β expression and sensitizes cancer cells to retinoid treatment.³⁰ This hypothesis may be supported by recent reports that a substantial induction of RAR- β and p21^{CIP1} produces positive feedback effects on the expression of RAR- β .^{5,12}

Acyclic retinoid has an agonistic activity for both RAR and RXR.² Therefore, the reduction of the dominant-negative effect of RXR- α phosphorylation and the induction of the RAR- β expression by the combination of ACR plus BCAA might exert a significant inhibition of growth in the HCC xenografts. Because this study shows the possibility that the combination treatment consisting of ACR plus BCAA is an effective regimen for the treatment of HCC, we presume that this combination might also be useful for the prevention of HCC. In order to confirm

this prediction, future studies are required to determine whether this combination treatment prevents the development of HCC using chemically-induced liver carcinogenesis in a rodent model with, for example, diethylnitrosamine.

In conclusion, this study, as well as prior ones,^{5,11–14} indicates that the combination chemoprevention using ACR as a key agent might be an effective strategy for the prevention and treatment of HCC. Among such regimens, particularly combining ACR with BCAA might hold promise as a clinical modality for the chemoprevention of HCC because clinical trials have shown that both of these agents can significantly prevent the development of HCC without causing any adverse effects.^{3,15}

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Role of Acyclic Retinoid in the Chemoprevention of Hepatocellular Carcinoma: Basic Aspects, Clinical Applications, and Future Prospects

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Abstract: The poor prognosis for hepatocellular carcinoma (HCC) is associated with its high rate of recurrence in the cirrhotic liver. Therefore, development of effective strategies for preventing recurrence and secondary tumors will improve the clinical outcome of HCC patients. A malfunction of the retinoid X receptor- α (RXR α) due to phosphorylation by the Ras-MAPK signaling pathway is profoundly associated with liver carcinogenesis, and thus, may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), which inhibits Ras-MAPK activation and RXR α phosphorylation, successfully prevents HCC recurrence, thus improving patient survival. The fundamental concept of HCC chemoprevention by ACR is “clonal deletion,” which is defined as the removal of latent malignant clones from the liver before they expand into clinically detectable HCC. “Combination chemoprevention” using ACR as a key drug holds great promise of a new effective strategy for the prevention of HCC because of its synergism. ACR is also expected to prevent the development of HCC in obese people, who are at an increased risk to HCC, because this agent significantly inhibits obesity-related liver tumorigenesis in the rodent model. Here, we review the detailed effects of ACR on preventing HCC development, especially based on the results of our basic and clinical research.

Keywords: Acyclic retinoid, chemoprevention, clonal deletion, combination therapy, HCC, obesity, phosphorylated RXR α .

INTRODUCTION

Retinoids, a group of structural and functional derivatives of vitamin A, have fundamental effects on cellular activities, including growth, differentiation, and apoptosis, as well as on morphology [1-3]. Because of this, loss of retinoid activity or responsiveness is linked to carcinogenesis, including the development of hepatocellular carcinoma (HCC). Targeting retinoid abnormalities may therefore be an effective strategy for the prevention and/or treatment of certain types of malignancies [4, 5]. We have reported in clinical trials that the administration of acyclic retinoid (ACR), which is the same substance as NIK-333 and Peretinoin (Kowa Pharmaceutical Co., Tokyo, Japan; Fig. 1), reduces the incidence of post-therapeutic recurrence of HCC, and thus, improves patient survival from this malignancy (for the most recent results, see Ref. 9) [6-9]. Many experimental studies have revealed the pleiotropic effects of ACR in the prevention of HCC and suppression of cancer cell growth [10-12]. Among these effects, targeting the phosphorylation of retinoid X receptor- α (RXR α), which is closely involved in liver carcinogenesis [13], is one of the critical mechanisms of inhibition of HCC development by ACR [14-17].

The aim of this article is to review the evidence that a malfunction of RXR α due to phosphorylation plays a critical role in liver carcinogenesis, and that ACR prevents the development of HCC by targeting this retinoid receptor. We also review the concept of “clonal deletion” therapy, a practical approach to preventing HCC development, and the

possibility of “combination chemoprevention,” which uses ACR as a key drug. In addition, the possibility that ACR can inhibit the development of liver tumorigenesis associated with HCC risk factors such as obesity and insulin resistance is also discussed.

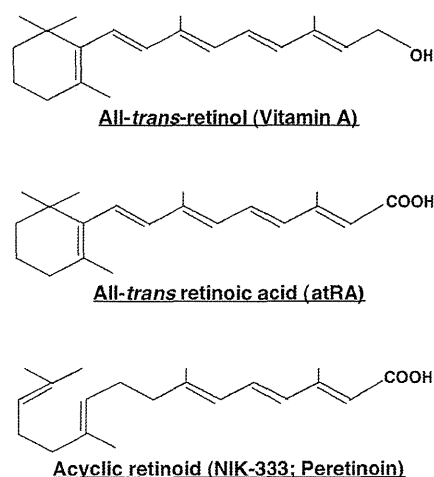


Fig. (1). The chemical structures of all-*trans* retinol (vitamin A), all-*trans* retinoic acid (atRA), and acyclic retinoid (ACR). ACR is the same substance as NIK-333 and peretinoin.

RETINOIDS AND THEIR RECEPTORS

Retinoids consist of natural and synthetic molecules. Natural retinoids include retinyl esters, retinol, and retinoic acid (RA). Large numbers of synthetic retinoids, including ACR, have also been developed (Fig. 1). Retinoids exert

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their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs. RARs bind to all-*trans*-RA (atRA), a drug for acute promyelocytic leukemia [18], and 9-*cis*-RA (9cRA) with similar affinities, whereas RXRs bind only to 9cRA. Both RAR and RXR are composed of 3 subtypes (α , β , and γ), which are characterized by a modular domain structure [1-3].

Like other members of the nuclear receptor superfamily, the retinoid receptors are ligand-dependent transcription factors. After ligand binding, RXRs form homodimers as well as heterodimers with RARs, which interact with the retinoid X response element (RXRE) or the retinoic acid receptor responsive element (RARE), located in the promoter region of target genes, thereby modulating gene expression [1-3]. In addition to forming a heterodimer with RARs, RXRs can also form heterodimers with several other nuclear receptors, thus indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors [3]. Therefore, RXRs play an essential role in controlling normal cell proliferation, differentiation, and death (regulation of apoptosis), and act as master regulators of nuclear receptors [3]. On the other hand, these facts also suggest that altered expression and function of retinoids and their receptors, especially RXRs, are strongly associated with deviation from normal cell proliferation and death, which are key factors for cancer development [4, 5].

CLINICAL CHARACTERISTICS OF HCC

HCC is a major healthcare problem worldwide, accounting for 750,000 annual cases; approximately the same number of individuals (700,000) die from this malignancy each year [19]. HCC development is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by a persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [20, 21]. Patients with viral liver cirrhosis have a high risk for HCC with an annual rate of carcinogenesis of approximately 7%. The frequency of HCC recurrence after curative treatment is also very high in cirrhotic patients; the recurrence rate 5 years after definitive therapy may exceed 70% [22-25], suggesting that curative treatment for HCC is difficult once this malignancy has developed. In addition, no effective and established chemotherapeutic agents are currently available for this malignancy. These facts indicate the limited improvement in HCC prognosis, and therefore, there is an urgent need to develop more effective strategies for chemoprevention and chemotherapy of this malignancy. In particular, cancer chemoprevention, an approach wherein a natural or synthetic chemical compound arrests or reverses premalignant cells *via* physiological pathways [26], is one of the most promising and practical strategies for the treatment of HCC because the high-risk groups of this cancer, such as hepatitis virus-positive patients, are easily identified; thus, the strategy targets good candidates for intervention.

RXR α PHOSPHORYLATION AND LIVER CARCINOGENESIS

Abnormalities in retinoid signaling are prominently involved in the development of HCC. In a rodent model of

liver carcinogenesis, retinol is locally deficient in the HCC but not in the adjacent normal liver tissues [27]. The RAR β gene, which is regarded as a tumor suppressor gene because of its ability to regulate cell growth and apoptosis [28], is an HBV integration site, and its expression is markedly decreased in human HCC [29, 30]. In a rat model of chemically-induced liver carcinogenesis, the expression levels of RAR β protein and mRNA are also decreased in HCC [31]. On the other hand, RAR γ is overexpressed in the HCC tissues and cells; this is associated with growth of HCC cells, which suggests the oncogenic potential of this retinoid receptor in liver carcinogenesis [32].

Among retinoid receptors, RXR α alterations are particularly implicated in the development and progression of HCC. The expression of RXR α is decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion, in a rat model of chemically-induced liver carcinogenesis. These findings indicate that the repression of RXR α occurs even in the early stage of liver carcinogenesis [31]. In addition, we have previously shown that RXR α protein is anomalously phosphorylated at the serine and threonine residues, and that it accumulates in both surgically resected human HCC tissues and HCC cell lines [13, 33]. The activated extracellular signal-regulated kinase (ERK) is highly expressed in HCC cells, and constitutive phosphorylation at the serine at position 260 of RXR α , a mitogen-activated protein kinase (MAPK)/ERK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and promotion of cancer cell growth; in turn, the abrogation of phosphorylation by a MAPK inhibitor restores the degradation of RXR α in a ligand-dependent manner [13, 14]. The phosphorylated form of RXR α (p-RXR α) is resistant to ubiquitination and proteasome-mediated degradation in human HCC cells, resulting in an accumulation of this phosphorylated protein within the HCC tissues [34]. In addition, p-RXR α abolishes its ability to form heterodimers with RAR β , and this is associated with uncontrolled cell growth and resistance to retinoids [35]. The analogous effects of phosphorylated RXR α in the negative modulation of its heterodimeric binding partners have also been reported in several studies [36-38]. These findings, therefore, suggest that the accumulation of p-RXR α , which is regarded as the non-functional form of RXR α , may interfere with the function of normal (unphosphorylated) RXR α in a dominant-negative manner, and thus, play a critical role in HCC development (Fig. 2). The impact of RXR α phosphorylation on retinoid resistance, anti-apoptosis, and activation of cell growth is also revealed in other types of human malignant cells, including colon cancer and leukemia cells [39-41].

MECHANISMS OF ACR IN HCC CHEMOPREVENTION: RESULTS FROM EXPERIMENTAL STUDIES

As mentioned above, liver carcinogenesis is accompanied by the accumulation of p-RXR α because aberrant phosphorylation of RXR α abolishes its function as the master regulator of the nuclear receptor superfamily [13, 33-35]. Therefore, inhibition of RXR α phosphorylation and the associated restoration of its heterodimeric activity with other

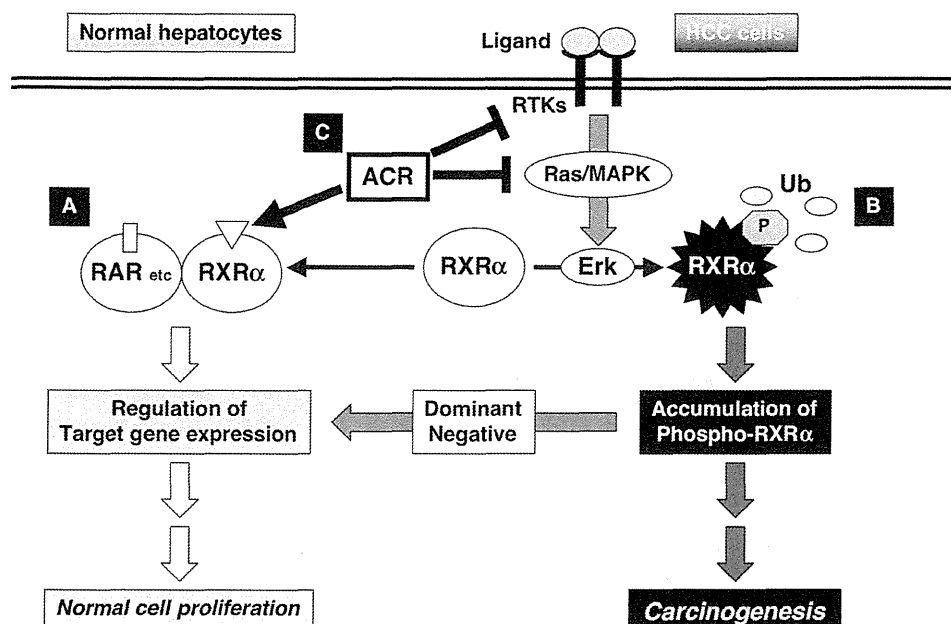


Fig. (2). Retinoid refractoriness due to phosphorylation of RXR α , and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXR α in normal hepatocytes, it forms homo- and/or heterodimers with other nuclear receptors, including RARs, resulting in expression of the target genes that regulate normal cell proliferation and differentiation (A). In HCC cells, the Ras-MAPK pathway is highly activated and phosphorylates RXR α at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor. Furthermore, non-functional phosphorylated RXR α (p-RXR α) is sequestered from ubiquitin/proteasome-mediated degradation and accumulates in liver cells, interfering with the physiological function of the remaining unphosphorylated RXR α in a dominant negative manner, thereby playing a critical role in liver carcinogenesis (B). ACR is not only a ligand for RXR α but also a suppressor of the Ras-MAPK signaling pathway; it inhibits RXR α phosphorylation, thereby restoring the function of the receptor and activating the transcriptional activity of the responsive element. ACR also inhibits, directly or indirectly, the ligand (growth factor)-dependent RTK activities, which contribute to the inhibition of ERK and RXR α phosphorylation and suppression of growth in HCC cells (C).

nuclear receptors may be an effective and important strategy for the prevention and treatment of HCC. ACR, which was initially developed as an agonist for both RXR and RAR [42, 43], is a possible candidate for this goal [10-12]. ACR activates the promoter activity of RXRE and RARE; controls the expression of target genes including RAR β , p21^{CIP1}, and cyclin D1; induces apoptosis; and causes cell cycle arrest in G₀-G₁, thus, inhibiting the growth of human HCC-derived cells [14-16, 44-52]. This agent inhibits both chemically-induced liver carcinogenesis in rats and spontaneously occurring HCC in mice [27, 53-55]. The anti-tumor effects of ACR are also associated with inhibition of angiogenesis and repression of oxidative stress [56, 57]. A number of beneficial effects of ACR on the prevention of HCC development and inhibition of growth of HCC cells are summarized in Fig. (3).

Increasing evidence has indicated that, in addition to retinoid receptors, several types of growth factors and their corresponding receptor tyrosine kinases (RTKs), which play a role in the activation of the Ras-MAPK signaling pathway, are critical targets of ACR to inhibit cancer cell growth [46, 54-56, 58, 59]. These reports are important because aberrant activation of certain types of RTKs such as the epidermal growth factor receptor and the downstream Ras-MAPK pathway are closely associated with liver carcinogenesis and,

therefore, are regarded as critical targets for HCC treatment [60, 61]. The activated Ras-MAPK pathway phosphorylates RXR α , and this may contribute to liver carcinogenesis [13, 33]. On the other hand, ACR restores RXR α function by inactivating the Ras-MAPK signaling system, leading to dephosphorylation of RXR α , although 9cRA is incapable of suppressing ERK and RXR α phosphorylation [14]. ACR inactivates Ras activation in human HCC and pancreatic cancer cells [15, 62]. In the liver of diethylnitrosamine (DEN)-treated *db/db* mice, ACR also inhibits the activation of Ras and the phosphorylation of ERK and RXR α proteins [17]. These findings suggest that ACR impedes the development of HCC and inhibits cancer cell growth, at least in part, by targeting the RTK-Ras-MAPK signaling pathway and subsequent RXR α phosphorylation. The role of RXR α phosphorylation in liver carcinogenesis and its inhibition by ACR are schematically represented in Fig. (2).

CHEMOPREVENTION OF HCC BY ACR: RESULTS FROM CLINICAL TRIALS

Findings from numerous preclinical experiments strongly indicate that ACR is a promising agent for the chemoprevention of HCC (Figs. 2 and 3). Therefore, an early-phase randomized controlled clinical trial was conducted to test the chemopreventive effect of ACR on

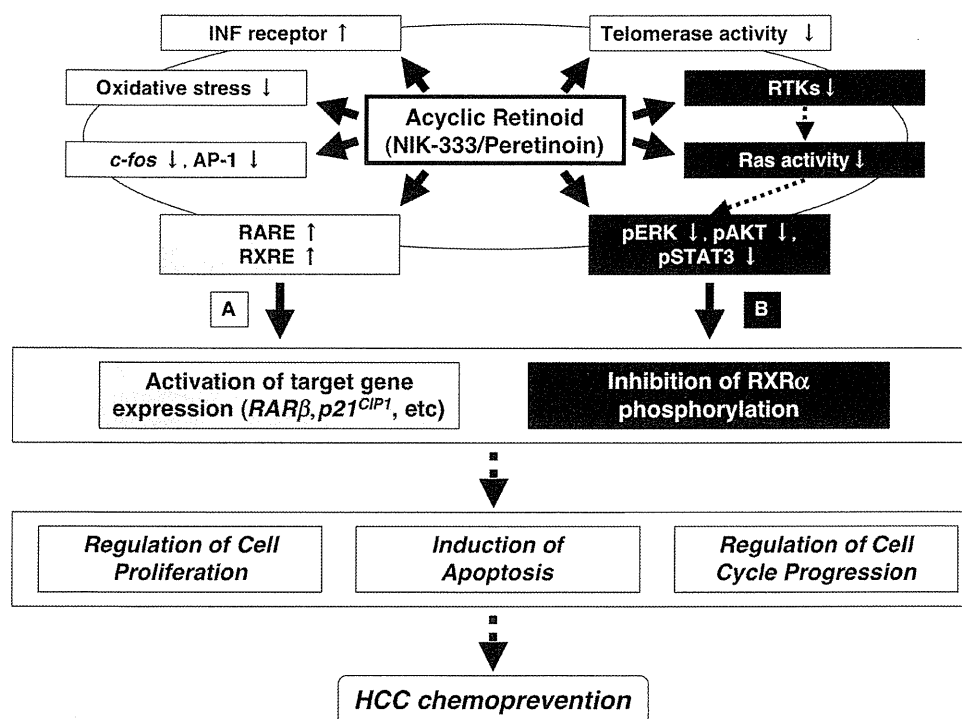


Fig. (3). Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes such as *RARβ* and *p21^{CIP1}* by upregulating the promoter activity of RARE and RXRE (A). In addition, ACR suppresses cancer cell growth by inhibiting the activation and expression of some types of RTKs, including EGFR, HER2, VEGFR-2, and FGFR, which contribute to the subsequent inhibition of Ras-MAPK activation and RXRα phosphorylation (B). ACR inhibits phosphorylation of Akt and Stat3 proteins. Induction of *RARβ* and restoration of RXRα function due to dephosphorylation by ACR leads to cooperative regulation of cell proliferation, cell cycle progression, and induction of apoptosis, thus preventing HCC development. ACR also induces the expression of the IFN receptor (INFR), inhibits the transcriptional activity of *c-fos* and AP-1 promoters, and downregulates telomerase activity in the HCC and squamous cell carcinoma cells. ACR also suppresses liver tumorigenesis by repressing oxidative stress.

secondary HCC in patients who underwent potentially curative treatment for initial HCC [6-8]. We randomly assigned 89 patients to undergo either ACR treatment (44 patients, 600 mg/day) or placebo (45 patients) for 12 months. In this trial, oral administration of ACR significantly reduced the incidence of recurrent or new HCCs after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC as compared with 22 patients (49%) in the placebo group ($P = 0.04$) [6]. After a further follow-up to 62 months, ACR improved both the recurrence-free survival ($P = 0.002$) and overall survival ($P = 0.04$) [7]. The relative risk of the development of secondary HCC and death were 0.31 (95% confidence interval [CI], 0.12 – 0.78) and 0.33 (95% CI, 0.11 – 0.79), respectively [6, 7]. Moreover, the preventive effects of ACR lasted up to 50 months after randomization or 38 months after completion of ACR administration, indicating that administration for only 12 months conferred a long-term effect over several years [8]. No severe side effects were observed in this trial.

A phase II/III clinical trial of ACR confirmed its effectiveness in preventing second primary HCCs in HCV-positive patients, who received curative treatment for primary or the first recurrence of HCC, in a multicenter large-scale ($n = 401$) randomized placebo-controlled trial

with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) was tolerated, and it had a strong effect on the prevention of second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07 – 0.96) 2 years after treatment. Cumulative recurrence-free survival rates in the ACR-treated group were higher than those in the placebo group (during the first year: ACR, 71.9%; placebo, 66.0% and during 3 years: ACR, 43.7%; placebo, 29.3%), indicating that ACR reduced the recurrence of HCC, especially after 2 years of treatment [9]. Therefore, the results of these clinical trials [6-9, 63] suggest that ACR is a novel first-line therapy to reduce the development of second primary HCC. Application for governmental approval of this agent as an “HCC chemopreventive drug” in patients with liver cirrhosis is under progress in Japan.

“CLONAL DELETION” THERAPY: THE FUNDAMENTAL CONCEPT OF HCC CHEMOPREVENTION

It should be noted that, in an early-phase clinical trial [6-8], the serum levels of lectin-reactive α -fetoprotein factor 3 (AFP-L3), which indicates the presence of latent (*i.e.*, invisible) malignant clones in the remnant liver, were significantly reduced after ACR administration for 12 months. This agent also prevented the appearance of serum

AFP-L3 in patients whose AFP-L3 levels were negative at trial enrollment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group, and these patients had a significantly higher risk of developing secondary HCC [63]. These observations suggest the following 2 possibilities: (1) ACR deletes the AFP-L3-producing premalignant clones from the remnant liver before they expand into clinically detectable tumors (“clonal deletion”) and (2) ACR also actively inhibits the development of AFP-L3-producing clones, which have the potential to become HCC (“clonal inhibition”). From these results, we have proposed new concepts of HCC chemoprevention, “clonal deletion” and “clonal inhibition,” which are defined as the removal or inhibition, respectively, of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the chronically damaged liver when it is in a hyper-carcinogenic state. We believe that ACR prevents the development of HCC by implementing this concept, which is a key to HCC chemoprevention (Fig. 4). Once the malignant clones are eliminated or inhibited from the remnant liver by ACR through the induction of apoptosis and cell differentiation [14-16, 42, 44-52], it takes several years for the clinical appearance of the

next HCC clones. Therefore, only short-term administration (12 months) of ACR could exert long-term preventive effect on HCC development for several years after termination of treatment [8]. A recent phase II/III trial also demonstrated a similar result emphasizing the preventive effect of ACR on the development of second primary HCC 2 years after administration of this agent [9]. This finding suggests that ACR may mainly suppress *de novo* carcinogenesis in the cirrhotic liver, and we presume that this phenomenon is associated with the implementation of “clonal deletion” therapy by ACR.

FUTURE PROSPECTS OF ACR – 1: “COMBINATION CHEMOPREVENTION” OF HCC USING ACR AS THE KEY DRUG

There is growing interest in the combination therapy involving ACR with a variety of other agents because such a therapy often provides the potential for synergistic effects on growth inhibition in cancer cells. We have initially found that the combination of ACR and interferon (IFN)- β synergistically inhibits cell growth and induces apoptosis in HCC cells, and that this is associated with an ACR-induced

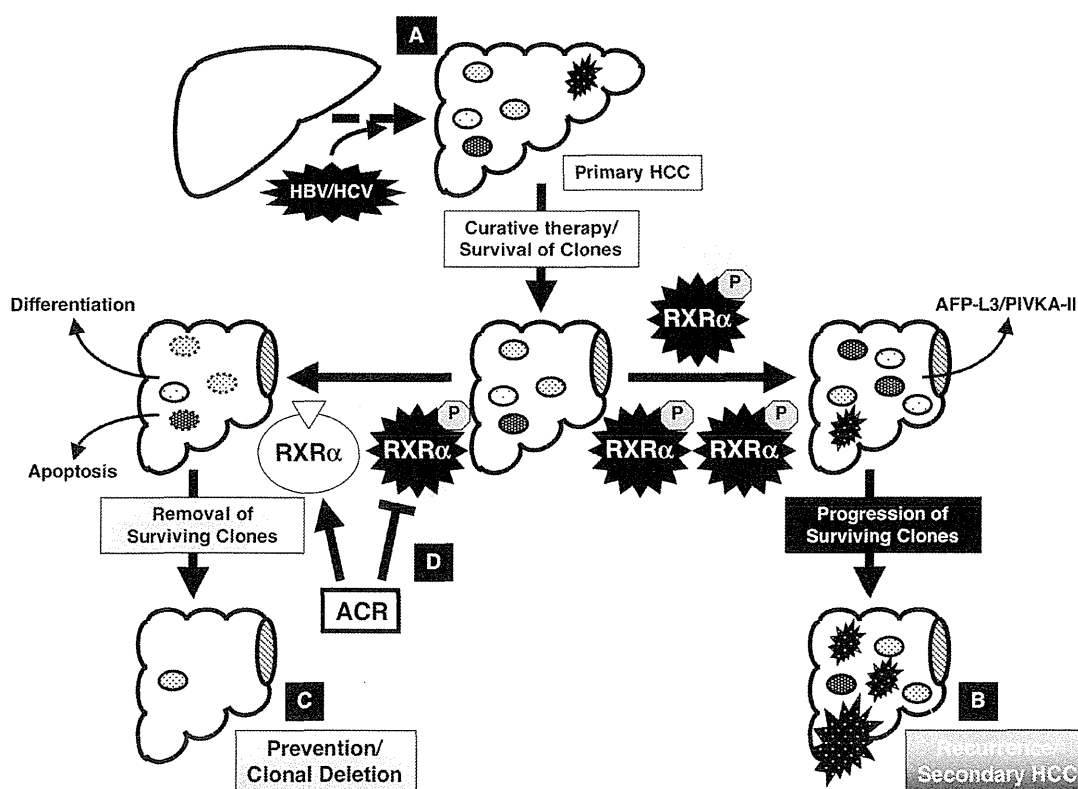


Fig. (4). Concept of “clonal deletion” therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a precancerous setting, which consists of multiple latent malignant clones that can, at some point, develop into HCC (A). Even after early detection and removal of the primary HCC, the remaining clones survive in the liver and grow into secondary HCC lesions, which is a major cause of the poor prognosis for patients with this malignancy (B). Therefore, one of the most promising strategies to prevent secondary HCC is the deletion of such transformed clones by inducing cell differentiation or apoptosis before they expand into clinically detectable tumors (the concept of “clonal deletion”) (C). ACR, which targets phosphorylated RXR α , prevents the recurrence and development of secondary HCC *via* the mechanism described by this concept (D).

upregulation of the expression of type-1 IFN receptor and Stat1 [48]. ACR also acts synergistically with vitamin K₂ (VK₂) in suppressing growth and inducing apoptosis in the human HCC cells [15]. These findings are significant when considering the clinical use of ACR because both IFN and VK₂ are expected to exert preventive effects on the development and recurrence of HCC [64-67]. Therefore, in the near future, "combination chemoprevention" using ACR as the key agent, such as ACR plus IFN or VK₂, may become one of the effective strategies to prevent the development of HCC. Synergistic growth inhibition caused by ACR plus VK₂ treatment is also observed in HL-60 human leukemia cells [68].

The expected mechanisms of how the combination of ACR and other specific agents synergistically exerts chemopreventive effects in HCC cells are schematically summarized in Fig. (5). Among these mechanisms, dephosphorylation of RXR α by targeting the Ras-MAPK signaling pathway or its upstream RTKs seems to be one of the most crucial approaches of ACR for inducing synergistic growth inhibition in HCC cells. Indeed, ACR and VK₂ cooperatively inhibit the activation of the Ras-MAPK signaling pathway, and thus, suppress the phosphorylation of RXR α and the growth of HCC cells [15]. Trastuzumab is a humanized monoclonal antibody against human epidermal growth factor receptor-2 (HER2), a member of the RTK

family, and the combination of 9cRA [69] or ACR (unpublished data) and this antibody synergistically inhibits the activation of HER2, ERK, and Akt, subsequently dephosphorylating RXR α , and thus inhibiting growth and inducing apoptosis in the HCC cells. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, also acts synergistically to induce apoptosis and G₀-G₁ cell cycle arrest in the HCC cells by inhibiting phosphorylation of RXR α , ERK, Akt, and GSK-3 β proteins [16]. In addition to HCC, the combination of ACR plus gemcitabine synergistically inhibits cell growth and induces apoptosis by inhibiting Ras activation in pancreatic cancer cells [62].

In addition to targeting RXR α phosphorylation, induction of nuclear receptors that dimerize with RXR, such as RAR and PPAR [39, 70], and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. For instance, both valproic acid [16] and OSI-461 [50], a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of RAR β , thereby markedly increasing the RARE and RXRE promoter activity and inducing apoptosis in the HCC cells. These findings suggest that, among the nuclear receptors, RAR β is considered the most preferable heterodimeric partner for RXR α in ACR-based combination chemoprevention.

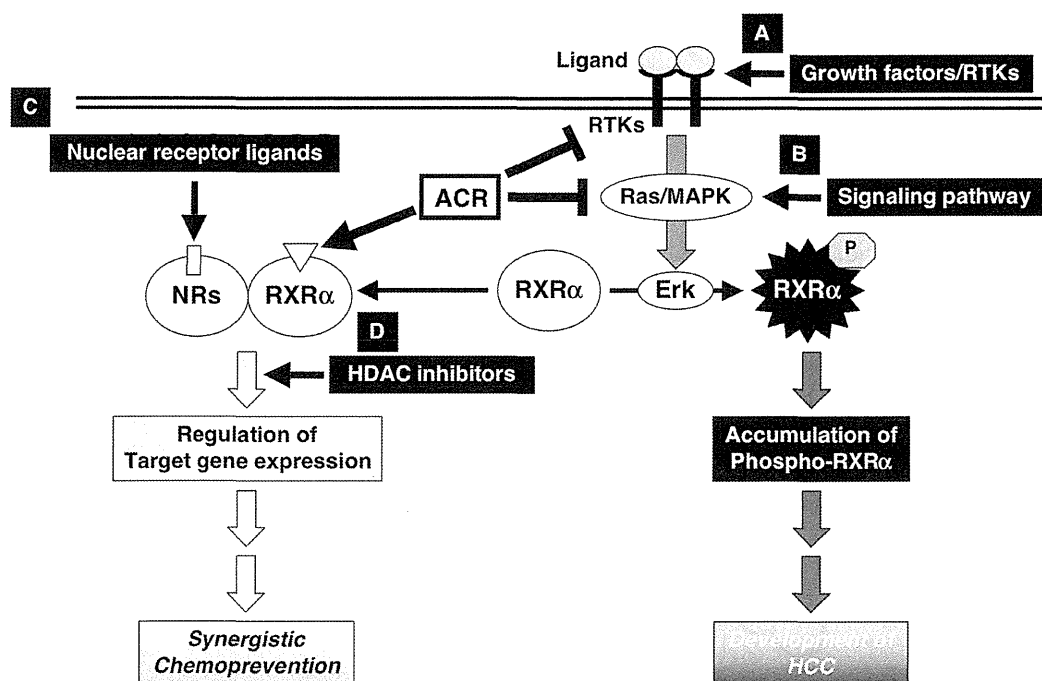


Fig. (5). "Combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXR α and subsequent restoration of the function of this nuclear receptor are critical to prevent HCC development. Therefore, agents that target growth factors and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK that phosphorylates RXR α , may be good candidates to synergize with ACR for the chemoprevention of HCC. The ligands for the nuclear receptors (NRs) that heterodimerize with RXRs, such as RARs and PPARs (C), are also capable of enhancing the chemopreventive effect of ACR through the activation of target gene expression. HDAC inhibitors increase the expression of ACR-target genes by remodeling the chromatin template and increasing histone acetylation, suggesting that the combination of ACR and HDAC inhibitors may be a promising regimen for HCC chemoprevention (D).

FUTURE PROSPECTS OF ACR – 2: PREVENTION OF OBESITY-RELATED LIVER CARCINOGENESIS BY ACR

Recent studies have revealed that the risk of HCC is elevated in patients with metabolic syndrome, which is commonly associated with obesity and diabetes mellitus [20, 71, 72]. Several pathophysiological mechanisms linking obesity and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade [73]. Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and thus, are regarded as considerable targets for HCC chemoprevention. On the other hand, improving metabolic abnormalities such as insulin resistance and chronic inflammation by nutritional or pharmaceutical intervention might be an effective and attractive strategy to inhibit obesity-related liver carcinogenesis [74]. We have reported that the administration of branched chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, and (-)-epigallocatechin-3-gallate, which is a major biologically active component of green tea, significantly prevents liver tumorigenesis in obese and diabetic *db/db* mice by targeting insulin resistance and improving chronic inflammation [75, 76]. A recent clinical trial also revealed that supplementation of food with BCAA reduced the risk of HCC in obese patients with chronic viral liver disease [72].

ACR effectively prevents the development of obesity-related liver carcinogenesis by inhibiting the activation of Ras and phosphorylation of ERK and RXR α in the liver of DEN-treated *db/db* mice. In this study, ACR administration also improved liver steatosis and insulin sensitivity, while attenuating the chronic inflammation induced by excessive fatty deposits [17]. Obesity and metabolic syndrome are the major healthcare problems in the present society, and the influences of metabolic abnormalities, in particular, such as promotion of cancers including HCC, are critical issues awaiting resolution. The results of this preclinical experiment [17] may encourage the clinical use of ACR for cirrhotic patients with obesity and diabetes, who are at a notably higher risk of developing HCC [20, 71, 72].

CONCLUSION

HCC prevention is an urgent issue demanding attention across the globe. One of the most practical approaches to prevent HCC is realization of the concept of “clonal deletion,” and ACR is a promising candidate for this purpose because it may accomplish this concept. Experimental studies strongly suggest that RXR α phosphorylation is profoundly involved in liver carcinogenesis, and ACR mainly exerts chemopreventive effects on HCC *via* inhibition of RXR α phosphorylation. ACR-based combination chemoprevention, which is expected to exert synergism, holds a great possibility to perform a central role in HCC chemoprevention. Obese individuals, who are at an increased risk for HCC, might also be critical targets of ACR to prevent the development of this malignancy.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

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ABBREVIATIONS

ACR	=	acyclic retinoid
AFP-L3	=	lectin-reactive α -fetoprotein factor 3
BCAA	=	branched chain amino acids
DEN	=	diethylnitrosamine
ERK	=	extracellular signal-regulated kinase
GSK-3 β	=	glycogen synthase kinase-3 β
HBV	=	hepatitis B virus
HCC	=	hepatocellular carcinoma
HCV	=	hepatitis C virus
HDAC	=	histone deacetylase
HER2	=	human epidermal growth factor receptor-2
IFN	=	interferon
MAPK	=	mitogen-activated protein kinase
PIVKA-II	=	protein induced by vitamin K absence or antagonist-II
PPAR	=	peroxisome proliferator-activated receptor
RA	=	retinoic acid
RAR	=	retinoic acid receptor
RARE	=	retinoic acid receptor responsive element
RTK	=	receptor tyrosine kinase
RXR	=	retinoid X receptor
RXRE	=	retinoid X response element
VK ₂	=	vitamin K ₂

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Review Article

Acyclic retinoid in chemoprevention of hepatocellular carcinoma: Targeting phosphorylated retinoid X receptor- α for prevention of liver carcinogenesis

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Abstract

One of the key features of hepatocellular carcinoma (HCC) is the high rate of intrahepatic recurrence that correlates with poor prognosis. Therefore, in order to improve the clinical outcome for patients with HCC, development of a chemopreventive agent that can decrease or delay the incidence of recurrence is a critical issue for urgent investigation. Acyclic retinoid (ACR), a synthetic retinoid, successfully improves HCC patient survival by preventing recurrence and the formation of secondary tumors. A malfunction of the retinoid X receptor- α (RXR α) due to phosphorylation by the Ras-MAPK signaling pathway plays a critical role in liver carcinogenesis, and ACR exerts chemopreventive effects on HCC development by inhibiting RXR α phosphorylation. Here, we review the relationship between retinoid signaling abnormalities and liver disease, the mechanisms of how RXR α phosphorylation contributes to liver carcinogenesis, and the detailed effects of ACR on preventing HCC development, especially based on the results of our basic and clinical research. We also outline the concept of "clonal deletion and inhibition" therapy, which is defined as the removal and inhibition of latent malignant clones from the liver before they expand into clinically detectable HCC, because ACR prevents the development of HCC by implementing this concept. Looking toward the future, we discuss "combination chemoprevention" using ACR as a key drug since it can generate a synergistic effect, and may thus be an effective new strategy for the prevention of HCC.

Keywords: Acyclic retinoid, chemoprevention, clonal deletion and inhibition, combination therapy, HCC, RXR α phosphorylation

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers in the world. The number of

new cases per year is estimated to be 750,000; approximately the same number (700,000) of people die from this malignancy each year.^[1,2] Chronic inflammation and subsequent cirrhosis of the liver, most cases of which are induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), contribute to the development of HCC.^[2,3] In order to improve the prognosis of HCC, several effective strategies to prevent the development of primary HCC and intrahepatic recurrence of this malignancy have been demonstrated in clinical trials. A meta-analysis reported that antiviral treatment reduces the risk of HBV-related HCC recurrence and decreases liver-related mortality as well as overall mortality.^[4]

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For patients with HCV-related HCC, the effectiveness of interferon therapy for preventing the recurrence of HCC has been shown by meta-analyses.^[5,6]

In addition to studies of antiviral treatment, other important trials using specific agents have been conducted to seek ways for preventing HCC development. For instance, supplementation with branched-chain amino acids reduces the risk of HCC in cirrhotic patients who are obese.^[7] We reported the results of our prospective randomized study,^[8-10] in which the oral administration of acyclic retinoid (ACR), a synthetic retinoid, inhibited the development of a second primary HCC and thus improved patient survival. The pleiotropic effects of ACR in the prevention of HCC and suppression of cancer cell growth have also been revealed in many experimental studies.^[11-13] The aim of this article is to review the detailed effects of ACR in preventing the development of HCC, based on our clinical and basic research. In particular, we focus on the effects of ACR in targeting the phosphorylation of retinoid X receptor- α (RXR α), because malfunction of this nuclear receptor due to aberrant phosphorylation is closely involved in liver carcinogenesis.^[14] We also review the concept of “clonal deletion and inhibition” therapy, a practical approach to preventing HCC development. Finally, we discuss the possibility of “combination chemoprevention,” which uses ACR as a key drug and is expected to be an effective strategy that takes advantage of pharmacologic synergism to prevent the formation of HCC.

Retinoids and their receptors

Retinoids, a group of structural and functional derivatives of vitamin A, have fundamental effects on cellular activities, including growth, differentiation, and apoptosis, as well as on morphology.^[15-17] Retinoids exert their biologic functions primarily by regulating gene expression through two distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs, which are ligand-dependent transcription factors. Both RARs and RXRs are composed of three subtypes (α , β , and γ), which are characterized by a modular domain structure.^[15-17] RARs can be activated by both all-*trans*-retinoic acid and 9-*cis*-retinoic acid with similar affinities, whereas RXRs are exclusively activated by 9-*cis*-retinoic acid.

After ligand binding, RXRs form homodimers as well as heterodimers with RARs, which interact with the retinoid X response element (RXRE) or the retinoic acid receptor responsive element (RARE), located in the promoter region of target genes, thereby modulating gene expression.^[15-17] In addition to forming a heterodimer with RARs, RXRs can also form heterodimers with several other nuclear receptors, indicating that RXRs act

as common heterodimerization partners for various types of nuclear receptors.^[16] For instance, RXRs interact with peroxisome proliferator-activated receptors (PPARs), which are receptors for fatty acids, thus regulating PPAR-mediated pathways and controlling energy homeostasis.^[18] Therefore, RXRs function as auxiliary factors, determining the effects of other hormones, and acting as master regulators of nuclear receptors.^[16]

Retinoid abnormalities and liver disease

The liver is one of the most important target organs for retinoid actions. Hepatocytes play central roles in the uptake and processing of dietary retinol in the liver and secrete retinol-binding protein into the plasma. Hepatic stellate cells (HSCs) are critically involved in the storage of retinoids in the liver, suggesting that the development of hepatic disease is highly correlated with impaired hepatic retinoid metabolism and storage.^[19,20] In alcoholic patients, diminished hepatic retinoid storage is associated with progressively worsening stages of hepatic disease.^[21] The loss of hepatic retinyl ester stores from the lipid droplets within HSCs leads to HSC activation and the development of liver fibrosis.^[22,23] A progressive decrease in serum retinol levels has also been observed in patients diagnosed with liver cirrhosis compared with healthy subjects, and those patients with both cirrhosis and HCC had significantly lower levels than patients with cirrhosis alone.^[24,25]

Several experimental studies using genetically engineered mice have revealed the pivotal effects of retinoids on fat metabolism in the liver. Yanagitani *et al.*^[26] reported that RAR α dominant-negative form transgenic mice developed steatohepatitis through the downregulation of hepatic mitochondrial β -oxidation activity of fatty acids. Studies in hepatocyte RXR α -deficient mice also demonstrated that RXR α plays vital roles in fatty acid and cholesterol metabolism in the liver.^[27,28] These observations suggest that retinoids and their receptors are involved in the mediation of normal hepatic lipid metabolism.

Retinoid abnormalities and liver carcinogenesis

As previously noted, retinoids and their receptors, especially RXRs, play an essential role in controlling normal cell proliferation, differentiation, metabolism, and death (regulation of apoptosis).^[15-17] On the other hand, these facts also suggest that the loss of retinoid activity or responsiveness is linked to deviation from normal cell proliferation and death, which are key factors for cancer development.^[29,30] Indeed, it is well established that abnormalities in retinoid signaling are prominently involved in carcinogenesis in several organs, including the liver. In a rodent model of liver carcinogenesis,^[31] retinol was observed to be locally deficient

in HCC lesions, but not in the adjacent normal liver tissues. Functional loss of retinoic acid leads to the occurrence of cellular dysplasia and cancer in the liver.^[26] Overexpression of cellular retinoic acid-binding protein-II, which shows a high affinity for all-*trans*-retinoic acid, is associated with induction of retinoic acid resistance in HCC cells.^[32] On the other hand, lecithin:retinol acyltransferase knockout mice, which possess increased retinoid signaling in the liver, are less susceptible to diethylnitrosamine (DEN)-induced hepatocarcinogenesis.^[33] These findings suggest that supplementation with additional retinoids and improvement of retinoid signaling may be a promising strategy for the prevention and/or treatment of HCC.

In addition to retinoid depression, abnormalities in retinoid receptors are also associated with liver carcinogenesis and the growth of HCC cells. The *RARβ* gene, a tumor suppressor gene, is an HBV integration site, and the expression of this gene is markedly decreased in human HCC.^[34,35] *RARβ* expression is also suppressed in liver cancer cell lines.^[36,37] We have previously reported that the levels of *RARβ* protein and mRNA were decreased in HCC lesions in a rat model of chemically induced liver carcinogenesis.^[38] On the other hand, *RARγ* is overexpressed in HCC tissues and cells, which is associated with the growth of HCC cells.^[39] It has been reported that *RARγ* often resides in the cytoplasm of HCC cells and interacts with the p85α regulatory subunit of phosphatidylinositol 3-kinase, resulting in the activation of Akt and nuclear factor-κB, which are critical regulators of the growth and survival of cancer cells.^[39,40] These reports support an oncogenic potential for *RARγ* in liver carcinogenesis.

RXRα phosphorylation and HCC

Among retinoid receptors, *RXRα* is most abundant in the liver; therefore, its alterations are particularly implicated in the development and progression of HCC. *RXRα* is reported to bind to the enhancer element of HBV and modulate viral replication.^[41] The expression of *RXRα* is decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion, as seen in a rat model of chemically induced liver carcinogenesis.^[38] These findings indicate that repression of *RXRα* occurs even in the early stage of liver carcinogenesis.

Moreover, we have shown that aberrant phosphorylation of *RXRα* is critically involved in liver carcinogenesis. Initially, we revealed that the *RXRα* protein is anomalously phosphorylated at the serine and threonine residues and that it was seen to accumulate in both surgically resected human HCC tissues and HCC cell lines, whereas in normal

hepatocytes, *RXRα* is unphosphorylated and is broken into smaller peptides.^[14,42] We previously reported that the phosphorylated form of *RXRα* protein was present in higher concentrations in HCC tissues than in noncancerous surrounding and normal liver tissues among all 10 cases examined.^[14] Activated extracellular signal-regulated kinase (ERK) is highly expressed in HCC cells, and constitutive phosphorylation at the serine at position 260 of *RXRα*, a mitogen-activated protein kinase (MAPK)-ERK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and promotion of cancer cell growth. In turn, the abrogation of phosphorylation by an MAPK inhibitor or transfection with unphosphomimic mutant *RXRα* restores the degradation of *RXRα* in a ligand-dependent manner.^[14,43] Phosphorylated *RXRα* abolishes its ability to form heterodimers with *RARβ*, and this is associated with uncontrolled cell growth and resistance to retinoids.^[44] Moreover, phosphorylated *RXRα* is resistant to proteolytic degradation via the ubiquitination/proteasome-mediated pathway in human HCC cells, resulting in an accumulation of this phosphorylated protein within the HCC tissues.^[45] Therefore, in HCC tissues and cells, the accumulation of phosphorylated *RXRα*, which is regarded as the nonfunctional form of *RXRα*, may interfere with the function of normal (unphosphorylated) *RXRα* in a dominant-negative manner. Our observations suggest that not only retinoid depletion, but also malfunction of retinoid receptors, especially phosphorylation of *RXRα*, may play a critical role in HCC development [Figure 1].

Mechanisms of ACR in HCC chemoprevention

ACR, which is the same substance as NIK-333 and Peretinoin (Kowa Pharmaceutical Co., Tokyo, Japan), inhibits both chemically induced liver carcinogenesis in rats and mice and spontaneously occurring HCC in mice.^[31,46-49] ACR was initially developed as an agonist for both *RXR* and *RAR*.^[50,51] Therefore, this agent activates the promoter activity of RXRE and RARE and controls the expression of target genes, including *RARβ*, p21^{CIP1}, and cyclin D1, which results in induction of apoptosis, cell cycle arrest in G₀-G₁, and growth inhibition in human HCC-derived cells.^[43,52-62] These findings suggest that ACR suppresses HCC, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially *RARβ* and p21^{CIP1}.

On the other hand, many experimental studies have shown that ACR exerts chemopreventive effects in HCC cells by inhibiting *RXRα* phosphorylation. In human HCC cells, ACR restores *RXRα* function by inactivating the Ras-MAPK signaling system, leading to dephosphorylation of *RXRα*.^[43] ACR also suppresses cancer cell growth by inhibiting the activation and expression of several

types of growth factors and their corresponding receptor tyrosine kinases (RTKs).^[47,48,56,63-65] These findings are significant because RTKs play a role in the activation of Ras–MAPK signaling and the subsequent phosphorylation of RXR α , which may contribute to liver carcinogenesis. In addition, we have recently reported that ACR inhibits the activation of Ras and the phosphorylation of ERK and RXR α proteins in the liver of DEN-treated obese mice.^[49] The inhibitory effects of ACR on Ras activation are also observed in human HCC and pancreatic cancer cells.^[52,66] These findings may indicate that activation of the RTK–Ras–MAPK signaling pathway and subsequent RXR α phosphorylation are critical targets of ACR for the inhibition of HCC development and cancer cell growth [Figure 1].

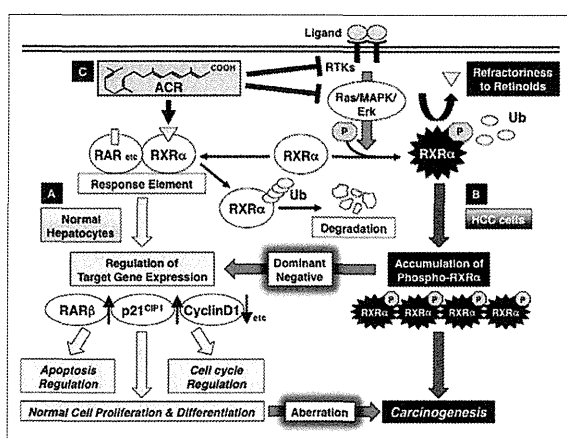


Figure 1: Retinoid refractoriness due to phosphorylation of retinoid X receptor alpha (RXR α), and its restoration by acyclic retinoid (ACR) in liver carcinogenesis. (a) In normal hepatocytes, when ACR binds to and activates RXR α , it forms homo- and/or heterodimers with other nuclear receptors, including retinoic acid receptors (RARs). This results in expression of the target genes, such as RAR β , p21^{CIP1}, and cyclin D1, which regulate normal cell proliferation and differentiation, as well as controlling the induction of apoptosis and cell cycle progression. Thereafter, RXR α is rapidly ubiquitinated (Ub) and degraded via the proteasome pathway. (b) In hepatocellular carcinoma (HCC) cells, the Ras–mitogen-activated protein kinase (MAPK) pathway is highly activated and phosphorylates RXR α at serine residues, impairing dimer formation and the subsequent transactivation functions of the receptor (refractoriness to retinoids). Furthermore, nonfunctional phosphorylated RXR α is sequestered from ubiquitin/proteasome-mediated degradation and accumulates in liver cells. This interferes with the physiologic function of the remaining unphosphorylated (ie, functional) RXR α in a dominant-negative manner, causing a deviation from normal cell proliferation and differentiation, thereby playing a critical role in liver carcinogenesis. (c) ACR is not only a ligand for RXR α , but also a suppressor of the Ras–MAPK signaling pathway; it inhibits RXR α phosphorylation, thereby restoring the function of the receptor and activating the transcriptional activity of the responsive element. ACR also inhibits, directly or indirectly, the ligand (growth factor)-dependent RTK activities, which contribute to the inhibition of ERK and RXR α phosphorylation and suppression of growth in HCC cells

Chemoprevention of HCC by ACR

In order to test whether ACR can reduce the incidence of recurrent and second primary HCC, an early phase randomized controlled clinical trial was conducted in patients who underwent potentially curative treatment for initial HCC.^[8-10] In this trial, treatment with ACR (administered to 44 patients, 600 mg/day) for 12 months significantly reduced the incidence of recurrent or new HCCs compared with placebo (administered to 45 patients) after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC compared with 22 patients (49%) in the placebo group ($P = 0.04$).^[8] After further follow-up to 62 months, ACR was also found to improve both recurrence-free survival ($P = 0.002$) and overall survival ($P = 0.04$). The estimated 6-year overall survival was 74% in the ACR group and 46% in the placebo group.^[9] The relative risks for the development of secondary HCC and death were 0.31 [95% confidence interval (CI), 0.12–0.78] and 0.33 (95% CI, 0.11–0.79), respectively.^[8,9] Moreover, the preventive effects of ACR lasted up to 50 months after randomization, or 38 months after completion of the drug.^[10] The results of these reports suggest that administration of ACR for only 12 months exerts a long-term effect on the prevention of second primary HCC without causing severe adverse effects from retinoid.

The preventive effect of ACR on the development of second primary HCC in HCV-positive patients, who underwent curative therapy for initial HCC or first recurrence, has also been confirmed by a multicenter large-scale ($n = 401$) randomized placebo-controlled trial with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) exerted a strong effect on the prevention of second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07–0.96) at 2 years after treatment. Cumulative recurrence-free survival rates in the ACR-treated group were higher than those in the placebo group (after the first year: ACR, 71.9%; placebo, 66.0% and at 3 years: ACR, 43.7%; placebo, 29.3%), indicating that ACR reduced the recurrence of HCC, especially after 2 years of treatment.^[67] In addition, subgroup analysis of this study showed the significant result that ACR powerfully prevented the development of a second primary HCC, with a hazard ratio of 0.38 (95% CI, 0.20–0.71) in patients who were Child–Pugh A and had small tumors (size < 20 mm).^[68] The results of these clinical trials suggest that ACR inhibits *de novo* carcinogenesis and is therefore a novel first-line therapy to reduce the development of second primary HCC, especially for patients with well-preserved liver function who have undergone curative therapy for a small tumor. Common treatment-related adverse events observed in this trial were albuminuria, hypertension, and headache; however, these adverse events were tolerated. The safety of ACR was also

determined in a phase I pharmacokinetics clinical trial. In that trial, doses of 300 and 600 mg/day did not result in any adverse effects or dose-limiting toxicities, whereas a dose of 900 mg/day resulted in grade 3 hypertension as a dose-limiting toxicity.^[69]

Concept of “clonal deletion and inhibition” therapy

The annual incidence of HCC reaches approximately 3% in HBV- and 7% in HCV-infected cirrhotic patients. More serious is that the frequency of HCC recurrence after curative treatment is very high in cirrhotic patients; the annual incidence rises to approximately 20%–25% and the recurrence rate at 5 years after definitive therapy exceeds 70%.^[70-73] This typical clinical course of patients with HCC is associated with the clinical characteristic mode of liver carcinogenesis, multicentric carcinogenesis, which is also expressed by the term “field cancerization.” Once a liver is exposed to a continuous carcinogenic insult, such as hepatitis virus infection, the whole liver is regarded as a precancerous field that possesses multiple as well as independent premalignant or latent malignant clones. Based on this characteristic, a curative treatment for HCC is difficult once this malignancy has developed; therefore, we believe that one of the most promising and practical strategies for HCC treatment is the removal and inhibition of latent malignant clones from the chronically damaged liver that is in a hypercarcinogenic state before the latent malignant clones expand into a clinically detectable tumor. We have proposed this new concept of “clonal deletion and inhibition” therapy for HCC chemoprevention.^[74] We believe that ACR prevents the development of HCC through implementation of this concept for the following reasons.

First, the serum levels of lectin-reactive α -fetoprotein factor 3 (AFP-L3) and protein induced by vitamin K absence or antagonist-II (PIVKA-II), both of which indicate the presence of latent (ie, invisible) malignant clones in the remnant liver, were significantly reduced after ACR administration for 12 months in an early-phase clinical trial.^[8-10] Next, ACR was found to prevent the appearance of serum AFP-L3 in patients whose AFP-L3 levels were negative at trial enrolment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group, and these patients had a significantly higher risk of developing secondary HCC.^[74] These observations are explained by the implementation of “clonal deletion and inhibition” therapy with ACR; namely, ACR eliminates the AFP-L3- and PIVKA-II-producing premalignant clones from the remnant liver before they expand into clinically detectable tumors (“clonal deletion”). At the same time, ACR inhibits the development of such clones, which have the potential to become HCC, in the liver (“clonal inhibition”).

Once the malignant clones are eliminated or inhibited from the remnant liver by ACR, it takes several years for *de novo* HCC to develop in the cirrhotic liver. Therefore, as demonstrated in an early-phase clinical trial,^[10] short-term administration (12 months) of ACR could exert a long-term (ie, several years) preventive effect on HCC development even after termination of treatment. The roles of ACR in the implementation of “clonal deletion and inhibition” therapy are schematically represented in Figure 2.

“Combination chemoprevention” of HCC with ACR

In order to establish more effective strategies to prevent HCC development, we have conducted a study of “combination chemoprevention” using ACR as a key agent. Combination chemoprevention with ACR provides an opportunity to take advantage of the synergistic effects of ACR on growth inhibition in HCC cells. We have initially found that the combination of ACR and interferon- β synergistically inhibits cell growth and induces apoptosis in HCC cells.^[58] This finding is significant when considering the clinical use of ACR in the near future because interferon exerts a chemopreventive effect against the recurrence of HCC.^[5,6]

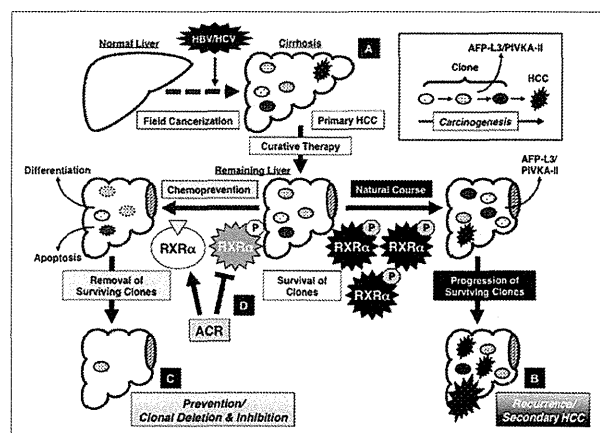


Figure 2: Concept of “clonal deletion and inhibition” therapy for hepatocellular carcinoma (HCC) chemoprevention and the effects of acyclic retinoid (ACR) on implementation of this concept. (a) Persistent inflammation caused by hepatitis viral infection transforms the liver into a precancerous field (“field cancerization”), which contains of multiple latent malignant clones that can, at some point, develop into HCC. **(b)** Even after early detection and removal of the primary HCC, the remaining clones survive in the remaining liver and grow into secondary HCC lesions (natural course), which is a major cause of the poor prognosis for patients with this malignancy. **(c)** Therefore, one of the most promising strategies to prevent secondary HCC is the deletion and inhibition of such transformed clones by inducing cell differentiation or apoptosis before the clones expand into clinically detectable tumors. This is the concept of “clonal deletion and inhibition” therapy for HCC chemoprevention. **(d)** ACR, which binds to RXR α and inhibits phosphorylation of this nuclear receptor, prevents the recurrence and development of secondary HCC via the mechanism described by this concept