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COMPARISON OF OPIOID PROPERTIES BETWEEN D-ARG-CONTAINING DIPEPTIDES AND TETRAPEPTIDES

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Abstract—Since the D-Arg-containing dipeptides, H-Tyr-D-Arg-OMe (TDA) and H-Tyr(Et)-D-Arg-OMe, and D-Arg^2-substituted dermorphin N-terminal tetrapeptide analogues, H-Tyr-D-Arg-Phe-Gly-OEt (TDAPG) and H-Tyr(Et)-D-Arg-Phe-Gly-OEt gave different pharmacological responses *in vivo*, opioid interaction and structure–activity relationships have been investigated *in vitro*. In the isolated guinea-pig ileum assay, the tetrapeptides were potently inhibitory, their activity markedly exceeding that of the dipeptides. In particular, the first tetrapeptide had twice the activity of morphine, while the potency of the dipeptides was less than one twentieth that of morphine. Also in the opioid receptor binding assay, tetrapeptides had a higher affinity than the dipeptides. IC_{50} values of tetrapeptides were 8.46 and 23.7 nM, respectively, which were lower than that of morphine. Ethylation of the Tyr residue of TDA much increased the opioid activity whereas that of TDAPG greatly decreased it. All peptides used were extremely stable to aminopeptidase-M and carboxypeptidase-Y and had an inhibitory effect on enkephalin (EK)-degrading enzymes. From these results, it appears that the effects of the tetrapeptides are due mainly to specific interaction with opioid receptors, whereas the dipeptides do not act specifically on the opioid receptors, but are involved in non-opioid mechanisms. The resistance to enzymes and inhibitory effect of the peptides used on the EK-degrading enzymes may also account for their potent and long-lasting opioid-like activities.

Of all the peptides occurring in amphibian skin, which is a rich source of biologically active peptides, dermorphin (DM‖) has the highest peripheral and central opioid-like activity [1]. This heptapeptide, which has the sequence Tyr-Ala-Phe-Gly-Tyr-Pro-Ser amide, is notable for having an Ala in position 2 that is in the D-configuration [2]. DM is synthesized via precursor molecules which contain up to five copies of its sequence and it has been suggested that the L-amino acid could be converted to the D-isomer in the peptide linkage by a novel post-translational reaction [3]. The structure–pharmacological activity relationship studies of DM have demonstrated the crucial role of the D-Ala^2 moiety and the minimal structural requirement of the N-terminal tetrapeptide [4]. In fact, a synthetic DM analogue whose D-Ala is replaced with L-Ala is devoid of activity [4], and the activity increases from tetra to the parent heptapeptide [4,5].

In another type of endogenous opioid peptide, Tyr-Arg [6], the antinociceptive activity produced by intracisternal or i.c.v. administration can be prolonged by the use of an analogue, Tyr-D-Arg [7, 8]. This parallels the D-Ala^2 of DM and DM-like peptides.

Accordingly, we have been investigating the structure–antinociceptive activity relationships of D-Arg^2 substituted dipeptides and tetrapeptides, and have already reported that: (1) an i.c.v. administered dipeptide with its side-chain hydroxyl group ethylated [Tyr(Et)-D-Arg] showed antinociceptive activity equipotent to morphine, which was not completely antagonized with naloxone as assayed by the tail-flick and digitus-pinching tests in rats [9]; (2) T(Et)DA possessed more potent antinociceptive activity than TDA in the tail-pressure and PBQ writhing tests in mice when administered i.c.v. [10]; (3) In tetrapeptides, TDAPG showed a much more potent antinociceptive activity than T(Et)DAPG, which was as potent as morphine. The activity was diminished significantly by pretreatment with naloxone administered i.c.v. and also s.c. [11, 12]; (4) The effect of the dipeptide on spontaneous motor activity in mice was the exact opposite of that of the tetrapeptide; and (5) H-Tyr-D-Arg-Phe-Gly-OH possessed high affinity with opioid receptors [13].

On the basis of these reports, the present investigation was undertaken to determine biochemically the difference in the interaction with opioid receptors between the D-Arg-containing dipeptides and D-Arg^2-substituted tetrapeptides. In addition, we estimated the enzymatic stability to APase-M and CPase-Y, and the inhibitory effect on EK-degrading enzymes of these peptides.

MATERIALS AND METHODS

Chemicals and materials. The dipeptides [TDA and T(Et)DA], tetrapeptides [TDAPG and T(Et)DAPG] and DM were synthesized by the

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‖ Abbreviations: TDA, H-Tyr-D-Arg-OMe; T(Et)DA, H-Tyr(Et)-D-Arg-OMe; TDAPG, H-Tyr(Et)-D-Arg-Phe-Gly-OEt; T(Et)DAPG, H-Tyr(Et)-D-Arg-Phe-Gly-OEt; DM, dermorphin; EK, enkephalin; AHPA, (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoic acid; PBQ, phenylbenzoquinone; GPI, guinea pig ileum; i.c.v. intracerebroventricular(ly); i.t., intrathecal(ly); APase-M, aminopeptidase-M; Pase-Y, carboxypeptidase-Y.

conventional liquid-phase method in our laboratory. Details of the synthesis have been reported in separate papers [14, 15]. Bestatin and D-Phe-AHPA were generously donated by Dr T. Aoyagi (Institute of Microbial Chemistry, Tokyo, Japan). Captopril was a generous gift from the Sankyo Co. Ltd. (Tokyo, Japan) and morphine was from Takeda Chemical Industries (Osaka, Japan). [^{3}H]Naloxone (sp. act. 40.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and APase-M, CPase-Y and [Met^5]EK were from Protein Research Foundation (Osaka, Japan).

Isolated organ assay. The longitudinal muscle of male Hartley guinea pig (250–300 g) was mounted in an organ bath prepared for electrical stimulation (0.1 Hz, 0.5 msec, 10 V) in Krebs–Henseleit solution according to the method described by Kosterlitz *et al.* [16]. The sensitivity of the tissue preparation to opioids was checked using morphine as a standard substance. The IC_{50} values were determined with log-linear plots and were expressed in nanomolar concentrations (mean ± SE). At least four points were used in the construction of each dose–effect curve; each experiment was repeated using ileum strips from at least five animals.

Radioreceptor assay. Crude brain membranes extracted from the whole brains of male Wistar rats (150–200 g) without cerebellum were prepared using the method of Chang and Cuatrecasas [17]. The binding assay was carried out, in principle, according to the method described by Terenius [18], but included a filtration method due to Pert and Snyder [19]. For routine assay, the mixture of [^{3}H]-naloxone (100 μL, 1.0 nM final concentration), tissue preparation (100 μL) and sample solution (25 μL) was incubated at 25° for 30 min in the presence and absence of 1 μM naloxone. The incubation was terminated by filtration under vacuum over a Whatman glass fibre filter (GF/C). After filtration, the filters were washed twice with 1 mL of 5% sodium dodecyl sulfate (Sigma Chemical Co., St Louis, MO, U.S.A.) in scintillation vials for 60 min. After the addition of 7 mL of ACS II (Amersham Corp., Arlington Heights, IL, U.S.A.), radioactivity was determined using a liquid scintillation counter (Beckman LS 7800). All assays were performed in triplicate and specific binding represents the bound radioactivity displaceable with a large excess of unlabeled naloxone.

Evaluation of the stability to APase-M or CPase-Y. Peptide (2 μM) and APase-M (60 μM) were incubated in a final volume of 1 mL of 0.1 M Tris–HCl buffer (pH 7.6) at 37°. In the case of CPase-Y digestion, according to the report [20] that CPase-Y hydrolyses peptides containing basic amino acids at more alkaline regions than neutral peptides, the incubation was performed in a final volume of 1 mL of 0.1 M pyridine acetate buffer (pH 6.1) or 0.1 M Tris–HCl buffer (pH 7.6) at 25° using the same quantity of CPase-Y. At different times, 100-μL aliquots of reaction mixture were lyophilized after immediate freezing with dry ice-MeOH bath and free amino acids were measured with the amino acid analyser (HITACHI 835). After the incubation of control analysis (enzyme alone), the values of Tyr, Gly (from tetrapeptides), D-Arg (from dipeptides) and Met (from [Met^5]EK) liberated by APase-M and CPase-Y were used for the calculation of hydrolysis rates.

Table 1. Inhibitory effects of peptides on the electrically evoked contractions of the GPI

Materials used	IC_{50} ± SE (nM)	N	Relative potency (± SE)
Morphine	214 ± 27.45	12	1.00
TDA	42760 ± 4657	10	0.0056 ± 0.0006
T(Et)DA	4950 ± 514.30	10	0.0471 ± 0.0043
TDAPG	108 ± 8.09	12	2.10 ± 0.14
T(Et)DAPG	1561 ± 157.7	12	0.15 ± 0.01
DM	3.75 ± 0.18	18	59.56 ± 3.02
[Met^5]EK	151.80 ± 6.17	10	1.43 ± 0.01

Evaluation of inhibitory effects of peptides on EK-degrading enzymes. EK-degrading enzymes were extracted from the brains of male Wistar rats (150–200 g) according to the method of Gorenstein and Snyder [21]. Peptide (200 μL), solubilized enzymes (200 μL) and [Met^5]EK (5×10^{-4} M, 600 μL) were incubated at 37° for 60 min. One hundred microliters of the mixture were placed in a small round-bottomed flask (LABCONCO) at each time-point and freeze-dried quickly by a freeze drier (LABCONCO 75035). Then, 0.5 mL of 0.05 N HCl was added to stop the reaction and free amino acids were measured. The 200 μL of solubilized enzymes is an amount that causes 100% of Tyr, Phe and Met to be liberated from [Met^5]EK when incubated for 60 min. Therefore, the inhibitory effects of peptides were compared with bestatin, captopril and D-Phe-AHPA. The IC_{50} values were determined with the inhibitory percentages calculated after incubation for 60 min. No amino acid was detected even at 1 hr after the incubation of peptides tested with 200 μL of solubilized enzymes.

RESULTS

Isolated organ assay

As shown in Table 1, the IC_{50} values of TDA, T(Et)DA, TDAPE and T(Et)DAPG were 42760, 4950, 108 and 1561 nM, respectively, on the electrically induced contractions of the GPI. The inhibitory effect of TDAPG was twice that of morphine, whereas the dipeptide effect was less than one twentieth that of morphine. In dipeptides, the ethylation of Tyr residue resulted in an 8.6 times increased potency, whereas in the tetrapeptides, it reduced the potency to one fourteenth. The parent heptapeptide was the most active of all the peptides tested, with an IC_{50} value of 3.75 nM, which was about 60 times more potent than morphine. [Met^5]-EK showed equipotent activity to morphine.

Radioreceptor assay

Table 2 shows the IC_{50} values and affinity ratios of peptides in the opioid receptor binding assay using [^{3}H]naloxone as a tracer ligand.

Table 2. Comparison of potency in competing with the binding of [^{3}H]naloxone using rat brain homogenates

Materials used	IC_{50} (nM)	Affinity ratio
Morphine	27.50	1
TDA	7000	0.0039
T(Et)DA	9100	0.003
TDAPG	8.46	3.25
T(Et)DAPG	23.7	1.16
DM	3.85	7.14
[Met^5]EK	13.15	2.09

TDAPG was found to possess three times the affinity of morphine, while the dipeptides had affinity less than 0.4% of that of morphine. It is of interest that the T(Et)DAPG, which was not as active in the GPI assay, showed equipotent affinity to morphine.

In contrast to the results with the isolated organ assay, the O-alkylation of Tyr residue reduced the affinity for opioid receptors both in the dipeptides and tetrapeptides.

DM also showed relatively high affinity for the opioid-binding site, which was 7.14 times the affinity of morphine and 2.2 times that of TDAPG.

Evaluation of stability of peptides to APase-M or CPase-Y

The stability of the peptides tested to enzymatic degradation by APase-M or CPase-Y is summarized in Tables 3 and 4. Incubated for 1 hr with APase-M, T(Et)DA and T(Et)DAPG were hydrolysed 2.1 and three times more slowly than TDA and TDAPG, respectively. The Tyr release from T(Et)DA or T(Et)DAPG was 10 or 8% slower than that from the respective parent peptide after incubation for 24 hr. Even at 48 hr, T(Et)DA showed 1.4 times and T(Et)DAPG 3.6 times more resistance to APase-M than the respective parent peptide. In this connection, 94% of Tyr was cleaved from H-Tyr-D-Ala-Phe-Gly-OH by 48 hr and nearly all of the Tyr was released from [Met^5]EK within 1 hr (Table 3).

It became clear that the side chain hydroxyl group-ethylated peptides were also more stable to CPase-Y under both sets of conditions. When these peptides were treated with CPase-Y at pH 6.1, T(Et)DA and T(Et)DAPG were hydrolysed approximately 1.5 and 1.3 times more slowly than TDA and TDAPG, respectively, during each incubation period (Table 4). At pH 7.6, they were cleaved in the same manner, whereas CPase-Y was not as selective for Gly as for D-Arg or Met (Table 4). Moreover, the Tyr-L-Arg bond of H-Tyr-L-Arg-OMe or H-Tyr-L-Arg-Phe-Gly-OH was rapidly and almost completely hydrolysed by both APase-M and CPase-Y within 3 hr (data not shown).

Evaluation of inhibitory effects of peptides on EK-degrading enzymes

As seen in Table 5, in the inhibition of Tyr release at the N-terminal of [Met^5]EK, that is, the inhibitory effect on APase, TDAPG had an IC_{50} value of 8.30 μM. Though the IC_{50} values tended to be increased by the ethylation of Tyr residue, there was no significant difference to those of the parent peptides. Bestatin, a specific APase inhibitor [22], showed a potent selectivity for APase, as reported previously [23]. On the other hand, dipeptidyl CPase (enkephalinase A) [24] and angiotensin I-converting enzyme [25] have been introduced as EK-degrading enzymes that cleave its Gly^3-Phe^4 bond. Of the four peptides, TDAPG had the lowest IC_{50} value, which was 2.45 μM. Comparing the four IC_{50} values, the inhibitory effects seemed to be approximately the same and no significant difference owing to the ethylation of Tyr residue was recognized. The ED_{50} for captopril, generally known as a specific angiotensin I-converting enzyme inhibitor [26], was 0.38 μM and that for D-Phe-AHPA, reported as a enkephalinase A inhibitor [27], was more than 100 μM. In addition, no amino acid was detected when any of the peptides was incubated with the membrane fraction, whereas [Met^5]EK was cleaved completely within 10 min.

DISCUSSION

In this paper, we have demonstrated that TDA, T(Et)DA, TDAPG and T(Et)DAPG interacted with opioid receptors using the GPI and the radioreceptor

Table 3. Stability of each peptide to enzymatic degradation with APase-M

Peptides used	APase-M digestion: Liberation of Tyr (%) (hr) 1	5	24	48
TDA	10.1 ± 2.15	17.3 ± 2.98	29.3 ± 0.98	40.7 ± 0.40
T(Et)DA	4.5 ± 1.50	8.9 ± 3.64	19.8 ± 3.20	28.6 ± 0.60
TDAPG	0.6 ± 0.40	6.6 ± 1.08	15.8 ± 2.42	21.2 ± 2.36
T(Et)DAPG	0.2 ± 0.20	2.2 ± 0.86	7.8 ± 1.77	9.3 ± 1.44
H-Tyr-D-Ala-Phe-Gly-OH	15.0 ± 1.84	37.4 ± 2.38	81.8 ± 1.74	94.0 ± 1.52
[Met^5]EK	99.4 ± 0.40	—	—	—

Values are means ± SE.

Table 4. Stability of each peptide to enzymatic degradation with CPase-Y

Peptides used	CPase-Y digestion: liberation of Gly (%) pH 6.1			pH 7.6	
	1	3 (hr)	5	1	3 (hr)
TDA*	55.2 ± 3.76	72.0 ± 3.90	85.4 ± 4.38	68.2 ± 1.53	80.4 ± 2.86
T(Et)DA*	37.6 ± 2.38	49.6 ± 2.32	59.0 ± 2.21	50.2 ± 1.77	60.6 ± 2.29
TDAPG	71.2 ± 1.43	86.2 ± 1.50	94.8 ± 0.97	12.0 ± 1.38	28.4 ± 1.21
T(Et)DAPG	55.6 ± 1.72	68.8 ± 1.16	80.4 ± 1.33	6.8 ± 1.07	20.6 ± 1.33
H-Tyr-D-Ala-Phe-Gly-OH	86.0 ± 1.92	91.4 ± 3.57	98.2 ± 0.92	16.2 ± 2.42	39.2 ± 1.88
[Met^5]EK†	99.2 ± 0.49			98.0 ± 0.95	

* Liberation of D-Arg was measured.
† Liberation of Met was measured.
Values are means ± SE.

Table 5. Inhibitory effects of peptides on EK-degrading enzymes

Materials used	Cleavage of Tyr^1-Gly^2 IC_{50} (μM)	Cleavage of Gly^3-Phe^4 IC_{50} (μM)
Morphine	>100	>100
TDA	21 (11.05–39.09)	5.2 (2.77–9.78)
T(Et)DA	38 (20–72.2)	7.6 (4.02–14.29)
TDAPG	8.3 (4.46–15.44)	2.45 (1.28–4.7)
T(Et)DAPG	29 (14.95–56.26)	4.9 (2.58–9.31)
Bestatin	0.1 (0.06–0.17)	<0.05
Captopril	3.3 (1.98–5.51)	0.38 (0.23–0.64)
D-Phe-AHPA	>100	>100

assays. Good agreement was found between the results obtained by the two assays. TDAPG showed high opioid activity surpassing that of morphine and [Met^5]EK in both assays. Since it is reported that the myenteric plexus of the GPI has μ- and κ-receptors [28] and naloxone cannot be used reliably as a selective μ-antagonist at concentrations higher than half its K_1 value for binding at the κ- and δ-sites, for instance at not more than 10 nM in bioassays with the GPI [29], the relationship between the peptides tested and opioid receptors might be clarified further by bioassays with the vasa deferentia of rabbits which have only κ-receptors or radioreceptor assays with [D-Ala^2, MePhe^4, Gly-ol^5] EK as a selective μ-ligand [30] and U-69593 as a selective κ-ligand [31].

The results (Tables 3 and 4) revealed that these peptides were rather stable not only to APase-M or CPase-Y but also to the extracted enzymes from the rat brain. It seems clear that the enzymatic stability results in the prolonged antinociceptive duration we have reported previously [10, 12]. Furthermore, it was demonstrated that these peptides had inhibitory effects on the EK-degrading enzymes. Some peptidase inhibitors have been found to possess antinociceptive effects themselves, e.g. bestatin [32] and D-Phe-AHPA [27], or facilitate the antinociceptive activities of other materials, e.g. captopril [33, 34]. Zhang *et al.* [35] have suggested that the peptidase inhibitors protect endogenous [Met^5]EK from inactivation by endopeptidases and that the accumulation of [Met^5]EK close to the receptor may elicit an antinociceptive response. This hypothesis might be applicable to the peptides we have studied.

In the structure–pharmacological activity relationship, O-alkylation of the Tyr residue enhances by 8.6 times the opioid activity of TDA in the GPI assay. Also in the radioreceptor assay, the side-chain hydroxyl group-ethylated tetrapeptide, T(Et)DAPG had a lower affinity for opioid receptors than the parent tetrapeptide. As regards the enzymatic stability, O-alkylation of the Tyr residue increased the resistance to APase-M or CPase-Y in both the di- and tetrapeptides tested. These results support our previous results. We have reported that, in Tyr-Arg, O-alkylation of the Tyr residue and substitution of Arg with homo-Arg contribute greatly to the higher and longer lasting activity [9]. Our previous findings [10–12] with the dipeptides [TDA vs T(Et)DA] show that O-alkylation of the Tyr residue enhances and prolongs the activity. However, in tetrapeptides [TDAPG vs T(Et)DAPG], despite the prolonged duration, O-alkylation not only causes the activity to decrease but also increases the naloxone resistance.

As seen in Table 5, the inhibitory effect on EK-degrading enzymes tended to be diminished by ethylation of the Tyr residue. It seems that APase can not recognize the amino radical because Tyr^1 is modified by O-alkylation. In our previous report, the antinociceptive effect of T(Et)DAPG was 3.4 times in the tail-pressure test and 2.8 times in the PBQ writhing test more potent than that of morphine in mice [10]. There is no obvious difference in opioid receptor binding site affinity between T(Et)DAPG and morphine, therefore the inhibitory effect may account for the potent antinociceptive activity of T(Et)DAPG. Moreover, the prolonged duration may originate from resistance to enzymes.

DM was found to possess more pronounced opioid-like activity than any of the naturally occurring or most of the synthetic opioid peptides. It has been reported already that the D-Ala^2 moiety and N-terminal tetrapeptide sequence of DM were of crucial importance for its activity [4]. We, however, suggested that D-Ala^2 could be replaced with D-Arg with great success as reported with the EK analogues, indicating that the D-Ala residue as a D-amino acid at the second position is not always necessary for opioid activity [36]. On the other hand, we also demonstrated that the N-terminal tripeptide in the structure of [D-Arg^2]DM must be the active core for the manifestation of the full intrinsic opioid-like antinociceptive activity, because the relative potencies of [D-Arg^2]DM, [D-Arg^2]DM(1-6), [D-Arg^2]DM(1-5), [D-Arg^2]DM(1- 4), [D-Arg^2]DM(1-3) and [D-Arg^2]DM(1-2) to morphine were 52.2, 96.0, 59.4, 116.5, 5.7 and 0.025, respectively, in the tail pressure test in mice [37].

We have reported that i.c.v. administered TDAPG had an antinociceptive activity 96, 48 and 166 times more potent than that of morphine in the tail-pressure, PBQ writhing and tail-flick tests in mice, respectively [10, 12]. In addition, it showed five, six and 11 times more potent antinociceptive activity than did morphine in each assay even after s.c. administration [11]. These potent antinociceptive activities were antagonized completely by pre-treatment with naloxone.

There are some differences in pharmacological response both *in vivo* and *in vitro* between dipeptides [TDA and T(Et)DA] and tetrapeptides [TDAPG and T(Et)DAPG]. As we have reported, the antinociceptive effects of tetrapeptides were significantly reversed even by low doses of naloxone, while high doses of naloxone were required to antagonize those of dipeptides [10]. For the effect on locomotor activity in mice, the tetrapeptides elicited locomotor hyperactivity following an initial locomotor suppression, while spontaneous motor activity was lowered by the dipeptides throughout the observation period, which was scarcely antagonized by naloxone [12]. These results led us to conclude that the dipeptides must act in a different manner to that of the tetrapeptides.

The previous and present results indicate that while the effects of the tetrapeptides used must be due to interaction with opioid receptors, the effects of the dipeptides used may be mediated non-specifically through various systems including the opioid receptors. The opioid interaction would be substantiated further by radioreceptor assay using other opioid ligands selective for each receptor. Except for the interaction with opioid receptors, Met-EK or other neurotransmitters-releasing action by Tyr-Arg has already been demonstrated by Shiomi *et al.* [38] and Janicki *et al.* [39].

Our previous data [40] suggest that the mechanism of the antinociceptive effect of [D-Arg^2]DM(1-4) differs from that of morphine and that it may possess higher affinity for opioid receptors than does morphine especially in the spinal cord: the dose ratios (i.c.v./i.t.) of [D-Arg^2]DM(1-4) and morphine, which were calculated from the AD_{50} (antinociceptive dose) values, were 5.8 and 1.46, respectively. On the other hand, a derivative of Tyr-Arg showed the most potent antinociceptive effect when administered into the lateral cerebroventricle in the tail-flick test in rats and into the 3rd cerebroventricle in the digitus pinching test in rats, while the administration of this dipeptide into the spinal subarachnoid space was without effect; morphine showed the most potent antinociceptive effect when administered i.t. [9]. From these points of view, the differences in mechanism between the dipeptides and tetrapeptides seem to be clear.

As mentioned above, these tetrapeptides possess potent antinociceptive activity when administered i.c.v. or s.c. Even [D-Arg^2]DM(1-4) administered orally displays equipotent antinociceptive activity to morphine with a much longer duration than that of morphine [40]. No less important is the fact that the intensity of physical dependence is much weaker with the D-Arg^2-containing tetrapeptide derivatives than with morphine [41]. Therefore, on balance, there is a strong possibility that they might be exploited clinically. Therefore, we are investigating how to prevent their digestion and make them be absorbed efficiently from digestive tracts, other mucosae or skin, i.e. how to improve their bioavailability.

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