云 南 植 物 研 究 2003，25 (6)：724～729
Acta Botanica Yunnanica

从天然提取物或分离部位中以碳酸酐酶 II 为靶点的抗骨质疏松活性筛选*

付 祥[1]，谭宁华[1**]，姜立花[1]，贾锐锐[1]，嵇长久[1]，
LANDRO James[2]，FEKETE Alexander[2]，MUELLER Hartwig[3]，
HENKEL Thomas[3]
（1 中国科学院昆明植物研究所植物化学与西部植物资源持续利用国家重点实验室，云南 昆明 650204；
2 Bayer Corporation，IRT，West Haven CT 06516，美国；3 Bayer AG，PH－R－EU，D－42096 Wuppertal，德国）

摘要：目的是以碳酸酐酶 II（CA II）为靶点筛选其抑制剂，以期寻找抗骨质疏松活性样品。实验是在 96 孔酶标板上对来源于 178 个科、608 个属、1020 种动植物 2919 个提取物或分离部位样品在 CA-II 模型上进行了批量筛选。结果表明在 10 μg/ml 浓度下发现了来源于 40 个科、61 个属、72 个种的 100 个样品有活性，其中 5 个样品的 IC_{50} 小于 2.50 μg/ml，22 个样品的 IC_{50} 在 2.51～5.00 μg/ml 范围，73 个样品的 IC_{50} 在 5.01～10.00 μg/ml 范围。通过以上工作我们认为以碳酸酐酶 II 为分子靶点的体外筛选方法稳定、方便、快速、微量、有效，特别适用于天然产物的抗骨质疏松活性筛选。

关键词：抗骨质疏松活性筛选；碳酸酐酶 II；天然产物；提取物；分离部位
中图分类号：Q 946　　**文献标识码**：A　　**文章编号**：0253－2700(2003)06－0724－06

Searching for Antiosteoporotic Activities of Extracts and Fractions Derived from Natural Sources Targeting Carbonic Anhydrase II*

FU Xiang[1]，TAN Ning-Hua[1**]，JIANG Li-Hua[1]，JIA Rui-Rui[1]，JI Chang-Jiu[1]，
LANDRO James[2]，FEKETE Alexander[2]，MUELLER Hartwig[3]，HENKEL Thomas[3]
（1 *State Key Laboratory of Phytochemistry and Plant Resources in West China，Kunming Institute of Botany，Chinese Academy of Sciences*，Kunming 650204，China；2 *Bayer Corporation，IRT*，West Haven CT 06516，U.S.A.；
3 *Bayer AG，PH－R－EU*，D－42096 Wuppertal，Germany）

Abstract：To discover inhibitors of carbonic anhydrase II (CA II) with antiosteoporotic activity a batch of

* Foundation item：Project supported by the Agreement between Kunming Institute of Botany (Chinese Academy of Sciences，China) and Bayer AG (Germany) (BK－9501)，the Ministry of Science & Technology of China (863：2001AA234011－02)，Chinese Academy of Sciences (KSCX1－09－03－1，KSCX1－SW－11) and Kunming Institute of Botany.
** Correspondence to Prof. Dr. TAN Ning-Hua. Tel：86－871－5223800. Fax：86－871－5223228.
E－mail：nhtan@mail.kib.ac.cn.
收稿日期：2003－05－08，2003－06－26 接受发表
作者简介：付祥（1976－）男，云南人，硕士，主要从事生物活性筛选研究。

2919 extract or fraction samples prepared from plants and animals belonging to 178 families, 608 genera and 1020 species were tested on carbonic anhydrase II bioassay (CA-II) in 96-well microtiterplates. Finally 100 samples, which belong to 40 families, 61 genera and 72 species, showed inhibitory activity at 10 μg/ml, in which 5 samples showed IC_{50} at = < 2.50 μg/ml, 22 samples showed IC_{50} at 2.51 – 5.00 μg/ml and 73 samples showed IC_{50} at 5.01 – 10.00 μg/ml. It is indicated that this *in-vitro* bioassay is convenient, stable, rapid, sensitive and effective in searching for antiosteoporotic activity samples from natural sources.

Key words: Antiosteoporotic activity screening; Carbonic anhydrase II (CA II); Natural products; Extracts; Fractions

Osteoporosis is one of important diseases caused by the imbalance in bone skeletal turnover because bone resorption exceeds bone formation. Bone resorption is the unique function of the osteoclast so that dysfunction of the osteoclast by some inhibitors may be showed antiosteoporotic activity. The osteoclast is a specialized macrophage polykaryon which is formed from the macrophage through determination, proliferation and differentiation stages that are up-regulated by PU.1, M-CSF, RANKL, c-fos, NFκB, PTH and down-regulated by OPG. Bone resorption by the osteoclast divides into polarization and resorption stages that are affected by αvβ3, TRAF6, c-Src, Carbonic anhydrase II, H^+-ATPase and Cathepsins, in which αvβ3, Carbonic anhydrase II, H^+-ATPase and Cathepsins may be some attractive targets for anti-osteoporosis therapy (Teitelbaum, 2000).

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc enzymes which catalyze the reversible conversion of carbon dioxide and water into bicarbonate and protons: $CO_2 + H_2O <===> HCO_3^- + H^+$. In mammals there are seven isozymes i.e. CA I, CA II, CA III, CA IV, CA V, CA VI and CA VII which have different tissue specificities and probably varying physiological roles. In humans, CA II is abundant in ostoeoclasts and an essential enzyme in bone resorption by osteoclasts, which is a zinc enzyme of single chain polypeptide with about 30 000 Da molecular weight containing about 260 amino acid residues (Shapiro *et al*, 1989). After formation of ruffled border bone resorption depends on the secretion of protons and lysosomal proteinases from osteoclasts into the extracellular microenvironment. In osteoclasts protons are generated by CA II and actively transported by the proton pump driven by vacuolar-type H^+-ATPase at the ruffled border into the lacuna. Now several studies suggest that CA II inhibitors, such as acetazolamide, inhibit bone resorption in vivo and in vitro (Rousselle *et al*, 2002; Ohba *et al*, 1996).

In this paper we describe testing of natural extract or corresponding fraction samples with the CA-II bioassay in order to discover new antiosteoporotic activity samples from natural products.

Materials and methods

Materials and instruments Carbonic anhydrase II (CA II), p-nitrophenyl acetate (PNPA), acetazolamide and 3-[N-Morpholino] propanesulfonic acid (MOPS) were purchased from SIGMA. Other reagents and solvents used in the experiments are of biological, analytic and reagent grades.

2919 samples tested are extracts or fractions prepared from plants and animals belonging to 178 families, 608 genera and 1020 species. They are a part of the sample library of the Lab. for Screening within the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, China.

SPECTRAmax 340 96-well microtiterplate reader from Molecular Devices (USA) was used for end point measurement.

Sample preparation 20 mg sample of extracts or fractions was dissolved in 2 ml of Me_2SO as sample stock solution (10 mg/ml). The final concentration of sample for pre-test was 10 μg/ml, in which 5 μl sample solution (diluted to 200 μg/ml by adding Me_2SO) was added to microtiterplate wells as appropriate.

CA-II bioassay Five μl Me_2SO solvent was distributed in Blank wells (Bl) and Substrate wells (Sub). Five μl sample solution was filled in Sample wells (Sam) and Sample Blank wells (Samb). Five μl 100 μmol/L acetazolamide solution was added to Control wells (Con). Ninety-five μl 50 mmol/L MOPS buffer mixture (pH 6.9) were added to Blank wells and Sample Blank wells, and sixty μl 50 mmol/L MOPS buffer mixture (pH 6.9) were added to Substrate wells, Control wells and Sample wells. Thirty-five μl bioassay mixture, which contains 25 μl of 6 mmol/L PNPA and 10 μl of 1 μmol/L CA II were added to Substrate wells, Control wells and Sample wells. After incubation at 25℃ for 2 h OD values at 405 nm were measured by a microtiterplate reader.

Sample testing was divided into three steps: 1) Pre-test: Samples were screened in one well at the concentration of 10 μg/ml. Samples with ≥40% inhibition at 10 μg/ml were selected for Follow-up test; 2) Follow-up test: Samples were screened in duplicates at a concentration of 10 μg/ml. Samples with ≥50% inhibition at 10 μg/ml were selected for Evaluation-test; 3) Evaluation-test: Samples were screened in triplicates at four concentrations of 10.00, 5.00, 2.50 and 1.25 μg/ml. IC_{50} (μg/ml) of active samples were calculated using the following formula:

$$IC_{50} = \frac{Concentration_L\ (I_H - 50) + Concentration_H\ (50\text{-}I_L)}{I_H - I_L}$$

Templates of Pre-test, Follow-up test and Evaluation-test are presented in Figs. 1, 2 and 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Samb1	Samb2	Samb3	Samb4	Samb5	Samb6	Samb7	Samb8	Samb9	Samb10	Samb11
B	Bl	Sam1	Sam2	Sam3	Sam4	Sam5	Sam6	Sam7	Sam8	Sam9	Sam10	Sam11
C	Sub	Samb12	Samb13	Samb14	Samb15	Samb16	Samb17	Samb18	Samb19	Samb20	Samb21	Samb22
D	Sub	Sam12	Sam13	Sam14	Sam15	Sam16	Sam17	Sam18	Sam19	Sam20	Sam21	Sam22
E	Sub	Samb23	Samb24	Samb25	Samb26	Samb27	Samb28	Samb29	Samb30	Samb31	Samb32	Samb33
F	Con	Sam23	Sam24	Sam25	Sam26	Sam27	Sam28	Sam29	Sam30	Sam31	Sam32	Sam33
G	Con	Samb34	Samb35	Samb36	Samb37	Samb38	Samb39	Samb40	Samb41	Samb42	Samb43	Samb44
H	Con	Sam34	Sam35	Sam36	Sam37	Sam38	Sam39	Sam40	Sam41	Sam42	Sam43	Sam44

Fig. 1 Template for Pre-test.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Sub	Con	Samb7	Sam7	Sam7	Samb15	Sam15	Sam15	Samb23	Sam23	Sam23
B	Bl	Sub	Con	Samb8	Sam8	Sam8	Samb16	Sam16	Sam16	Samb24	Sam24	Sam24
C	Samb1	Sam1	Sam1	Samb9	Sam9	Sam9	Samb17	Sam17	Sam17	Samb25	Sam25	Sam25
D	Samb2	Sam2	Sam2	Samb10	Sam10	Sam10	Samb18	Sam18	Sam18	Samb26	Sam26	Sam26
E	Samb3	Sam3	Sam3	Samb11	Sam11	Sam11	Samb19	Sam19	Sam19	Samb27	Sam27	Sam27
F	Samb4	Sam4	Sam4	Samb12	Sam12	Sam12	Samb20	Sam20	Sam20	Samb28	Sam28	Sam28
G	Samb5	Sam5	Sam5	Samb13	Sam13	Sam13	Samb21	Sam21	Sam21	Bl	Sub	Con
H	Samb6	Sam6	Sam6	Samb14	Sam14	Sam14	Samb22	Sam22	Sam22	Bl	Sub	Con

Fig. 2 Template for Follow-up test.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B1	$Samb_{11}$	$Samb_{12}$	$Samb_{13}$	$Samb_{14}$	$Samb_{31}$	$Samb_{32}$	$Samb_{33}$	$Samb_{34}$	$Samb_{51}$	$Samb_{52}$	B1
B	Sub	Sam_{11}	Sam_{12}	Sam_{13}	Sam_{14}	Sam_{31}	Sam_{32}	Sam_{33}	Sam_{34}	Sam_{51}	Sam_{52}	Sub
C	Sub	Sam_{11}	Sam_{12}	Sam_{13}	Sam_{14}	Sam_{31}	Sam_{32}	Sam_{33}	Sam_{34}	Sam_{51}	Sam_{52}	Sub
D	Sub	Sam_{11}	Sam_{12}	Sam_{13}	Sam_{14}	Sam_{31}	Sam_{32}	Sam_{33}	Sam_{34}	Sam_{51}	Sam_{52}	Sub
E	B1	$Samb_{21}$	$Samb_{22}$	$Samb_{23}$	$Samb_{24}$	$Samb_{41}$	$Samb_{42}$	$Samb_{43}$	$Samb_{44}$	$Samb_{53}$	$Samb_{54}$	B1
F	Con	Sam_{21}	Sam_{22}	Sam_{23}	Sam_{24}	Sam_{41}	Sam_{42}	Sam_{43}	Sam_{44}	Sam_{53}	Sam_{54}	Con
G	Con	Sam_{21}	Sam_{22}	Sam_{23}	Sam_{24}	Sam_{41}	Sam_{42}	Sam_{43}	Sam_{44}	Sam_{53}	Sam_{54}	Con
H	Con	Sam_{21}	Sam_{22}	Sam_{23}	Sam_{24}	Sam_{41}	Sam_{42}	Sam_{43}	Sam_{44}	Sam_{53}	Sam_{54}	Con

Fig. 3 Template for Evaluation-test.

Results and Discussion

Screening of 2919 samples 2919 samples were screened in Pre-test on 67 96-well microtiterplates and samples were selected for Follow-up test with $\geqslant 40\%$ inhibition. 805 samples were screened in Follow-up test on 29 96-well microtiterplates and 192 samples were selected for Evaluation-test with $\geqslant 50\%$ inhibition. 192 samples were screened in Evaluation-test on 39 96-well microtiterplates and 100 samples showed inhibitory activities on CA II. Hit rate of active samples is 3.43%. Five samples showed IC_{50} at less than 2.50 μg/ml, 22 samples showed IC_{50} at 2.51 – 5.00 μg/ml and 73 samples showed IC_{50} at 5.01 – 10.00 μg/ml, respectively.

Activity-sample source relationship 2919 samples of extracts or fractions were prepared from plants and animals belonging to 178 families, 608 genera and 1020 species, notably, plants from Annonaceae, Compositae, Euphorbiaceae, Labitae, Liliaceae, Papilionaceae, Ranunculaceae, Rosaceae, Rubiaceae, Rutaceae and Umbelliferae. 100 samples, which belong to 40 families, 61 genera and 72 species, showed inhibitory activity at 10 μg/ml, notably, samples from Boraginaceae, Celastraceae, Dilleniaceae, Euphorbiaceae, Geraniaceae, Lauraceae, Moraceae, Palmae, Papilionaceae, Pinaceae, Polygonaceae, Rosaceae, Saxifragaceae, Vitaceae (Table 1).

Table 1 Plant Family and Genus distribution of active samples.

No.	Chinese Family Name	Latin Family Name	Chinese Genus Name	Latin Genus Name	No.	CA-II
1	铁线蕨科	Aiantaceae		*Adiantum*	1	+
2	漆树科	Anacardiaceae	黄连木属	*Pistacia* L.	2	+
			漆树属	*Rhus* L.	3	+
3	夹竹桃科	Apocynaceae	毛车藤属	*Amalocalyx Pierre*	4	+
4	蛇菰科	Balanophoraceae	蛇菰属	*Balanophora* J. R. et G. Forst.	5	+
5	紫草科	Boraginaceae	驴臭草属	*Onosma* L.	6	+
6	云实科	Caesalpiniaceae	决明属	*Cassia* L.	7	+
7	忍冬科	Caprifoliaceae	接骨木属	*Sambucus* L.	8	+
8	卫矛科	Celastraceae	南蛇藤属	*Celastrus* L.	9	+
			卫矛属	*Euonymus* L.	10	+
			雷公藤属	*Tripterygium* Hook. f.	11	+

续表 1

No.	Chinese Family Name	Latin Family Name	Chinese Genus Name	Latin Genus Name	No.	CA-II
9	山茱萸科	Cornaceae	四照花属	*Dendrobenthamia* Hutch.	12	+
10	第伦桃科	Dilleniaceae	锡叶藤属	*Tetracera* L.	13	+
11	鳞毛蕨科	Dryopteridaceae		*Cyrtomium*	14	+
12	柿科	Ebenaceae	柿属	*Diospyros* L.	15	+
13	大戟科	Euphorbiaceae	重阳木属	*Bischofia* Bl.	16	+
			大戟属	*Euphorbia* L.	17	+
			水柳属	*Homonoia* Lour.	18	+
			叶下珠属	*Phyllanthus* L.	19	+
14	壳斗科	Fagaceae	栎属	*Quercus* L.	20	+
15	牻牛儿苗科	Geraniaceae	老鹳草属	*Geranium* L.	21	+
16	买麻藤科	Gnetaceae	买麻藤属	*Gnetum* L.	22	+
17	樟科	Lauraceae	樟属	*Cinnamomum* Trew	23	+
			山胡椒属	*Lindera* Thunb.	24	+
			新樟属	*Neocinnamomum* H. Liou	25	+
18	百合科	Liliaceae	沿阶草属	*Ophiopogon* Ker-Gawl.	26	+
19	桑寄生科	Loranthaceae	离瓣寄生属	*Helixanthera* Lour.	27	+
20	野牡丹科	Melastomataceae	金锦香属	*Osbeckia* L.	28	+
			偏瓣花属	*Plagiopetalum* Rehd.	29	+
21	楝科	Meliaceae	楝属	*Melia* L.	30	+
22	桑科	Moraceae	榕属	*Ficus* L.	31	+
			桑属	*Morus* L.	32	+
23	杨梅科	Myricaceae	杨梅属	*Myrica* L.	33	+
24	紫金牛科	Myrsinaceae	紫金牛属	*Ardisia* Sw.	34	+
25	桃金娘科	Myrtaceae	番石榴属	*Psidium* L.	35	+
26	棕榈科	Palmae	桄榔属	*Arenga* Labill.	36	+
			黄藤属	*Daemonorops* Bl.	37	+
27	蝶形花科	Papilionaceae	山蚂蝗属	*Desmodium* Desv.	38	+
			密花豆属	*Spatholobus* Hassk.	39	+
28	松科	Pinaceae	油杉属	*Keteleeria* Carr.	40	+
			松属	*Pinus* L.	41	+
29	罗汉松科	Podocarpaceae	罗汉松属	*Podocarpus* L'Her. ex Pers.	42	+
30	蓼科	Polygonaceae	荞麦属	*Fagopyrum* Mill.	43	+
			山蓼属	*Oxyria* Hill	44	+
			蓼属	*Polygonum* L.	45	+
31	毛茛科	Ranunculaceae	芍药属	*Paeonia* L.	46	+
32	蔷薇科	Rosaceae	山楂属	*Crataegus* L.	47	+
			委陵菜属	*Potentilla* L. = *Dasiphora*	48	+
			绣线菊属	*Spiraea* L.	49	+
33	茜草科	Rubiaceae	九节属	*Psychotria* L.	50	+
34	清风藤科	Sabiaceae	清风藤属	*Sabia* Colebr.	51	+
35	虎耳草科	Saxifragaceae	落新妇属	*Astilbe* Buch.-Ham.	52	+
			鬼灯擎属	*Rodgersia* A. Gray	53	+
36	菝葜科	Smilacaceae	菝葜属	*Smilax* L.	54	+
37	茶科	Theaceae	茶梨属	*Anneslea* Wall.	55	+
38	延龄草科	Trilliaceae	重楼属	*Paris* L.	56	+
39	荨麻科	Urticaceae	冷水花属	*Pilea* Lindl.	57	+
40	葡萄科	Vitaceae	蛇葡萄属	*Ampelopsis* Michx.	58	+
			火筒树属	*Leea* L.	59	+
			崖藤属	*Tetrastigma* Planch.	60	+
			葡萄属	*Vitis* L.	61	+

Carbonic anhydrase II (CA II) catalyzes the hydration of carbon dioxide and ester such as p-nitrophenyl acetate (PNPA), in the latter which produces yellow p-nitrophenol (PNP) that can be detected quantitatively in the microtiterplate reader at 405 nm. OD value decreases if certain samples inhibit CA II reflecting a reduction in PNP liberation.

After screening 2919 samples we found that the *in-vitro* bioassay (CA-II) described above is a convenient, stable, rapid, sensitive and effective model in searching for antiosteoporotic activity samples from natural sources.

Acknowledgments: We thank all sample providers in the Sample Library, especially our colleagues in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences. We also thank Prof. LIU Ji-Kai for assistance.

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〔上接 723 页〕

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