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AG-041R, a novel indoline-2-one derivative, induces systemic cartilage hyperplasia in rats

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Abstract

AG-041R (3*R*-1-(2,2-diethoxyethyl)-3-((4 methylphenyl)aminocarbonylmethyl)-3-((4-methylphenyl) ureido)-indoline-2-one) is a novel small compound synthesized as a cholecystokinin-2 (CCK_2)/gastrin receptor antagonist. In the course of the development of this compound, we discovered unexpectedly that oral administration of a high dose for 4 weeks markedly induced systemic cartilage hyperplasia. This change was histologically observed in the auricles, the trachea, the marginal region of the femoral condyle, the xiphoid process and intervertebral disks in rats. Daily intraarticular injections of AG-041R into rat knee joints for 3 weeks also caused cartilage hyperplasia in the marginal region of the femoral condyle, but no hyperplasia was observed in any other cartilage. We have confirmed that chondrogenic activity of AG-041R is an intrinsic property of the compound, and is not due to its CCK_2/gastrin receptor antagonistic actions. These results indicate that AG-041R is a novel stimulator of chondrogenesis, and can be expected to be a potent therapeutic agent for cartilage disorders. © 2001 Published by Elsevier Science B.V.

Keywords: Systemic cartilage hyperplasia; Indoline-2-one derivative

1. Introduction

AG-041R (3R-1-(2,2-diethoxyethyl)-3-((4 methylphenyl) aminocarbonylmethyl)-3-((4-methylphenyl) ureido)-indoline-2-one), a novel indoline-2-one derivative, was originally synthesized as a CCK_2/gastrin receptor antagonist (Ding et al., 1997; Fukui et al., 1998). This compound had the most potent activity ($IC_{50} = 1.1$ nmol/l) and bioavailability of the numerous indoline derivatives we synthesized, and was selected as a clinical candidate. It showed therapeutic efficacy in animal models of gastric ulcer on oral administration (Baba et al., 1995).

In a preclinical toxicological study of AG-041R, we discovered unexpectedly a unique and intense chondrogenic activity in animals. Daily oral administration of a high dose of AG-041R (2000 mg/kg) for 4 weeks in rats markedly induced systemic cartilage hyperplasia in the auricles, the trachea, the xiphoid process, the marginal region of the femoral condyle and intervertebral disks. Cartilage hyperplasia was also shown by intraarticular administration of AG-041R, but this was restricted to the knee where the injection was carried out. We have confirmed that the chondrogenic activity of AG-041R is not due to its CCK_2/gastrin receptor antagonistic activity.

Although chondrogenic and cartilage-repairing activities of many growth factors, such as transforming growth factors (TGF-β), bone morphogenic proteins (BMP), basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I) have been reported (Cuevas et al., 1988; Glansbeek et al., 1998; Sellers et al., 2000; Shida et al., 1996; Tsukazaki et al., 1994; Van Beuningen et al., 1998), the clinical application of these factors has not been achieved. One of the main reasons for this situation is the formulation difficulty due to the poor stability and short half-life of growth factors in vivo (Putney and Burke, 1998). Therefore, chondrogenic small molecules, such as AG-041R, which can overcome these problems, are expected to be powerful alternative to growth factors.

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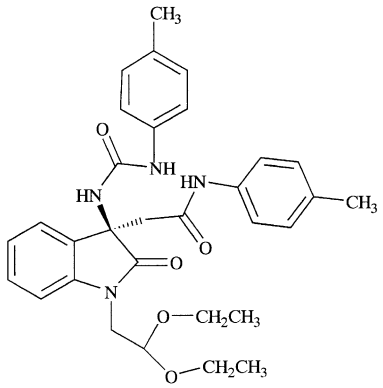

Fig. 1. Chemical structure of AG-041R. Molecular weight: 544.65.

We now report on the chondrogenic activity of AG-041R in vivo, demonstrated with a histological method. AG-041R is the first chemically synthesized small compound that induces systemic cartilage hyperplasia on oral administration.

2. Materials and methods

2.1. Materials

AG-041R was synthesized in our laboratory (Ding et al., 1997; Fukui et al., 1998). The chemical structure of AG-041R is shown in Fig. 1.

2.2. Animals

Specific pathogen-free, 6-week-old, male Sprague–Dawley rats (SLC, Japan) and specific pathogen-free, 10-week-old, male Sprague–Dawley rats (Charles River Japan, Japan) were used for oral (p.o.) and intraarticular (i.a.) administration experiments, respectively. Both types of animal were used after a 7-day acclimation period. All animal experiments were performed in accordance with the animal care guidelines of Chugai Pharmaceutical.

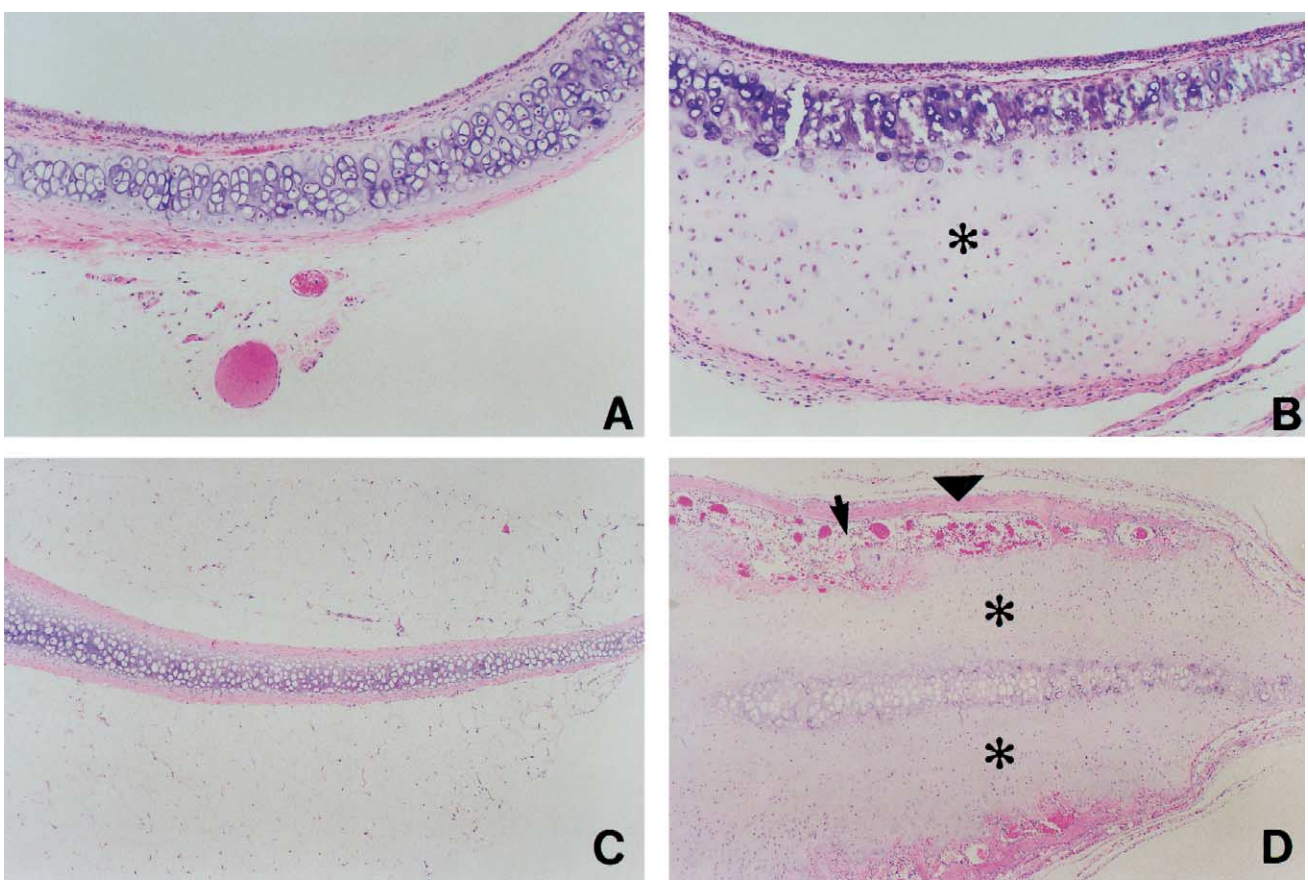

Fig. 2. Photomicrograph of trachea (A, B), xiphoid process (C, D, E), femoral condyle (F, G), auricle (H, I) and ventral part of intervertebral disk (J, K). A, C, F, H, J: control. B, D, E, G, I, K: AG-041R 2000 mg/kg. Hyaline cartilage hyperplasia (*) was observed in AG-041R-treated rats (B, D, F, K). Differentiation of chondrocytes from perichondrium was stimulated by AG-041R in trachea (B, *), auricle (I, arrowhead) and intervertebral disk (K, *). In the xiphoid process, bone (arrowhead) and bone marrow (arrow) were also formed with AG-041R(D, E). In the auricle, the content of lipid droplets in the chondrocytes cytoplasm was decreased, and hyaline cartilage matrix was increased (arrow). In F and G, ac: articular cartilage, gp: growth plate. HE, original magnification: ×25 (A, B, C, D, H, I, J, K), ×40 (E, F, G).

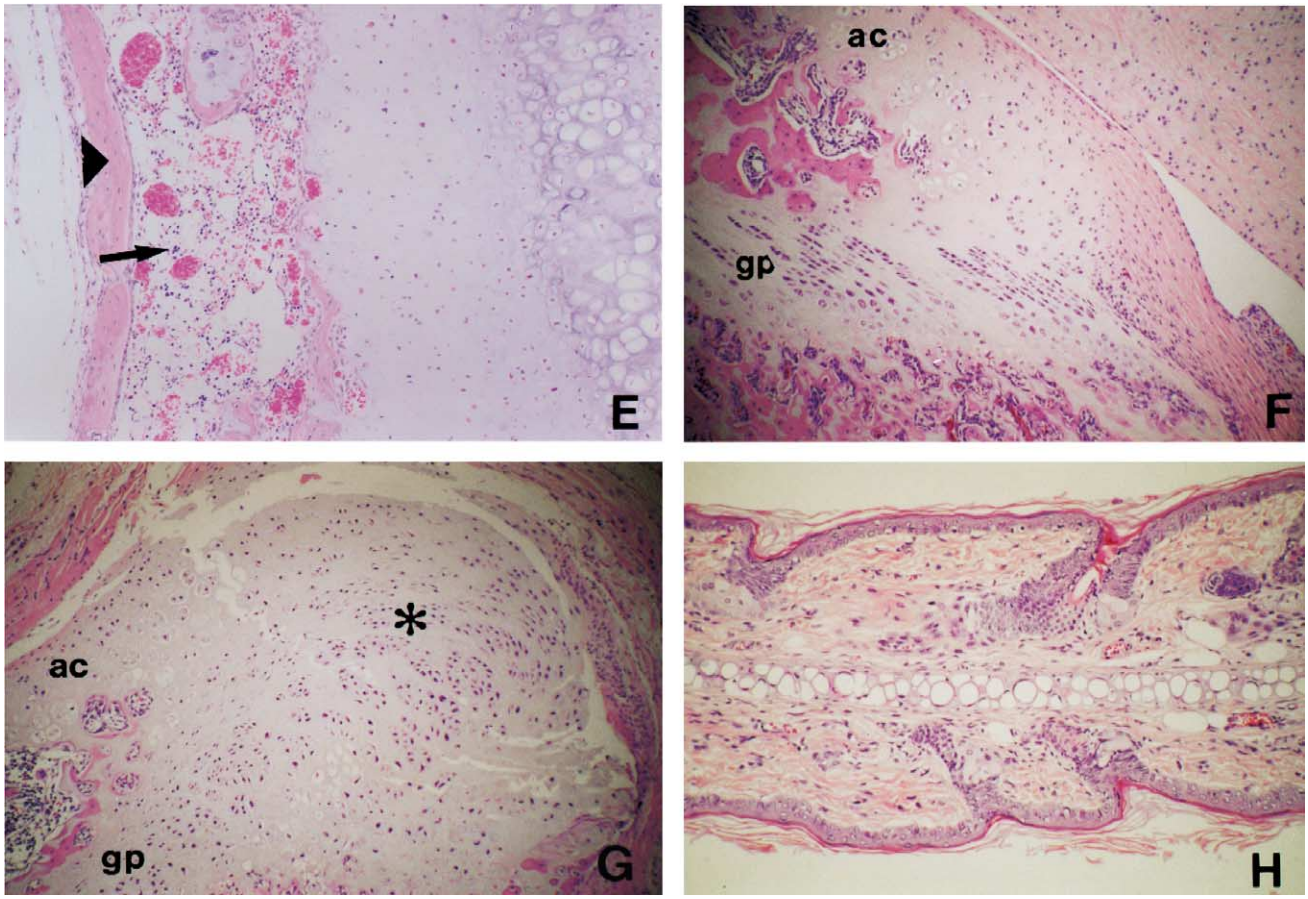

Fig. 2 (*continued*).

2.3. p.o. administration

A suspension of AG-041R in 3% gum arabic solution (Wako, Japan) was administered orally for 4 weeks, daily, at 2000 mg/kg. A 3% gum arabic solution was administered as the vehicle control.

2.4. i.a. administration

Fifty microliters of AG-041R solution (1.0 mmol/l) in 50% dimethylsulfoxide (DMSO, Junsei Chemical, Japan) in saline (Otsuka Pharmaceutical, Japan) was injected daily into the knee joints of rats, for 3 weeks, under ether anesthesia. The AG-041R administered amounted to 27.3 μg/day. Fifty microliters of 50% DMSO in saline was administered as the vehicle control.

2.5. Histopathological examination

The rats were killed after administration for 4 weeks (p.o.) or 3 weeks (i.a.), by exsanguination under ether anesthesia. In the p.o. administration experiment, the following organs were fixed in 20% neutral buffered formalin: femora (diaphysis, distal metaphysis and epiphysis), whole sterna, whole auricles, whole thoracic spines, trachea separated from bronchi. In the i.a. administration experiment, the knee joints were fixed in 20% neutral-buffered formalin for 7 days. The auricles were cut longitudinally. The femora, sterna, thoracic spines and knee joints were cut longitudinally after decalcification in 20% EDTA (Wako) pH 7.5 for 2 weeks, and were embedded in paraffin. Sections which were 4 μm thick were stained with hematoxylin and eosin (Merck, Germany). In the i.a. study, sections were stained with safranin-O and fast green (Merck).

3. Results

3.1. p.o. study

Orally administered AG-041R induced marked hyaline cartilage hyperplasia in the trachea, xiphoid process, femoral condyle, auricle and intervertebral disks in rats. Tracheal cartilage was markedly thickened by AG-041R administration (Fig. 2B). In the xiphoid process, AG-041R induced marked hyaline cartilage hyperplasia, and also induced bone and bone marrow in the periphery (Fig. 2D,E). AG-041R induced chondrophytes in the marginal region of the femoral condyle, namely the junction of the

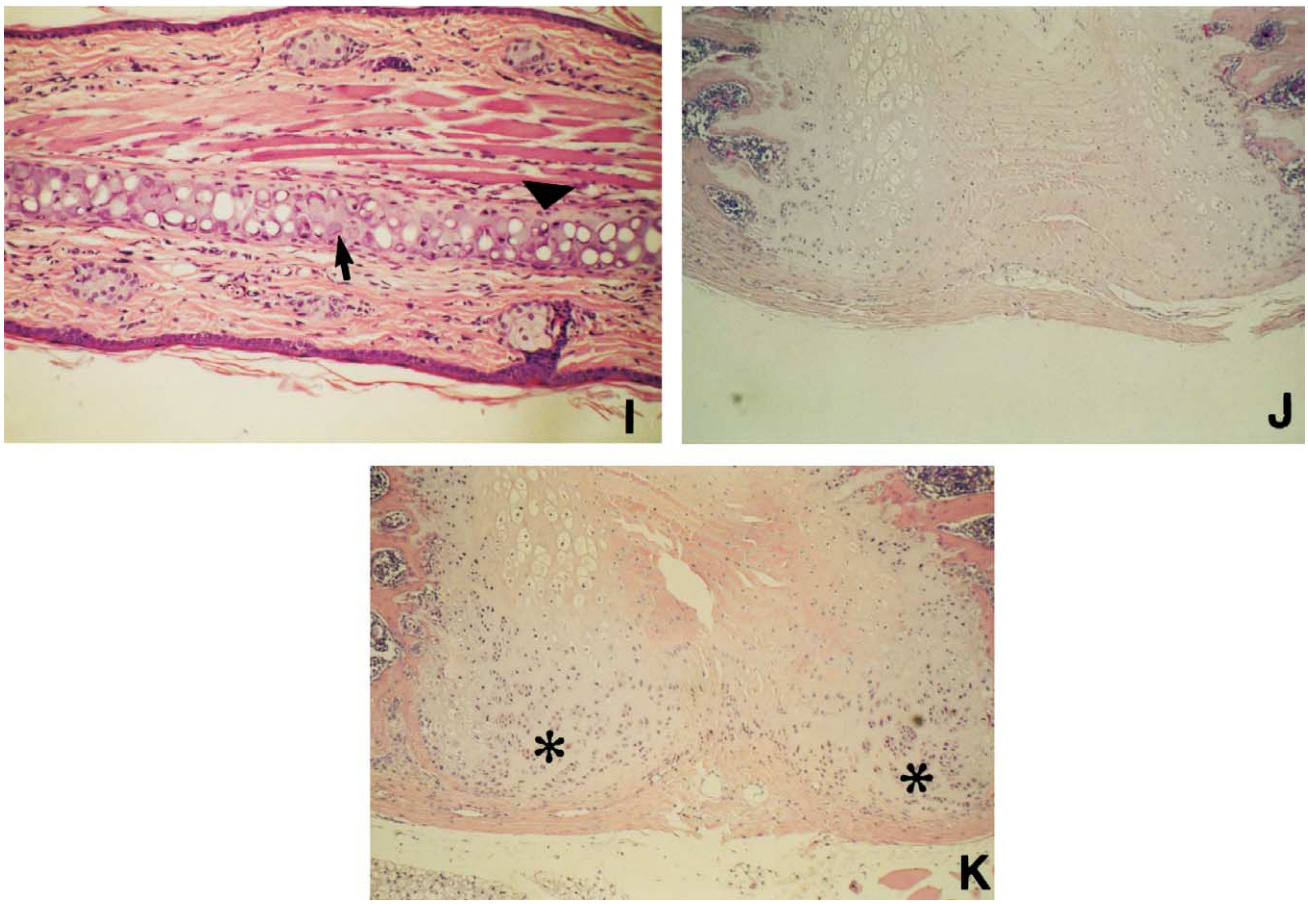

Fig. 2 (*continued*).

articular cartilage and the growth plate (groove of Ranvier, Fig. 2G). In this region, apparent subperiosteal cartilage growth was observed. In the auricles of AG-041R-treated rats, the content of lipid droplets in the chondrocyte cyto-

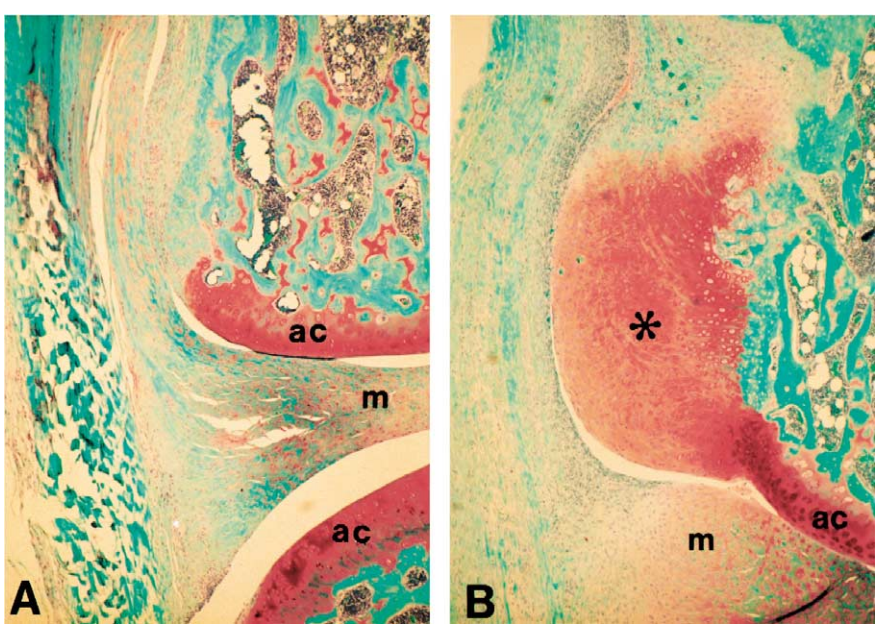

Fig. 3. Photomicrograph of femoral condyle after intraarticular injections of vehicle control (A, 50% DMSO, 50 μl/day) or AG-041R (B, 27.3 μg/day) for 21 days. Chondrophyte (*) caused by AG-041R had safranin-O-positive hyaline cartilage-like extracellular matrix. In chondrophytes, the arrangement of chondrocytes was somewhat irregular in comparison with that of normal articular cartilage. ac: articular cartilage, gp: growth plate, m: meniscus Safranin-O and fast green, original magnification: ×25.

plasm was decreased, and the hyaline cartilage matrix was increased (Fig. 2I). In the intervertebral disks, hyaline cartilage hyperplasia was induced by AG-041R in the dorsal and ventral marginal regions. In these tissues, chondrocyte recruitment from the chondroprogenitor was stimulated by AG-041R. Chondrocytes within hyperplastic cartilage were recruited from the perichondrium in the trachea (Fig. 2B), auricles (Fig. 2I) and intervertebral disks (Fig. 2K). In the femoral condyle of AG-041R-treated rats, chondrocytes were recruited from the periosteum contiguous to articular cartilage (Fig. 2G). No histopathological change was observed in the stomachs of these animals.

3.2. i.a. study

Intraarticular injections of AG-041R to rat knee joints resulted in the stimulation of chondrophyte formation in femoral condyle (Fig. 3B), as observed in the p.o. protocol. The chondrophyte induced by AG-041R was stained strongly by safranin-O, and histologically resembled hyaline cartilage. In chondrophyte induced by AG-041R, the chondrocyte arrangement was disarrayed in comparison with normal articular cartilage, in which there is a columnar arrangement of chondrocytes. In contrast to oral administration, no hyperplasia was observed in other permanent cartilage.

4. Discussion

In the present study, we clearly demonstrated that AG-041R, a novel indoline-2-one derivative, induced systemic cartilage hyperplasia. This chondrogenic activity was unexpectedly discovered in an oral chronic toxicological study in rats during the development of AG-041R as a gastric ulcer agent. With oral administration, no histopathological change was observed in the stomachs of AG-041R-treated rats. The maximal plasma level of AG-041R was 80.5 ± 6.3 μg/ml after administration of AG-041R for 27 days in the p.o. experiment. At this concentration, cartilage hyperplasia was accompanied by weight loss and elevation of several serum biochemical markers of hepatic injury such as glutamic–pyruvic transaminase.

AG-041R induced cartilage hyperplasia in permanent cartilage such as the auricles, trachea, marginal regions of the femoral condyle, xiphoid process and intervertebral disks, on oral administration. I.a. administration of AG-041R also induced cartilage hyperplasia, without change in any other untreated joints. On the basis of the histological observations, cartilage hyperplasia induced by AG-041R was probably due to both chondrocyte recruitment from chondroprogenitors and increase of peripheral hyaline cartilage matrix. These changes were not generalized, but occurred in specific sites of cartilaginous tissue such as the femoral groove of Ranvier, outer perichondrium of trachea, dorsal and ventral margin of intervertebral disks. Since the rats used in this study were growing, cartilage hyperplasia induced by AG-041R might have been a specific change in growing animals. Further examination in skeletally mature animals is needed to characterize the chondrogenic activity of AG-041R in vivo.

The chondrogenic activity of AG-041R on local administration suggested that AG-041R itself, rather than its metabolites, is the genuine active compound. The chondrogenic activity of AG-041R has also been shown in in vitro experiments, using rat articular chondrocytes (data not shown). Major metabolites of AG-041R identified in rats were the following three compounds: 1-(2,2-diethoxyethyl)-3-((4-hydroxymethylphenyl)aminocarbonylmethyl)-3-((4-methylphenyl)ureido)-indoline-2-one, 1-(2,2-diethoxyethyl)-3-((4-methylphenyl)aminocarbonylmethyl)-3-((4-hydroxymethylphenyl)ureido)-indoline-2-one, and 1-(2-oxoethyl)-3-((4-methylphenyl)aminocarbonylmethyl)-3-((4-methylphenyl)ureido)-indoline-2-one. Since these metabolites had no chondrogenic activity in vitro in an AG-041R-responsive murine chondrogenic cell line (data not shown), they probably did not contribute to the cartilage hyperplasia induced by AG-041R in vivo.

Since AG-041R was originally synthesized as a CCK_2/gastrin receptor antagonist (Ding et al., 1997; Fukui et al., 1998), we examined the involvement of this activity in chondrogenesis. There is a large discrepancy between doses of AG-041R effective in the gastric ulcer models (10 mg/kg) (Baba et al., 1995) and those effective in chondrogenesis (2000 mg/kg). Several derivatives of AG-041R showed chondrogenic activity, but no correlation with anti-gastrin activity (data not shown). The other gastrin receptor antagonists, 3*R*(+)-*N*-(2, 3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1, 4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl)urea (L-365,260, Konturek et al., 1991) and (*R*)-1-[2, 3-Dihydro-1-(2′-methyl-phenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)-urea (YM-022, Nishida et al., 1994), had no in vitro chondrogenic activity at any concentration tested (data not shown). These results indicate that CCK_2/gastrin receptor antagonistic activity is not related to the chondrogenic activity of AG-041R. Further studies are required to clarify the mechanisms of the chondrogenic actions of AG-041R.

Articular cartilage lesions are still a commonly encountered medical problem, because no satisfactory treatment has been established. AG-041R might be a potent therapeutic agent for cartilage disorders according to its chondrogenic activity. Its physicochemical properties of metabolic stability and lipophilicity might be great advantages for clinical application. These properties are suitable for a formulation that would achieve controlled release. This issue has been a major problem for clinical application with cartilage-repairing growth factors such as bFGF, BMP-2 and TGF-β, which have a short half-life in vivo (Cuevas et al., 1988; Sellers et al., 2000; Van Beuningen et al., 1998; Shida et al., 1996; Glansbeek et al., 1998; Putney and Burke, 1998). Since chondrophyte is an unde-

sirable change for a joint, localization of the chondrogenic effect of AG-041R inside lesions must be achieved for clinical application.

The chondrogenic activity of (2*R*, 4*S*)-(−)-*N*-[4-(Diethoxyphosphorylmethyl)phenyl]-1, 2, 4, 5-tetrahydro-4-methyl-7, 8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide (TAK-778) was reported recently (Akiyama et al., 1999). TAK-778 promotes cartilage repair when injected into knee joints. The chondrogenic activity of TAK-778 was restricted to the osteochondral defect region, and no cartilage hyperplasia in the marginal region of articular cartilage was reported. This suggested that the target cells of TAK-778 in the knee joint of the osteochondral defect model were bone marrow-derived mesenchymal cells within the defect region (Hunziker, 1999). In contrast, our histological findings suggested that the target cells of AG-041R were chondroprogenitor cells in the periosteum and chondrocytes in the knee joint. The difference between target cells suggests the different modes of action of these two compounds. Moreover, systemic cartilage hyperplasia caused by AG-041R administration showed that the target cells of AG-041R were distributed in various regions of permanent cartilage. Therefore, AG-041R is expected to be applicable to disorders in various regions of permanent cartilage, including the auricle and intervertebral disks, and not to be limited to the articular cartilage.

In conclusion, AG-041R is a novel small molecule with systemically chondrogenic activity. It is expected to be a potent therapeutic agent for cartilage disorders, and may also be a useful tool for the elucidation of regulatory mechanisms of chondrogenesis.

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