International Journal of
Rheumatic Diseases

International Journal of Rheumatic Diseases 2015; **18**: 495–501

REVIEW ARTICLE

The association of vitamin C, alcohol, coffee, tea, milk and yogurt with uric acid and gout

Patapong TOWIWAT[1] and Zhan-Guo LI[2]

[1]*Autoimmune Immunology and Rheumatology Division, Internal Medicine Department, Faculty of Medicine, Naresuan University Hospital, Naresuan University, Phitsanulok, Thailand, and* [2]*Department of Rheumatology and Immunology, Peking University People's Hospital, Beijing, China*

Abstract

About 2500 years ago, gout was observed by Hippocrates and many people suffered severe pain and deformity. Lifestyle and diet play a significant role in gout and serum uric acid levels. Epidemiological and research studies have supported this evidence. Many recommendations and guidelines from different parts of the world mention the impact of diet on gout. Recently, new research has shown associations between vitamin C, alcohol, coffee, tea, milk and yogurt with uric acid and the risk of gout. Our review summarizes recently published research regarding dietary impact on the risk of gout and serum uric acid levels.

Key words: Gout, uric acid, diet.

INTRODUCTION

Gout is an inflammatory arthritis and is associated with high serum uric acid (SUA).[1,2] It is relatively common in late middle-aged men[3,4] and women,[5,6] with increased rates in menopausal women.[4,7–9] In the USA, the incidence of gout is 8.4 per 10 000 persons/year.[10] Many factors contribute to gout risk, such as high SUA,[1] ethnicity,[11–13] underling disease,[14–17] age,[18] greater body mass index,[19] and so on. Many studies based on the Third National Health and Nutrition Examination Survey (NHANES-III) suggest that dietary consumption affects SUA levels which is parallel to the increase in the risk of gout.[20] The relationship between diet, drinking and gout was found a long time ago.[14,21–23] In this review, we will update the recent data of the association between vitamin C (VC), alcohol, tea, coffee, milk and yogurt with SUA and gout risk as shown in Table 1.

Correspondence: Professor Zhan-Guo Li, Department of Rheumatology and Immunology, Peking University People's Hospital, Beijing 100044, China.
Email: zgli99@aliyun.com

VITAMIN C

A recent cross-sectional study in Korea showed that the intake of VC is lower in subjects with hyperuricemia when compared with control groups.[24] The effects of VC on SUA and the uricosuric effect are evaluated in many studies.[25–32] One randomized controlled trial showed that SUA levels were significantly reduced with supplementation of 500 mg/day of VC for 2 months, and VC is beneficial in the prevention and management of gout.[30] In 2011, a meta-analysis of randomized controlled trials, concluded that the median dose of VC supplementation (500 mg/day) significantly lowered SUA.[31] In contrast, Stamp *et al.*[29] studied the effect of supplemental VC on SUA in patients with gout. This study was a pilot randomized controlled trial: 40 patients with gout and a SUA level of more than 6 mg/dL were included. This research did not observe any significant effect of VC when compared to allopurinol on the reduction of SUA, and the uricosuric effect was small in patients with gout, regardless of whether VC was administered alone or in combination with allopurinol. Reasonses for this result include: the uricosuric effect of VC is weak, the dose of VC was small, and there might be interactions with other medications (in

Diet	Serum uric acid		Risk of gout	
	Change	Condition	Change	Condition
Vitamin C	↓	500 mg/day[3]	↓	> 500 mg/day[4]
Coffee	↓	≥ 4 cups/day[5,6]	↓	≥ 4 cups/day[7,8]
Tea	–	Any drinks[6]	–	Any drinks[7,8]
Alcohol				
Beer†	↑	≥ 0.01 serving/day[9]	↑	≥ 2 servings/week[11]
Liquor/spirits†	↑	≥ 0.1 serving/day[9]	↑	≥ 1 servings/month[11]
Wine†	↓	< 1 serving/day[9]	–	Any drinks[11]
Sake	↑	≥ 20 drinks/week‡,[10]	No study	
Shochu	↑	Any drinks[10]	No study	
Milk	↓	≥ 1 serving/day[12]	↓	≥ 2–4 glass/week§,[13]
Yogurt	↓	≥ 0.5 serving/day¶,[12]	↓	≥ 2 cups/week††,[13]

Table 1 Recent data of the association between vitamin C, alcohol, tea, coffee, milk and yogurt with serum uric acid and the risk of gout

†Increased risk of gout attack; ‡1 drink is 11.5 g of ethanol for sake; §skim milk or low fat milk; ¶or ≥ once every other day; ††adjusted for age-adjusted relative risk. ↑, increase; ↓, decrease; –, no change.

particular, aspirin and diuretics). This paper does not support VC supplementation as a urate-lowering therapy in gout patients. Many studies suggested mechanisms of VC for reduced SUA. First, at the renal urate transporter URAT1 and a sodium-dependent anion co-transporter SLC5A8/A12, VC has a uricosuric effect.[31,33–35] Second, it increases renal fractional clearance of uric acid.[25] Finally, VC decreases free radical-induced damage to body cells, thereby reducing SUA.[32,36]

The relationship between VC and incidence of gout was assessed by one prospective study in 2009.[33] This study concluded that the incidence of gout decreased with increasing VC intake with up to a 45% lower risk at the top VC intake category of 1500 mg or more. So, higher VC intake is independently associated with a lower risk of gout, and supplemental VC intake may be beneficial in the prevention of gout.[33]

Although much of the research has studied the relationship between VC and SUA, no research has evaluated the role of VC in gout flare. Some authors are aware that taking large amounts of VC may trigger gout flare because of changes in SUA.[25]

COFFEE

Coffee is the most widely consumed beverage in the world. In the US, the relationship between coffee and the incidence of gout in men and women was shown in two cohort prospective studies.[37,38] The risk of gout in men whose intake of coffee was 4–5 cups/day and ≥ 6 cups/day was 40% and 59% lower, respectively, when compared with no coffee consumption. The association between total caffeine intake and risk of gout was not significant.[38] The risk of gout was 22% and 57% lower in women whose intake was 1–3 cups/day and ≥ 4 cups/day, respectively, compared with no coffee consumption, but, in contrast to men, there was a significantly inverse association between total caffeine intake and risk of gout.[37] So, long-term coffee consumption is associated with a lower risk of gout incident,[34,37,38] and components other than caffeine may inversely affect the association between coffee and gout.[37,38]

Moreover, coffee has not been found to affect SUA. In 1999, the first study of the relationship between coffee consumption and SUA concentration was published.[39] This study concluded that coffee drinking may be associated with lower concentrations of SUA. Based on 14 758 participants ages > 20 years in NHANES-III (1988–1994), Choi and Curhan[40] evaluated the relationship between coffee, tea and caffeine intake, and SUA levels. These findings suggest that coffee consumption is associated with lower SUA levels. Also, this study suggested that the inverse association with coffee appears to be due to factors other than caffeine. Another study in Japan showed inverse associations between coffee consumption and both SUA levels and hyperuricemia in men, but not in women, regardless of adjustment for the covariates. After allowance for confounding factors, women showed a statistically significant inverse association between coffee and SUA levels.[41] A study in Asia showed that coffee consumption (≥ 4 cups/day) is associated with lower SUA levels.[42] Several mechanisms have suggested that coffee consumption may affect the risk of gout and

SUA.[20,34,39–41,43,44] From the review study, we have concluded that coffee consumption (≥ 4 cups/day) has urate-lowering properties and reduces the risk of gout. There has been no research study between coffee and gout flare.

Because coffee intake is one of the independent risk factors for chronic kidney disease[45] and is associated with increased risk of fractures in women,[46] drinking coffee for lowering the risk of gout should be considered carefully.

TEA

Tea is a leaf of *Camellia sinensis*. There are many types of tea. It can be divided into six major types of reprocessed basic tea leaf depending on fermentation, such as green tea, blue tea, black tea, yellow tea, white tea and dark tea.[47]

In tea, phenolic compounds (mainly tea catechins) are the main chemical components.[48] Five tea catechins can inhibit the liver enzyme xanthine oxidase.[49] In contrast to coffee, the relationship between nonspecific types of tea and SUA and risk of gout is negative for both men and women.[37,38,40] For green tea, a recent study has shown that daily drinkers of green tea exhibited a dose-dependent and statistically significant four-fold increase with hyperuricemia,[42] but an earlier study has shown that green tea drinking did not show a statistically significant trend with SUA.[39] During fermentation of black tea, the oxidized derivatives of black tea catechins, theaflavin and thearubigins are formed.[50] Theaflavins can inhibit the activity of xanthine oxidase.[50,51] The relation between black tea and SUA was shown in 2010 by Bahorun *et al.*[52] This data showed that black tea intake significantly decreased SUA by 9.4% and 7.1% in male and female groups with highest baseline values, respectively.[52] In contrast, with recent data published in 2013, Teng *et al.*[42] showed that there was no significant association between consumption of black tea and SUA levels. In our opinion, differences in baseline SUA levels between the studies may be a cause of the conflicting results. There has been no research investigating the impact of tea on gout flare.

As mentioned above, there are many types of tea. Some studies do not classify the type of tea, and a few studies only showed the relationship between green tea or black tea and SUA. Different types of tea may give different results, other specific types of tea need to be evaluated in the future. There is conflicting data with black tea and SUA.

ALCOHOL

Alcohol has been recognized as a potential risk factor for increased SUA and gout flare. An association between increased alcohol intake and hyperuricemia and risk of gout has been shown in many studies.[53–57] In 2013, a meta-analysis with 42 924 participants assessed the relative risks (RRs) and dose response of gout risk to alcohol consumption. The RRs for light (≤ 1 drink/day), moderate (> 1 to < 3 drinks/day) and heavy drinking (≥ 3 drinks/day) versus no or occasional alcohol drinking were 1.16 (95% confident interval [CI], 1.07–1.25), 1.58 (95% CI, 1.50–1.66) and 2.64 (95% CI, 2.26–3.09), respectively. The results suggest that alcohol consumption is associated with increased risk of gout.[58]

Beer, liquor/spirits

Beer has a high content of purine guanosine, which can increase uric acid production.[21] In 2004, the relationship between intakes of beer, liquor and wine, and SUA levels was evaluated.[54] The results showed that SUA levels increased with beer or liquor intake, and the authors concluded that the effect of individual alcoholic beverages on SUA levels varies substantially: beer confers a larger increase than liquor, and increase in SUA with beer intake was more pronounced for women than for men. Yu *et al.*[59] also demonstrated that beer intake is independently associated with increased risk of hyperuricemia in men. In 2010, Gaffo *et al.*[55] showed that, compared with non-drinkers, significant associations between higher SUA concentrations and greater beer intake were observed among men and women. However, in men there were only significant findings in the higher category of intake at a median of 12 servings/week (one serving of beer is equal to a 12 ounce bottle, approximately 355 mL). An association between greater liquor intake and higher SUA concentrations was only found in men in the last year of the study.

In their study, Choi *et al.*[53] used questionnaires to investigate the relationship between alcohol consumption (including beer and spirits) and risk of gout incident in 47 150 male participants with no history of baseline gout. Beer consumption showed the strongest independent association with the risk of gout, and the multivariate RR increased with servings of beer per week and per day. Consumption of spirits was also significantly associated with gout. They concluded that alcohol intake is strongly associated with an increased risk of gout and varies substantially according to type of

alcoholic beverage: beer confers a larger risk than spirits, even though alcohol content per serving was less for beer than spirits. At present, there has been no research evaluating the relationship between SUA or risk of gout and 'light beer.' Hence, we conclude that beer and spirits increase the risk of gout.

Wine

Regarding the difference between wine and other alcoholic beverages in relation to SUA and risk of gout, Choi and Curhan[54] showed that SUA levels decrease with increasing wine intake, except for the top category (≥ 1 serving per day) in which the level was lower than that of no use. They concluded that moderate wine drinking (< 1 serving per day) does not increase SUA levels. However, these findings contrast with the study of Gaffo *et al.*[55] Their data showed that wine intake was not associated with SUA in either sex.[55] The relationship between risk of gout and wine has been prospectively confirmed. Data from a prospective study investigating alcohol intake and risk of gout incident in men showed that the RR for men who drank ≥ 2 glasses of wine per day compared with those who drank less than one glass per month was 1.05 (95% CI 0.64–1.72), and there was no significant trend with increasing levels of wine consumption (*P* for trend = 0.66). Regardless of the type of wine, this association still persisted. It can be concluded that in men, moderate wine drinking (118 mL serving per day) was not significantly associated with an increased risk of gout.[53] However, there have been no studies investigating the relationship between gout risk and wine drinking for women.

Sake and shochu

Sake is a Japanese rice wine containing ethanol. Shochu is a Japanese distilled spirit, also containing ethanol, that is made from barley, sweet potato, rice or any combination. For sake, a 6-year prospective study conducted by Nakamura *et al.*[56] showed a dose-response between drinking sake and risk of hyperuricemia. For shochu, a study by Sugie *et al.*[57] showed there was no statistical difference in the risk of hyperuricemia when compared with subjects who drank sake, beer and shochu. Both sake and shochu cause increased SUA, but there was no data for gout flare.

Although beer and liquor have been associated with the risk of gout incident, wine has not. However, in 2014, there was a prospective, internet-based, case-crossover study which examined the quantity and type of alcohol consumed and risk of recurrent gout attacks over the prior 24 h. This study showed a significant dose-response relationship between the amount of alcohol consumption and risk of recurrent gout attacks.[60] Consuming wine, beer or liquor was associated with significantly increased risk of gout attack. This study concluded that even a moderate amount of alcohol consumption, regardless of type of alcoholic beverage, was associated with an increased risk of recurrent gout attacks.[60] Many recommendations and guidelines suggest avoiding drinking alcohol.[61–66]

MILK AND YOGURT

A numbers of studies have shown an association between milk and yogurt with SUA and gout.[27,67–71] Choi *et al.* studied the relationship between SUA and dairy products, including milk and yogurt. This study showed a significant inverse relationship between those who consumed milk one or more times per day and SUA level compared with no milk drinking. It also found a significantly lower SUA level in those who consumed yogurt at least once every other day compared with those who did not consume yogurt.[27] Similarly, Zgaga *et al.*[70] showed that skimmed milk and low-calorie yogurt had a significant and inverse association with SUA concentration. For the association between yogurt and milk with risk of gout in men, a prospective study showed that for men who drank two or more glasses of skim milk per day as compared with men who drank less than one glass per month, the multivariate RR was 0.54 (95% CI, 0.40–0.73; *P* for trend < 0.001).[68] The risk of gout had a similar inverse association with the level of consumption of low-fat yogurt.[68] From this evidence, milk and low-fat yogurt may decrease SUA and the risk of gout. Milk products such as skim milk powder enriched with glycomacropeptide and G600 milk fat extract may reduce the frequency of gout flares.[67]

Multifactorial analysis can explain the association between milk and SUA and reduced gout flare. First, orotic acid in milk promotes renal urate excretion.[70] Second, milk contains casein and lactalbumin, both of which have been shown to decrease SUA via a uricosuric effect.[71,72] Third, both glycomacropeptide, the 64-amino acid carboxyterminal fragment of κ-casein, and G600 milk fat extract, a complex lipid fraction in which the phospholipids and gangliosides, particularly disialo ganglioside 3, have anti-inflammatory effects in acute gout and may reduce gout flares through inhibition of the inflammatory response to monosodium urate crystals in the joint.[67,69] Finally,

vitamin D is found in milk, and a study suggests that gout patients may have significantly lower levels of 1,25-(OH)2-vitamin D3. After administration of urate-lowering drugs in this study, there was a decrease in SUA which was associated with a significant elevation in the levels of 1,25-(OH)2-vitamin D3.[73] The supplementation of vitamin D to prevent hyperuricemia has not been studied.[74]

CONCLUSION

Although many studies show association between VC, alcohol, coffee, tea, milk and yogurt with SUA and gout, in the 2012 American College of Rheumatology Guidelines, they recommend only avoidance of alcohol overuse (defined as more than two servings/day and one servings/day for men and women, respectively) and limiting of alcohol, particularly beer, and also wine and spirits, in gout patients.[62] Other than that, they encourage only low-fat or non-fat dairy products in gout patients.[62] Beyond the scope of gout, we should consider other risk factors and possible benefits in other diseases.

AUTHOR CONTRIBUTIONS

Patapong Towiwat reviewed the literature and drafted the manuscript; Zhan-Guo Li proofed the manuscript.

REFERENCE

1 Choi HK, Mount DB, Reginato AM (2005) Pathogenesis of gout. *Ann Intern Med* **143** (7), 499–516.
2 Firestein GS, Kelley WN (2013) *Kelley's Textbook of Rheumatology*, 9th edn. Elsevier/Saunders, Philadelphia, PA.
3 Teh CL, Chew KF, Ling GR (2014) Acute Gout in Hospitalized patients in Sarawak General Hospital. *Med J Malaysia* **69** (3), 126–8.
4 Teh CL, Cheong YK, Ling HN, Chan PL, Chan T, Ling GR (2013) A profile of gout patients in Sarawak. *Rheumatol Int* **33** (4), 1079–82.
5 Kumar S, Gupta R, Suppiah R (2012) Gout in women: differences in risk factors in young and older women. *N Z Med J* **125** (1363), 39–45.
6 Harrold LR, Yood RA, Mikuls TR *et al.* (2006) Sex differences in gout epidemiology: evaluation and treatment. *Ann Rheum Dis* **65** (10), 1368–72.
7 Cornelius R, Schneider HJ (1988) Gouty arthritis in the adult. *Radiol Clin North Am* **26** (6), 1267–76.
8 Lally EV, Ho G Jr, Kaplan SR (1986) The clinical spectrum of gouty arthritis in women. *Arch Intern Med* **146** (11), 2221–5.
9 Puig JG, Michan AD, Jimenez ML *et al.* (1991) Female gout. Clinical spectrum and uric acid metabolism. *Arch Intern Med* **151** (4), 726–32.
10 Maynard JW, McAdams-DeMarco MA, Law A *et al.* (2014) Racial differences in gout incidence in a population-based cohort: Atherosclerosis Risk in Communities Study. *Am J Epidemiol* **179** (5), 576–83.
11 Krishnan E (2014) Gout in African Americans. *Am J Med* **127**, 858–64.
12 Winnard D, Wright C, Taylor WJ *et al.* (2012) National prevalence of gout derived from administrative health data in Aotearoa New Zealand. *Rheumatology (Oxford)* **51** (5), 901–9.
13 Lu X, Li X, Zhao Y, Zheng Z, Guan S, Chan P (2014) Contemporary epidemiology of gout and hyperuricemia in community elderly in Beijing. *Int J Rheum Dis* **17** (4), 400–7.
14 Roddy E, Doherty M (2010) Epidemiology of gout. *Arthritis Res Ther* **12** (6), 223.
15 Grodzicki T, Palmer A, Bulpitt CJ (1997) Incidence of diabetes and gout in hypertensive patients during 8 years of follow-up. The General Practice Hypertension Study Group. *J Hum Hypertens* **11** (9), 583–5.
16 Roubenoff R, Klag MJ, Mead LA, Liang KY, Seidler AJ, Hochberg MC (1991) Incidence and risk factors for gout in white men. *JAMA* **266** (21), 3004–7.
17 McAdams-DeMarco MA, Maynard JW, Baer AN, Coresh J (2012) Hypertension and the risk of incident gout in a population-based study: the atherosclerosis risk in communities cohort. *J Clin Hypertens (Greenwich)* **14** (10), 675–9.
18 Krishnan E, Bennett M, Chen L (2014) Aging, not menopause, is associated with higher prevalence of hyperuricemia among older women. *Menopause* **21** (11), 1211–6.
19 Aune D, Norat T, Vatten LJ (2014) Body mass index and the risk of gout: a systematic review and dose-response meta-analysis of prospective studies. *Eur J Nutr* **53**, 1591–601.
20 Hak AE, Choi HK (2008) Lifestyle and gout. *Curr Opin Rheumatol* **20**, 179–86.
21 Lee SJ, Terkeltaub RA, Kavanaugh A (2006) Recent developments in diet and gout. *Curr Opin Rheumatol* **18**, 193–8.
22 Andres M, Sivera F, Falzon L, Buchbinder R, Carmona L (2014) Dietary supplements for chronic gout. *Cochrane Database Syst Rev* (10), CD010156.
23 Kolasinski SL (2014) Food, drink, and herbs: alternative therapies and gout. *Curr Rheumatol Rep* **16** (4), 409.
24 Ryu KA, Kang HH, Kim SY *et al.* (2014) Comparison of nutrient intake and diet quality between hyperuricemia subjects and controls in Korea. *Clin Nutr Res* **3** (1), 56–63.
25 Stein HB, Hasan A, Fox IH (1976) Ascorbic acid-induced uricosuria. A consequency of megavitamin therapy. *Ann Intern Med* **84**, 385–8.

Antiviral Research 160 (2018) 55–63

Contents lists available at ScienceDirect

Antiviral Research

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

Vitamin D derivatives inhibit hepatitis C virus production through the suppression of apolipoprotein

Asako Murayama[a], Hiroshi Saitoh[b], Akiko Takeuchi[b], Norie Yamada[a], Takuya Matsumura[c], Masaaki Shiina[a,d], Masamichi Muramatsu[a], Takaji Wakita[a], Michio Imawari[e], Takanobu Kato[a,*]

[a] Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
[b] Teijin Institute for Bio-medical Research, Teijin Pharma Ltd., Tokyo, Japan
[c] Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan
[d] Department of Gastroenterology and Hepatology, Shin-Yurigaoka General Hospital, Kawasaki, Japan
[e] Research Institute for Gastrointestinal and Liver Diseases, Shin-Yurigaoka General Hospital, Kawasaki, Japan

ARTICLE INFO

Keywords:
HCV
VD
Apolipoprotein
Virus assembly

ABSTRACT

Supplementation with vitamin D (VD) has been reported to improve the efficacy of interferon-based therapy for chronic hepatitis C. We found that 25-hydroxyvitamin D_3 (25-(OH)D_3), one of the metabolites of VD, has antiviral effects by inhibiting the infectious virus production of the hepatitis C virus (HCV). In this study, to clarify the underlying mechanisms of the anti-HCV effects, we searched VD derivatives that have anti-HCV effects and identified the common target molecule in the HCV life cycle by using an HCV cell culture system. After infection of Huh-7.5.1 cells with cell culture-generated HCV, VD derivatives were added to culture media, and the propagation of HCV was assessed by measuring the HCV core antigen levels in culture media and cell lysates. To determine the step in the HCV life cycle affected by these compounds, the single-cycle virus production assay was used with a CD81-negative cell line. Of the 14 structural derivatives of VD, an anti-HCV effect was detected in 9 compounds. Cell viability was not affected by these effective compounds. The 2 representative VD derivatives inhibited the infectious virus production in the single-cycle virus production assay. Treatment with these compounds and 25-(OH)D_3 suppressed the expression of apolipoprotein A1 and C3, which are known to be involved in infectious virus production of HCV, and the knockdown of these apolipoproteins reduced infectious virus production. In conclusion, we identified several compounds with anti-HCV activity by screening VD derivatives. These compounds reduce the infectious virus production of HCV by suppressing the expression of apolipoproteins in host cells.

1. Introduction

Hepatitis C virus (HCV) infection is a major health problem and affects approximately 71 million people worldwide (WHO, 2017). In most cases, HCV establishes a persistent infection resulting in the development of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Interferon (IFN)-based therapy has contributed to both the control of disease progression and eradication of this virus (Feld and Hoofnagle, 2005; Pawlotsky, 2006). Although a number of novel direct-acting antivirals (DAAs) have been developed, it is still important to discover novel anti-HCV reagents and identify host factors that serve as a target of antivirals, because there are still many issues to be solved: medication cost, limited access to novel treatment regimens and emerging resistance-associated substitutions to these DAAs (Feld, 2014; Pawlotsky, 2013; Welzel and Zeuzem, 2014).

1α,25-dihydroxy vitamin D_3 (1α,25-$(OH)_2D_3$), the active form of vitamin D (VD), shows biological activities through interaction with the vitamin D receptor (VDR) and transcriptional activation inside the nucleus. This VDR-mediated signaling is called genomic effect (Whitfield et al., 1995). VD primarily exerts its action as a regulator of calcium and phosphorus, including bone homeostasis. Moreover, it is reported that 1α,25-$(OH)_2D_3$ also has regulatory roles in infectious diseases and acts as an immune modulator in both innate and adaptive immunities. Supplementation with VD has been demonstrated to improve the treatment efficacy of IFN-based therapy (Abu-Mouch et al., 2011; Kondo et al., 2013; Nimer and Mouch, 2012; Yokoyama et al., 2014); however, the mechanisms underlying this effect have not yet been fully elucidated. Previously, we reported that 25-hydroxyvitamin

* Corresponding author. Department of Virology II, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640, Japan.
E-mail address: takato@nih.go.jp (T. Kato).

https://doi.org/10.1016/j.antiviral.2018.10.014
Received 1 August 2018; Received in revised form 21 September 2018; Accepted 14 October 2018
Available online 16 October 2018
0166-3542/

D3 (25-(OH)D_3), which is a metabolite of VD and a proactive form of 1α,25-(OH)$_2D_3$, has an antiviral effect against HCV (Matsumura et al., 2012). In an *in vitro* HCV cell culture system, the administration of 25-(OH)D_3 reduced infectious virus production, although detailed mechanisms of this effect remain unclear.

In this study, to clarify the underlying mechanism of the anti-HCV effect of 25-(OH)D_3, we screened structural derivatives of 1α,25-(OH)$_2D_3$ and identified several compounds possessing an anti-HCV effect. By comparing these compounds with 25-(OH)D_3, we assessed the contribution of VD activity to the anti-HCV effects and identified the common target molecule.

2. Materials and methods

2.1. Cell culture

Huh-7.5.1 cells (Zhong et al., 2005), derived from HuH-7 hepatoma cells, were a kind gift from Dr. Francis V. Chisari (Scripps Research Institute, La Jolla, CA). Huh7-25 cells, which lack cell surface expression of the HCV receptor CD81, have been described previously (Akazawa et al., 2007). Cells were cultured at 37 °C in a 5% CO_2 environment using Dulbecco's Modified Eagle's Medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum. A human osteosarcoma cell line, HOS cells, were maintained in phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum at 37 °C in 5% CO_2.

2.2. Reagents

The structures of the VD derivatives evaluated in this study are depicted in Fig. 1. These compounds have been reported previously (Saito et al., 2013; Saitoh et al., 2011, 2015). To assess the cell viability, a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI) was used.

2.3. Plasmids

The plasmid encoding a chimeric full-length HCV RNA, pJ6/JFH-1, has been described previously (Murayama et al., 2016).

2.4. Cell culture-generated HCV

The production of cell culture-generated HCV (HCVcc) has been described previously (Sugiyama et al., 2014). In brief, JFH-1 RNA-transfected cells were cultured for a long period, and cell culture-adapted HCVcc was harvested and stocked for further infection studies.

2.5. Detection of the anti-HCV effects of VD derivatives

For the screening assay, Huh-7.5.1 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and infected with HCVcc (multiplicity of infection = 0.1). Four hours after infection, the cells were washed and placed in fresh media containing 0.1% dimethyl sulfoxide (DMSO) or in media containing 0.1 μM, 1 μM and 10 μM of VD derivatives. The culture media were collected at day 3 and inoculated into cultures of naive Huh-7.5.1 cells. The inoculated cells were cultured for another 3 days, and culture media were harvested to measure the HCV core antigen (Ag). Independent assays were performed in triplicate, and the percentage of DMSO control was calculated; the data are presented as the mean ± standard deviation.

For the validation assay, Huh-7.5.1 cells were seeded at 1×10^5 cells/well in 12-well plates and infected with HCVcc (multiplicity of infection = 0.1). Four hours after infection, cells were washed and placed in fresh media containing 0.1% DMSO or in media containing 1 μM, 3 μM and 10 μM of VD derivatives. The culture media and cells were harvested at day 3 to measure the HCV core Ag in culture media and cell lysates.

2.6. RNA synthesis, transfection, and determination of infectivity

RNA synthesis and transfection were performed as previously described (Kato et al., 2006; Murayama et al., 2010). The determination of the infectivity titer was also performed as previously described (Murayama et al., 2007, 2012a), with the infectivity expressed as the number of focus-forming units per milliliter (FFU/mL).

2.7. Quantification of the HCV core Ag

The concentration of the HCV core Ag in the culture media and cell lysates was measured using a chemiluminescent enzyme immunoassay (Lumipulse Ortho HCV antigen, Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions (Murayama et al., 2012b).

2.8. Single-cycle virus production assay

Ten micrograms of *in vitro*-transcribed HCV RNA was transfected into 3×10^6 Huh7-25 cells. Four hours after transfection, the cells were placed in fresh medium containing 0.1% DMSO or in medium containing 10 μM VD derivatives. Cells and culture media were harvested at day 3, and HCV core Ag levels and infectivity titers in the culture media and cells were determined.

2.9. Gene expression assay

Total cellular RNA was extracted using an RNeasy Mini kit (QIAGEN, Valencia, CA). cDNA was synthesized from total cellular RNA using Superscript VILO reverse transcriptase (Invitrogen). Quantitative PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) and gene-specific primer and probe sets (TaqMan Gene Expression Assay; Applied Biosystems) for apolipoprotein (Apo) A1 (Rh00985000_m1), ApoA2 (Rh02913017_m1), ApoC1 (Hs03037377_m1), ApoC2 (Rh04255409_g1), ApoC3 (Rh02794312_m1) and ApoE (Hs00171168_m1) in accordance with the manufacturer's instructions. The expression levels of these genes were normalized by using the 18S rRNA Endogenous Control (Applied Biosystems) and are expressed as the fold changes over the DMSO control level.

2.10. Knockdown of apolipoproteins

Huh-7.5.1 cells seeded at 2×10^6 cells/well were transfected with a negative control siRNA (Stealth RNAi siRNA Negative Control Lo GC Duplex #3, 12935111, Invitrogen) or siRNAs against Apolipoprotein A1 (Stealth siRNA HSS166917, Invitrogen) or Apolipoprotein C3 (Stealth siRNA HSS179749, Invitrogen) using RNAi MAX (Invitrogen).

2.11. Evaluation of the affinity to the vitamin D receptor

The binding affinity of VD derivatives to the vitamin D receptor (VDR) was evaluated by using a Polarscreen Vitamin D Receptor Competitor Assay Kit, Red (Thermo Fisher Scientific, Waltham, MA). VD derivatives and 1α,25-(OH)$_2D_3$ were diluted with DMSO in 10 concentrations (5×10^{-12} M - 1.5×10^{-7} M) and added to a 384-well white plate (Aurora Microplate, Whitefish, MT). Fluorescently labeled VDR ligand/hVDR complex was added to each well and incubated at 21 °C for 2 h. The degree of fluorescence polarization of each well was measured using a PHERAstar FS (BMG LABTECH, Ortenberg, Germany). Triplicate wells were prepared for each concentration of each VD derivative. For calculation of the inhibition rate, the degree of fluorescence polarization with DMSO was set as 0%, and the degree of fluorescence polarization with the minimum plateau concentration of 1α,25-(OH)$_2D_3$ was set as 100%. The 50% inhibitory concentration