Int J Diabetes & Metab (2011) 19:69-74

Dipeptidyl peptidase IV and adenosine deaminase inhibition by Armenian plants and antidiabetic drugs

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Abstract
Dipeptidyl peptidase IV (DPP-IV) converts glucagon-like peptide-1 (GLP-1)(7-36), responsible for glucose tolerance into inactive GLP-1(9-36). The pathogenic role of elevated adenosine deaminase (ADA) activity at hyperglycemic conditions was also suggested. Hence, the inhibition of both DPP-IV and ADA would be beneficial in the treatment of diabetes mellitus. Screening of the ability of water extracts of 23 Armenian Highland native plants to inhibit the enzymatic activity of DPP-IV and ADA was performed. Over 40% of DPP-IV and ADA activity inhibition was observed by 1 percent extracts from between 6 and 12 plants. Among them, the most effective inhibitor for DPP-IV inhibitor was the extract from the leaves of sea-buckthorn *(Hippophaë rhamnoides)* (80.5% ± 3.3; IC50 = 2.5mg/ml ± 0.03); and for ADA: from St. John's wort (*Hypericum perforatum*) (88.3% ±7.4; IC50 = 1.99mg/ml ± 0.3). Very slight ability of the known antidiabetic drugs, diabeton, glurenorm, siofor, glucovance, and the medicinal herbal mixture Arfazetin-A to inhibit the enzymes under study was observed. Moreover, the extracts from clove, cinnamon, green and black tea were highly effective in inhibiting DPP-IV and ADA. The obtained results show that selected Armenian Highland plants: blackberry, melilot; oregano, St. John's wort, sea-buckthorn leaves could be used, in combination with other antidiabetic drugs, for the treatment of diabetes mellitus.

Keywords: *type 2 diabetes mellitus, plant extracts, enzyme inhibition, dipeptidyl peptidase IV,adenosine deaminase, antidiabetic drugs*

Introduction

Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are incretin hormones, released from intestinal L- and K-cells in response to the ingestion of nutrients.[1] They are responsible for normal glucose tolerance after meal intake. GLP-1 (7-36) is characterized as the most potent insulinotropic hormone, and is considered a therapeutic agent for the treatment of type 2 diabetes.[2] The continuous infusion of this peptide decreases plasma glucose and improves β-cells function. However, active GLP-1 (7-36) is converted into inactive GLP-1 (9-36) by dipeptidyl peptidase IV.[3]

Dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) is a unique serine protease, removing N-terminal dipeptides from polypeptides and proteins, which contain proline or alanine at the penultimate position.[4,5] Because the enzyme inactivates GLP-1 (7-36), the necessity to develop alternative strategies, aiming to prolong the antidiabetic activity of the hormone has become a prime objective.[6] One of the approaches to prolong the half-life of GLP-1 is the application of DPP-IV inhibitors.[1] At first, Pauly and colleagues postulated the link between the benefits of DPP-IV inhibition and enhancement of the incretin effect.[7] Indeed, inhibition of DPPIV with vildagliptin improved the glycemic control of type 2 diabetes by enhancing the activity of GLP-1, GIP, and pituitary adenylate cyclase-activating peptide with concomitant improvement in □-cell function.[8] In clinical studies, DPP-IV inhibitors show improved efficacy over time.[9] Therefore, at present, the inhibitors of DPP-IV are under development in preclinical and clinical studies as potential drugs for the treatment of type 2 diabetes.[10,11,12] However, since DPP-IV is involved in the metabolism of a vast number of vital substrates (neuropeptides, chemokines, cytokines, etc.), the question remains as to the safety of its inhibition for life.[13] A greater potential may lie in combinatorial treatment with other antidiabetic drugs.

It is known that hyperglycemic conditions lead to increased activity of adenosine deaminase (ADA, EC 3.5.4.4), involved in the metabolism of purine nucleosides, and playing an essential roles in the immune, nervous and vascular systems.[14]The serum level of ADA in diabetes mellitus patients reliably increased compared to those of healthy subjects. It was suggested that the increased ADA activity may be a reflection of altered immunity in the pathology of type 2 diabetes mellitus.[15] The pathogenic role of elevated ADA activity at type 2 diabetes was suggested.[16] Consequently, the inhibition of ADA would also be beneficial in diabetes, and the regulation of both DPP-IV and ADA activities can be considered as possible tools in the treatment diabetes mellitus.

Medical plants play an important role in the management of diabetes mellitus. The effects of these plants may delay the

Received on: 16/04/2010
Accepted on: 21/06/2011

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development of diabetic complications and correct the metabolic abnormalities. Some of the new bioactive drugs isolated from plants showed antidiabetic activity with more efficacy than hypoglycaemic agents used in clinical therapy.[17] It is worth noting that the investigations of the last years manifested the inhibition of DPP-IV and ADA activities by plant extracts.[18,19,20] The administration of aqueous extract of sea-buckthorn seed residues[21] had shown hypoglycemic, hypotriglyceridemic and antioxidant effects in streptozotocin-induced diabetic rats, suggesting that its supplementation can be useful in preventing diabetic complications induced by hyperlipidemia and oxidative stress.

In the present study we tested the inhibitory ability of aqueous extracts of 28 plants of different origin, traditionally used in folk medicine and/or as food, aiming to offer natural origin of DPPIV and ADA inhibitors instead of using chemically synthesized medications. Hopefully, the obtained results will allow the consideration of some Armenian Highland plants in the treatment of diabetes mellitus.

MATERIALS AND METHODS

Chemicals

The enzymes, DPP-IV and ADA, were purified in our laboratory from kidney cortex and lung, respectively, as described earlier.[22,23] The substrates Gly-Pro p-nitroanilide toluenesulfonate salt (GPN-Tos) and adenosine were purchased from Sigma Ltd. USA. The antidiabetic drugs were purchased at state drugstores. Other reagents were of the highest purity.

Plant material

The plants, usually used as food and in folk medicine were collected from the ecologically pure flanks of Aragats and Geghama mountains and in Ararat valley of Armenia. A voucher specimen has been deposited at the herbarium of the Botanical Department of Yerevan State University (Dr. Narine Zaqaryan). The green, Ahmad Tea, and black, GENEX N 1, pure Ceylon tea, cinnamon and clove were of commercial samples.

Extraction

The leaves, flowers and/or overground parts were dried in the shade and ground. The five-percent (w/v) extracts of the dry plants in distilled water were prepared by incubating in water bath at 96˚C for 30 min. After cooling, the extracts were filtered through a sterile cheese cloth and glass filter and used within 24 hours.

Enzyme assays

The activity of DPP-IV was assayed using GPN-Tos as a substrate, as described previously[22]. Briefly, 0.5 ml of assay mixture contained 40 mM K, Na-phosphate buffer, pH 7.5, and an enzyme sample. The reaction was initiated by adding the substrate up to a final concentration of 0.24 mM, and stopped by adding 0.2 M acetic buffer, pH 5.5. The differential absorption at 390 nm was registered against an identical mixture without the enzyme, and the amount of depleted p-nitroaniline was evaluated from its extinction coefficient at this wavelength of 9.9 mM/cm^{-1}.[24] The ADA activity was assayed by determination of ammonia, liberated in the reaction of adenosine deamination, by phenol-hypochlorite colorimetric method, described earlier.[23]

The influence of a plant extract on the enzymatic activity

The enzymes were pre-incubated with the respective extract for 15 min before the initiation of the reaction by adding the substrate. The activity without any extract was taken as 100 %; the activity in the presence of a plant extract was expressed as percentage of the activity without any extract. The difference of percentages between the two activities showed the inhibition value.

The influence of antidiabetic drugs on the enzymatic activity

A tablet of a drug was crushed to fine powder; a definite amount was weighed and suspended in 1 ml of 0.2 M acetic buffer, pH 5.5. The suspensions were incubated for 1 hour at room temperature, diluted with distilled water to 3 ml, centrifuged at 12000g for 4 min. The obtained solutions were used for monitoring the drug influence on the activities of enzymes under study as described for plant extracts.

Evaluation of IC50

The activity of enzymes was determined at the presence of different amounts of plant extracts or drug solutions. The dependence of inhibition value on the inhibitor amount in the assay mixture was defined, and IC50 was determined as dry material concentration (mg/ml), necessary for inhibiting the enzyme activity down to 50% of the initial, using the computer program GraFit Version 5 (Leatherbarrow, 2001, Erithacus Software Ltd., Horley, U.K).

Statistical analysis

The statistical analyses of the data were performed using the computer program InStat version 3 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The data are presented as a mean of triplicate assays ± SE.

Results and Discussion

Inhibition of DPP-IV and ADA activities in the presence of extracts from Armenian Highland plants

In Table 1, the inhibition of DPP-IV and ADA activities are presented in the presence of one-percent extracts of 23 plants, collected from the Armenian Highland. The data in this Table show that the activity of DPP-IV was inhibited by water extracts of the plants in a rather broad range from 8.1 ± 0.2 for warmot *(Artemisia absinthium)* to 80.5 % ± 3.3 for sea-buckthorn leaves *(Hippophaë rhamnoides).* The activity of ADA is inhibited more effectively within the range from 17.8 % ± 1.2 for elecampane *(Inula helenium)* to 88.3% ±7.4 for St. John's wort (*Hypericum perforatum*).

Inhibiting of DPP-IV activity over 40% has been observed with the extracts from 6 plants: immortelle (*Helichrysum rubicundum),* sea-buckthorn leaves (*Hippophaë rhamnoides),* St. John's wort (*Hypericum perforatum),* melilot (*Melilotus officinalis),* oregano (*Origanum vulgare),* young and mature leaves of blackberry (*Rubus caesius).*

Table 1. Inhibition of DPP-IV and ADA activities in the presence of one-percent plant extracts

Plant species			Activity inhibition (% of control)*	
Latin name	Common name		ADA	DPPIV
Achillea millefolium	thousand-leaf		40.7 ± 0.2	9.4 ± 0.4
Arctium lappa	Burdock		75.4 ± 6.5	13.3 ± 0.7
Artemisia absinthium	Warmot		38.2 ± 0.3	8.1 ± 0.2
Chamomillae recutita	Chamomile		21.6 ± 0.7	36.6 ± 0.8
Cichorium intybus	Chicory		47.5 ± 0.9	21.1 ± 0.3
Helichrysum rubicundum	Immortelle		34.4 ± 0.8	50.2 ± 1.1
Hippophaë rhamnoides	sea-buckthorn, leaves		46.3 ± 1.7	80.5 ± 3.3
Hypericum perforatum	St. John's wort		88.3 ± 7.4	47.7 ± 0.9
Inula helenium	Elecampane		17.8 ± 1.2	20.2 ± 0.2
Leonurus cardiaca	Motherwort		28.5 ± 0.5	20.3 ± 0.6
Melilotus officinalis	Melilot		80.2 ± 3.9	40.5 ± 3.1
Mentha piperita	Mint		35.4 ± 1.1	38.2 ± 1.4
Origanum vulgare	Oregano		84.6 ± 3.9	44.9 ± 0.6
Onopordon Acanthium L.	Thistle		71.5 ± 1.9	19.1 ± 0.2
Plantago major	Plantain		50.8 ± 7.8	19.9 ± 0.7
Poligonum aviculare	pigweed		25.3 ± 0.5	24.4 ± 0.6
Rosa canina	rose hip		36.9 ± 1.1	16.6 ± 0.5
		Berry	47.7 ± 2	31.1 ± 1
Rubus caesius	blackberry	Young leaves	81.1 ± 4.1	72.7 ± 5.1
		mature leaves	51.3 ± 1.7	49.9 ± 1.2
Rúmex confértus wild	sorrel wild		62.4 ± 1.5	37.4 ± 1.6
Taraxacum officinale	Dandelion		39.3 ± 2.3	17.1 ± 0.5
Teucrium polium	Germander		29.5 ± 0.5	15.9 ± 0.7
Urtica dioica	Nettle		32.1 ± 0.5	27.7 ± 1.1
Zea mays	corn stigma		72.2 ± 1.5	22.2 ± 0.6

* Data represent the mean of triplicate assays ± standard error of the evaluated parameter. DPP-IV = Dipeptidyl peptidase IV; ADA = adenosine deaminase

Table 2: IC50* values for inhibition of DPPIV by the selected plant extracts

Plant species		IC 50, (mg/ml)
Latin name	Common name	DPPIV
Hippophaë rhamnoides	sea-buckthorn, leaves	2.5 ± 0.03
Rubus caesius	blackberry, young leaves	6.7 ± 0.5
Helichrysum rubicundum	Immortelle	9.4 ± 1.1
Origanum vulgare	Oregano	11.8 ± 0.5
Mentha piperita	Mint	13.6 ± 0.7
Hypericum perforatum	St. John’s wort	14.3 ± 0.2
Rúmex confértus wild	sorrel wild	16.3 ±1.3
Rubus caesius	blackberry, berry	19.3 ± 2
Inula helenium	Elecampane	20 ± 2.1
Chamomillae recutita	Chamomile	21 ± 0.2
Citrus limonia	lemon peel	26.9 ± 1.6
Urtica dioica	Nettle	29.6 ± 5.4
Plantago major	Plantain	31.3 ± 6.5
Arctium lappa	Burdock	32.3 ± 4.1

***** IC50 is expressed as the concentration of dried plant in the assay mixture calculated from the amount of 5 % (w/v) extract, necessary for inhibiting the enzyme activity down to 50 % of the initial. DPP-IV = Dipeptidyl peptidase IV. Data represent the mean of triplicate assays ± SE.

Inhibiting of ADA activity over 40 % has been observed for the extracts from 12 Armenian plants: thousand-leaf (*Achillea millefolium L);* burdock (*Arctium lappa);* chicory (*Cichorium intybus);* sea-buckthorn leaves (*Hippophaë rhamnoides)*; St. John's wort (*Hypericum perforatum)*; melilot (*Melilotus officinalis)*; thistle *(Onopordon Acanthium L.)*; oregano (*Origanum vulgare)*; plantain *(Plantago major)*; berry and leaves of blackberry (*Rubus caesius);* horse sorrel (*Rúmex confértus wild*); corn stigma (*Zea mays).*

The high inhibiting activity of both the enzymes had been observed with the extracts from young leaves of blackberry (*Rubus caesius):* 72.7 % ± 5.1 and 81.1% ± 4.1, for DPP-IV

Table 3: IC50* values for inhibition of ADA by the selected plant extracts

Plant species		IC 50 (mg/ml)
Latin name	Common name	ADA
Hypericum perforatum	St. John's wort	1.99 ± 0.3
Origanum vulgare	Oregano	2.78 ± 0.2
Melilotus officinalis.	Melilot	3.63 ± 0.3
Rubus caesius	blackberry, young leaves	5.23 ± 0.2
Onopordon sp.	Thistle	5.54 ± 0.3
Zea mays	corn stigma	5.97 ± 0.2
Rúmex confértus wild	sorrel wild	6.3 ± 0.7
Arctium lappa	Burdock	7.17 ± 0.4
Plantago major	Plantain	9.42 ± 0.2
Rubus caesius	blackberry, mature leaves	9.7 ± 0.6
Rubus caesius	blackberry, berry	10.5 ± 2.2
Hippophaë rhamnoides	sea-buckthorn, leaves	10.8 ± 0.7
Achillea millefolium	thousand-leaf	12.2 ± 0.6
Artemisia absinthium	Warmot	13.7 ± 0.3
Mentha piperita	Mint	14.7 ± 1.6
Taraxacum officinale	Dandelion	14.7 ± 1.4
Urtica dioica	Nettle	14.9 ± 1.5

***** IC50 is expressed as the concentration of dried plant in the assay mixture calculated from the amount of 5 % (w/v) extract, necessary for inhibiting the enzyme activity down to 50 % of the initial. Data represent the mean of triplicate assays ± SE.ADA = adenosine deaminase

and ADA activities, respectively. Significant are also the inhibiting abilities of both DPP-IV and ADA of extracts from: melilot (*Melilotus officinalis)* (respectively, 40.5 % ± 3.1and 87.2 % ± 3.9); oregano (*Origanum vulgare) (*44.9 % ± 0.6 and 84.6 % ± 3.9); sea-buckthorn leaves (*Hippophaë rhamnoides)* (80.5 % ± 3.3 and 46.3 % ± 1.7); St. John's wort (*Hypericum perforatum)* (47.7 % ± 0.9 and 77.3 % ± 7.4); and mature leaves of blackberry (*Rubus caesius)* (49.9 % ± 1.2 and 51.3 % ± 1.7).

In Tables 2 and 3, the values of IC50 for inhibition of DPP-IV and ADA, respectively, by the selected plant extracts are presented. Based on the amount of five-percent (w/v) extract, necessary for inhibiting the enzyme activity down to 50 % of the initial, IC50 is expressed as the concentration of dried plant in the assay mixture.

The value of this parameter for DPP-IV inhibition is rated from 2.5 mg/m l± 0.03 (sea-buckthorn leaves, *Hippophaë rhamnoides)* to 32.3 ± 4.1 mg/ml (burdock, *Arctium lappa*). This parameter for ADA is rated from 1.99 ± 0.3 mg/ml (St. John's wort, *Hypericum perforatum)* to 14.9 ± 1.5 mg/ml (nettle, *Urtica dioica*).

Inhibition of DPP-IV and ADA by tea and some spicery, recommended for type 2 diabetes mellitus patients

In Table 4, the parameters of inhibition of DPP-IV and ADA activities by extracts from tea and some spicery are presented.

It is interesting, that significant inhibition of the activity of both enzymes (more pronounced for DPP-IV) has been observed for one-percent extracts from the spicery usually recommended for type 2 diabetes mellitus patients: clove, 92.6% ± 1.3 for DPP-IV activity (IC50 1.25 mg/ml ± 0.05), and 64.4% ± 2.1 for ADA activity (IC50 6.23 mg/ml ± 0.43); and cinnamon, respectively, 55% ± 0.8 (IC50 10 mg/ml ± 0.8), and 49.1% ± 0.5 (IC50 10.2 mg/ml ± 0.6).

The commercial tea preparations, especially the green tea, also were highly effective in inhibiting these enzymes. The inhibition is more pronounced in case of DPP-IV. The DPP-IV and ADA activities were inhibited with the one-percent extract from black tea by 74.1 % ± 2.8 (IC50 6.4mg/ml ± 0.5) and 53.3% ± 0.4 (IC50 8.7mg/ml ± 0.3); and from green tea by 85.2% ± 1.9 (IC50 5.3mg/ml ± 0.4) and 81.4% ± 3.3 (IC50 4.03mg/ml ± 0.2), respectively.

Inhibition of DPPIV by antidiabetic drugs

Since the inhibitors of DPP-IV are shown as normalizing agents for glycemic deviation of type 2 diabetes, some interest arises as to the ability of traditional antidiabetic agents to inhibit DPP-IV. The earlier investigations have shown that some pharmacological agents (metformin, pioglitazone, and glyburide) diminish DPP-IV activity *in vivo* as well ex vivo, but not *in vitro*.[25] For example, DPP-IV activity in mouse plasma was concentration-dependently lowered after metformin administration, generating IC50 values of 38 μM for normal mice, and 29 μM for ob/ob mice.[26] However, *in vitro* studies [27] conclusively indicated that metformin did not act directly on DPP-IV. DPP-IV inhibiting ability of 6 drugs from 4 classes of antidiabetic drugs had been studied.[28] All of them possessed the ability to inhibit human plasma DPP-IV with different efficacies, the strongest being nateglinide. The authors suggested the usefulness of combination of nateglinide and GLP-1 in the treatment of diabetes therapy.

In the present work we also studied the ability of 4 traditional antidiabetic drugs: diabeton (Les Laboratoires Sevier, France, active ingredient gliklazid, 80 mg), glurenorm (Boehringer Ingelheim, Germany, active ingredient gliquidone, 30 mg), siofor (Berlin-Chemie Menarini, active ingredient metformin, 500 mg), and glucovance (Merck Sante s.a.s., France, active ingredients: metformin, 500 mg, and glybenclamide, 5 mg) to inhibit the enzymatic activities both of DPP-IV and ADA in *in vitro* conditions. In our study, diabeton and glurenorm failed to affect both enzymes. The inhibition by siofor and glucovance, calculated for the amount of metformin in these drugs, resulted in too high values of IC50 to consider physiologically significant. IC50 for DPP-IV inhibition by metformin in siofor and glucovance was 0.23 M ± 0.02 and 0.36 M ± 0.06, respectively; and IC50 for ADA inhibition by metformin in glucovance was 0.54 M ± 0.06.

Besides, a medicinal herbal mixture Arfazetin-A is recommended as antidiabetic remedy because it possesses hypoglycemic effect and intensifying the glycogen producing function of liver. It is produced by AR Phito-Farm Co. Ltd, Republic of Armenia, and contains dried extract of *Eleutherococcus senticosus Maxim, Vaccinium myrtilius L., Phaseolus vulgaris L., Flores Chamomillae,*

Table 4: Inhibition of DPPIV and ADA by some spicery, recommended to type 2 diabetes mellitus patients

Spicery	DPPIV		ADA	
	Inhibition by one-percent extract (% of control)	IC50* (mg/ml)	Inhibition by one-percent extract (% of control)	IC50* mg/ml
cinnamon	55 ± 0.8	10 ± 0.8	49.1 ± 0.5	10.2 ± 0.6
clove	92.6 ± 1.3	1.25 ± 0.05	64.4 ± 2.1	6.23 ± 0.43
green tea	85.2 ± 1.9	5.3 ± 0.4	81.4 ± 3.3	4.03 ± 0.8
black tea	74.1 ± 2.8	6.4 ± 0.5	53.3 ± 0.4	7 ± 0.3.2

IC50 is expressed as the concentration of dried plant in the assay mixture calculated from the amount of 5 % (w/v) extract, necessary for inhibiting the enzyme activity down to 50 % of the initial. Data represent the mean of triplicate assays ± SE. DPP-IV = Dipeptidyl peptidase IV; ADA = adenosine deaminase

Herba Hiperici, *Fructus Rosae*, *Eguisetum Arvence L*., *Sambucus nigra L.* We also studied the ability of this mixture to inhibit DPP-IV and ADA activities *in vitro*. One percent water extract of Arfazetin-A prepared as described in Materials and Methods inhibited the enzymes insignificantly: DPP-IV was inhibited by 22% ± 1.5; and ADA by 15% ± 1.2.

All of the above stated evidence showed that the use of DPP-IV inhibitors, in combination with antidiabetic drugs, is strongly recommended for the management of type 2 diabetes mellitus,. It is noteworthy that among the existing antidiabetic drugs, JANUMET, along with metformin hydrochloride, contains sitagliptin, an active inhibitor of DPP-IV.

Conclusions

At present, the inhibition of DPP-IV is considered as a potential approach in the treatment of type 2 diabetes. Moreover, the traditional antidiabetic drugs such as diabeton, glurenorm, siofor, and glucovance and many others as well as the medicinal herbal mixtures such as Arfazetin-A did not inhibit DPP-IV *in vitro*. If they do, the inhibition is insignificant.

The results obtained in our work showed that water extracts of several Armenian Highland plants including blackberry, melilot; oregano, St. John's wort, sea-buckthorn leaves, etc., effectively inhibit DPP-IV and ADA and could be recommended for combined use with other antidiabetic drugs in the treatment of type 2 diabetes.

The observed effective inhibition of the studied enzymes by the extracts from clove and cinnamon, usually recommended for type 2 diabetes mellitus patients, as well as by green and black teas, proved that they are of value to diabetic patients.

Acknowledgement

This work was supported by grant No. 442 from the Ministry of Education and Science of Armenia.

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