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Anti-invasive Activity of Alkaloids and Polyphenolics in Vitro

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Abstract—Invasiveness, the ability of certain tumour cells to migrate beyond their natural tissue boundaries, often leads to metastasis, and usually determines the fatal outcome of cancer. The need for anti-invasive agents has led us to search for possibly active compounds among alkaloids and polyphenolics. One hundred compounds were screened in an assay based on the confrontation of invasive human MCF-7/6 mammary carcinoma cells with fragments of normal embryonic chick heart in vitro. Anti-invasive activity was frequently found among chalcones having a prenyl group. Six compounds were found to inhibit invasion when added to the culture medium at concentrations as low as 1 μM. For at least three of them the anti-invasive effect could be associated with a cytotoxic effect on the MCF-7/6 cells, but not on the heart tissue. This selective cytotoxicity was substantiated by different methods, such as histology and growth assays (volume measurements, cell counts, MTT and sulforhodamine B assays). The anti-invasive effects of the compounds could neither be ascribed to induction of apoptosis nor to the promotion of cell–cell adhesion. Our data indicate that among the alkaloids and polyphenolics a number of molecules can inhibit growth and invasion of human mammary cancer cells via selective cytotoxicity. © 1997 Elsevier Science Ltd.

Introduction

The ability of certain plant molecules to interfere with targets implicated in carcinogenesis[1–3] and in tumour cell biology makes them interesting tools in cancer research. Many activities of tumour cells in vitro can be downregulated by flavonoids, and one of these activities is cell proliferation.[4,5] This effect is sometimes ascribed to inhibition of tyrosine kinases associated with growth factor receptors,[6,7] with oncogene products[8] and with intracellular signal transduction.[9–12] Other tumour cell activities, such as apoptosis, however, were found to be upregulated by flavonoids.[13,14]

Invasion is a tumour cell activity which is closely related to the fatal outcome of cancer.[15] Invasion leads to the local destruction of surrounding normal tissues and eventually to metastasis. A number of flavonoids can inhibit tumour invasion in vitro[16] and artificial metastasis in vivo.[17] Some anti-invasive flavonoids, such as 3,7-dimethoxyflavone[18] and (+)-catechin[19] appear to act via targets in the normal tissue confronting the tumour cells. (+)-Catechin, for instance, binds to laminin, an extracellular matrix glycoprotein.[20] The action mechanism of tangeretin, another anti-invasive flavonoid, is different since tangeretin alters the behaviour of the tumour cells—it slows down their motility[21] and strengthens their mutual cohesion via activation of the cell–cell adhesion molecule E-cadherin.[22]

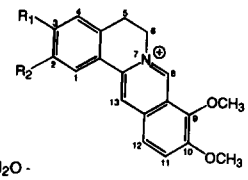
We have continued our search for additional anti-invasive flavonoids or flavonoid-like compounds that may reveal new action mechanisms. These compounds, both naturally occurring and synthetic, were tested in an organotypic assay for invasion in vitro.[23] In such confronting cultures, effects on the tumour cells can be discerned from effects on the normal host tissue (embryonic chick heart). We have used human MCF-7/6 breast carcinoma cells as test cells because they were invasive in this assay, and because they were shown earlier to be sensitive to the effect of a number of flavonoids.[13,18,24–26]

Results

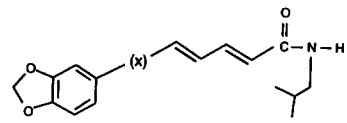
Preparation and isolation of compounds

The structural formulae of the compounds are shown in Figure 1.

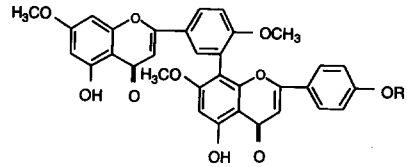
Compound **10**[27] was prepared by the Claisen–Schmidt reaction of 3,4-dimethoxybenzaldehyde with 6-acetyl-7-hydroxy-2,2-dimethylchroman,[28] while compound **31** was prepared by the reaction of 6-acetyl-7-hydroxy-2,2-dimethylchromene[29] with 2,4-dimethoxybenzaldehyde. Compounds **4** and **12** were isolated from *Piper falconeri*,[30] while the biflavones **6** and **7** were obtained from *Taxus baccata*.[31] The alkaloids **8**, **9** and **33** were



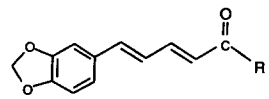
1. R_1 , R_2 = - OCH_2O -
2. R_1 = OH ; R_2 = OCH_3
3. R_1 = OCH_3 ; R_2 = OH

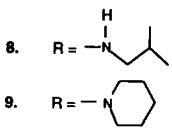


4. x = - CH_2CH_2 -
5. x = - CH = $CHCH_2CH_2$ -

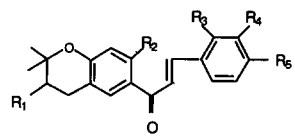


6. R = CH_3
7. R = H

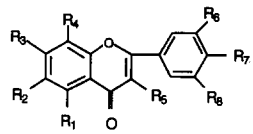




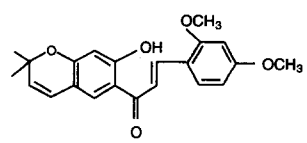
8. R =
9. R =

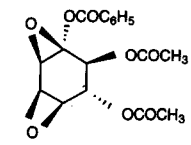


10. R_1 = R_3 = H ; R_2 = OH ; R_4 = R_5 = OCH_3
11. R_1 = OH ; R_2 = R_3 = R_5 = OCH_3 ; R_4 = H

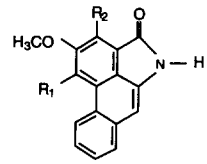


12. R_1 = OH ; R_2 = R_4 = R_5 = R_6 = R_8 = H ; R_3 = R_7 = OCH_3
13. R_1 = R_7 = OH ; R_2 = R_4 = R_5 = R_8 = H ; R_3 = R_6 = OCH_3
14. R_1 = R_3 = R_4 = R_7 = OCH_3 ; R_2 = R_6 = R_8 = H ; R_5 = OH
15. R_1 = OH ; R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = OCH_3
16. R_1 = R_3 = R_4 = R_6 = R_7 = R_8 = OCH_3 ; R_2 = R_5 = H
17. R_1 = OH ; R_3 = R_5 = OCH_3 ; R_2 = R_4 = R_6 = R_7 = R_8 = H
18. R_1 = R_2 = R_4 = H ; R_3 = R_5 = R_6 = R_7 = R_8 = OCH_3
19. R_1 = R_3 = R_7 = OCH_3 ; R_2 = R_4 = R_5 = R_6 = R_8 = H
20. R_1 = R_2 = R_4 = H ; R_3 = OH ; R_5 = R_6 = R_7 = R_8 = OCH_3
21. R_1 = R_4 = R_5 = R_8 = H ; R_2 = R_3 = OCH_3 ; R_6 , R_7 = - OCH_2O -
22. R_1 = R_3 = R_5 = OCH_3 ; R_2 = R_4 = R_6 = R_7 = R_8 = H
23. R_1 = OAc ; R_2 = R_5 = R_6 = R_7 = R_8 = H ; R_3 = R_4 = OCH_3
24. R_1 = OH ; R_2 = R_5 = R_6 = R_7 = R_8 = H ; R_3 = R_4 = OCH_3
25. R_1 = OH ; R_2 = R_3 = OCH_3 ; R_4 = R_5 = R_6 = R_7 = R_8 = H
26. R_1 = R_3 = R_4 = OCH_3 ; R_2 = R_5 = R_6 = R_7 = R_8 = H
27. R_1 = R_2 = R_3 = OCH_3 ; R_4 = R_5 = R_6 = R_7 = R_8 = H
28. R_1 = R_3 = OCH_3 ; R_5 = OH ; R_2 = R_4 = R_6 = R_7 = R_8 = H
29. R_1 = R_3 = OCH_3 ; R_2 = R_4 = R_6 = R_7 = R_8 = H ; R_5 = OAc
30. R_1 = OH ; R_2 = R_4 = R_6 = R_7 = R_8 = H ; R_3 = R_5 = OCH_3

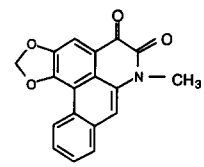
31. 



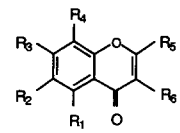
32.



33. R_1 = OH ; R_2 = OCH_3
34. R_1 = OCH_3 ; R_2 = OH

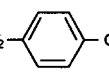


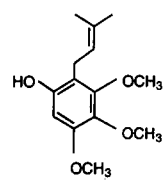
35.



36. R_1 = OH ; R_2 = R_4 = R_6 = H ; R_3 = OCH_3 ; R_5 = - $(CH_2)_{32}$ - CH_3

37. R_1 = H ; R_2 = R_4 = -  ; R_3 = OAc ; R_5 = CH_3 ; R_6 = $COCH_3$

38. R_1 = R_3 = OAc; R_2 = -CH_2-  OCH_3 ; R_4 = R_5 = R_6 = H



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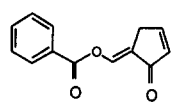

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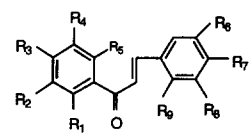
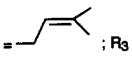
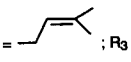
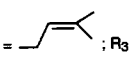
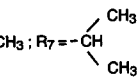


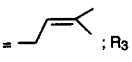
41. R_1 = R_2 = R_5 = R_6 = R_8 = R_9 = H ; R_3 = R_4 = OCH_3 ; R_7 = CH_3
42. R_1 = R_2 = R_5 = R_6 = R_8 = R_9 = H ; R_3 = R_4 = R_7 = OCH_3
43. R_1 = R_2 = R_5 = R_6 = R_8 = R_9 = H ; R_3 = R_4 = OCH_3 ; R_7 = 
44. R_1 = R_2 = R_5 = R_6 = R_8 = R_9 = H ; R_3 = R_4 = OCH_3 ; R_7 = NO_2
45. R_1 = R_2 = R_5 = R_7 = R_8 = R_9 = H ; R_3 = R_4 = OCH_3 ; R_6 = OPh
46. R_1 = R_4 = R_6 = R_8 = R_9 = H ; R_2 =  ; R_3 = OCH_3 ; R_5 = OH ; R_7 = OC_2H_5
47. R_1 = R_4 = R_6 = R_8 = R_9 = H ; R_2 =  ; R_3 = OCH_3 ; R_5 = OH ; R_7 = OPh
48. R_1 = R_4 = R_7 = R_8 = R_9 = H ; R_2 =  ; R_3 = OCH_3 ; R_5 = OH ; R_6 = NO_2

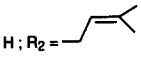
Figure 1. Structural formulae of 100 compounds tested for possible anti-invasive activity in vitro.

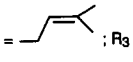
49. $R_1 = R_4 = R_6 = R_8 = R_9 = H$; $R_2 =$  ; $R_3 = R_5 = OCH_3$; $R_7 = NO_2$

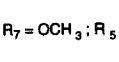
50. $R_1 = R_4 = R_8 = R_9 = H$; $R_2 =$  ; $R_3 = R_5 = R_6 = R_7 = OCH_3$

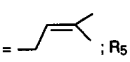
51. $R_1 = R_4 = R_6 = R_8 = R_9 = H$; $R_2 =$  ; $R_3 = R_5 = OCH_3$; $R_7 =$ 

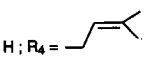
52. $R_1 = R_4 = R_6 = R_8 = H$; $R_2 =$  ; $R_3 = R_5 = R_7 = R_9 = OCH_3$

53. $R_1 = R_4 = R_6 = R_7 = R_8 = R_9 = H$; $R_2 =$  ; $R_3 = R_5 = OCH_3$

54. $R_1 = R_4 = R_7 = R_8 = R_9 = H$; $R_2 =$  ; $R_3 = R_5 = OCH_3$; $R_6 = OPh$

55. $R_1 = R_4 = R_6 = R_8 = R_9 = H$; $R_2 =$  ; $R_3 = R_7 = OCH_3$; $R_5 = OH$

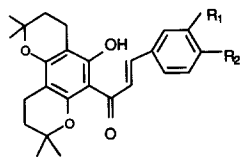
56. $R_1 = R_3 = OCH_3$; $R_2 = R_6 = R_7 = R_8 = R_9 = H$; $R_4 =$  ; $R_5 = OH$

57. $R_1 = R_3 = R_5 = OCH_3$; $R_2 = R_6 = R_7 = R_8 = R_9 = H$; $R_4 =$ 

58. $R_1 = R_2 = R_3 = OCH_3$; $R_5 = OH$; $R_4 = R_6 = R_7 = R_8 = R_9 = H$

59. $R_1 = R_3 = R_4 = OCH_3$; $R_5 = OH$; $R_2 = R_6 = R_7 = R_8 = R_9 = H$

60. $R_1 = R_2 = R_5 = R_6 = R_8 = H$; R_3 , $R_4 = -OCH_2O-$; $R_7 = R_9 = OCH_3$



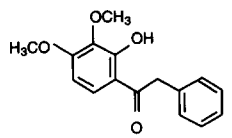
61. $R_1 = OC_7H_7$; $R_2 = OCH_3$

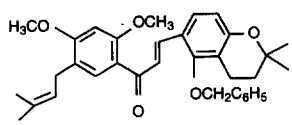
62. $R_1 = OPh$; $R_2 = H$

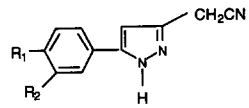
63. $R_1 = NO_2$; $R_2 = H$

64. $R_1 = H$; $R_2 =$ 

65. $R_1 = H$; $R_2 = CH_3$

66. 

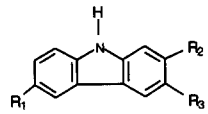
67. 



68. $R_1 = CH_3$; $R_2 = H$

69. $R_1 = Br$; $R_2 = H$

70. $R_1 = H$; $R_2 = OCH_3$

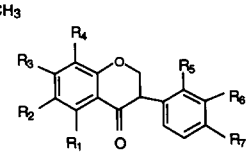


71. $R_1 = OCH_3$; $R_2 = H$; $R_3 = CH_3$

72. $R_1 = H$; $R_2 = OH$; $R_3 = CH_3$

73. $R_1 = H$; $R_2 = R_3 = OCH_3$

74. $R_1 = R_2 = H$; $R_3 = CH_3$



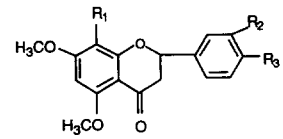
75. $R_1 = R_2 = R_4 = R_5 = R_6 = R_7 = H$; $R_3 = OCH_3$

76. $R_1 = R_2 = R_5 = R_6 = R_7 = H$; $R_3 = OH$; $R_4 = -CH_2CH = CH_2$

77. $R_1 = R_3 = R_5 = R_7 = OCH_3$; $R_2 = R_4 = R_6 = H$

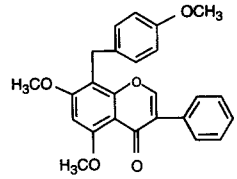
78. $R_1 = R_2 = R_5 = H$; $R_3 = OH$; $R_4 = -CH_2-CH = CH_2$; R_6 , $R_7 = -OCH_2O-$

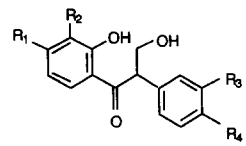
79. $R_1 = OH$; $R_2 = R_4 = R_6 = H$; $R_3 = R_5 = R_7 = OCH_3$



80. $R_1 =$  ; $R_2 = R_3 = H$

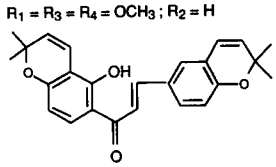
81. $R_1 = H$; $R_2 = R_3 = OCH_3$

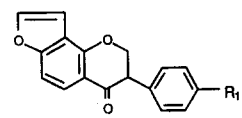
82. 



83. $R_1 = OCH_2OCH_2CH_3$; $R_2 = -CH_2CH = CH_2$; R_3 , $R_4 = -OCH_2O$

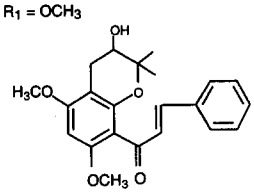
84. $R_1 = R_3 = R_4 = OCH_3$; $R_2 = H$

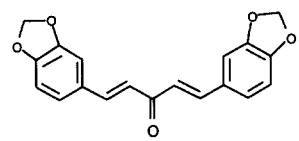
85. 

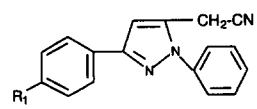


86. $R_1 = H$

87. $R_1 = OCH_3$

88. 

89. 

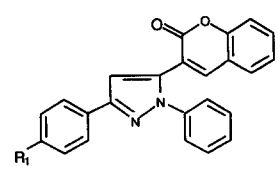


90. $R_1 = Br$

91. $R_1 = CH_3$

92. $R_1 = Cl$

93. $R_1 =$ 



94. $R_1 = Br$

95. $R_1 = CH_3$

Figure 1. *Continued.*

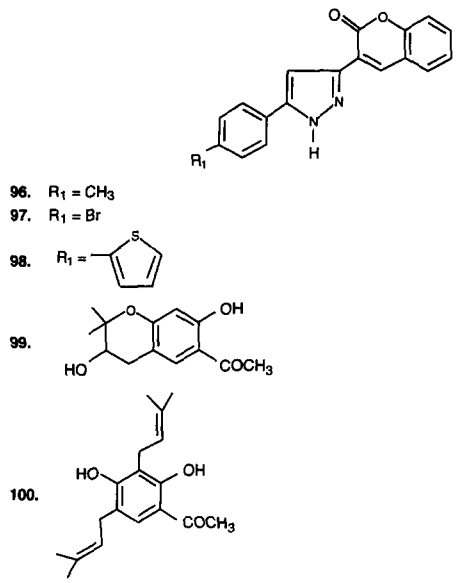


Figure 1. *Continued.*

isolated from *P. acutisleginum*,[32,33] while compounds **32** and **35** were obtained from *P. wightii*.[34] Velutin (**13**) and the prenylated phenol **39** were isolated from *P. clarkii*,[35] while the amides **5** and **34** were isolated from *P. longum*[36] and *P. argyrophylum*,[37] respectively. The novel chromone **36** was obtained from *Agave americana*,[38] and the compounds **11**, **14–20**, **22**, **30**, **37**, **38**, **55**, **57**, **60**, **66**, **71–89** and **99** were obtained from the collection of late Professor T. R. Seshadri, FRS in Delhi, India. The benzoic acid ester **40** was isolated from *Uvaria narum*,[39] and the chalcones **41–54**, **56**, **61–65** and **67** were prepared by the Claisen–Schmidt reaction of the appropriately substituted benzaldehyde and acetophenone derivatives.[40] The flavone **21** was prepared by the dehydrogenation of 6,7-dimethoxy-3,4-methylenedioxyflavanone using DDQ in dry benzene.[41] Compounds **23–29**, **58** and **59** were prepared by us using the previously published procedures.[42] The pyrazoles **68–70**, and **90–93** were prepared by refluxing the corresponding 3-cyano-4-thiomethyl-6-aryl-2*H*-pyran-2-ones with hydrazine or phenylhydrazine in methanol.[43,44] 3-[3-(4-Bromophenyl)-1-phenylpyrazol-5-yl]-2*H*-1-benzopyran-2-one (**94**) was prepared by condensing the pyrazole **90** with salicylaldehyde; its structure was confirmed by X-ray crystallography.[45] The pyrazolylcoumarins **95–98** were synthesized by the reaction of salicylaldehyde or its derivatives with the appropriate pyrazole derivatives in ethanol (unpublished data from our laboratories). The prenylated acetophenone **100** was prepared by the prenylation of β-resacetophenone with 2-methylbut-3-en-2-ol and boron trifluoride-etherate.[46] The berberine-type alkaloids **1–3** were isolated from the ethanol extract of the bark of *Mahonia leschenaultii*. These three compounds are known,[47,48] but the complete spectral data of **1** and **2** have not been reported.

Invasion

Table 1 summarizes the results of the assays for invasion. In control cultures treated with solvent only, the MCF-7/6 cells had invaded the PHF after eight

Table 1. Effect of 100 alkaloids and polyphenolics on invasion of MCF-7/6 cells in vitro

	μM				μM				μM				μM		
No.	**100**	**10**	**1**	**No.**	**100**	**10**	**1**	**No.**	**100**	**10**	**1**	**No.**	**100**	**10**	**1**
1	+	–	–	26	–	0	0	51	+	+	–	76	+	–	–
2	+	–	–	27	+	–	–	52	–	0	0	77	–	0	0
3	–	0	0	28	+	+	–	**53**	+	+	+	78	+	–	–
4	–	0	0	29	+	–	–	54	–	0	0	79	+	–	–
5	–	0	0	30	+	–	–	55	–	0	0	80	+	–	–
6	–	0	0	31	–	0	0	56	+	–	–	81	+	+	–
7	–	0	0	32	+	–	–	57	–	0	0	82	+	+	–
8	–	0	0	33	+	+	–	**58**	+	+	+	83	–	0	0
9	+	–	–	34	–	0	0	59	+	–	–	84	+	–	–
10	+	–	–	35	–	0	0	60	+	–	–	**85**	+	+	+
11	–	0	0	36	–	0	0	61	–	0	0	86	–	0	0
12	–	0	0	37	–	0	0	62	–	0	0	87	+	–	–
13	–	0	0	38	+	–	–	63	–	0	0	**88**	+	+	+
14	+	–	–	39	+	–	–	64	–	0	0	89	–	0	0
15	–	0	0	40	+	–	–	65	–	0	0	90	+	+	–
16	–	0	0	41	+	–	–	66	–	0	0	91	+	–	–
17	+	–	–	42	–	0	0	67	+	+	–	92	–	0	0
18	+	+	+	43	–	0	0	68	+	–	–	93	+	–	–
19	+	+	–	44	–	0	0	69	+	–	–	94	–	0	0
20	+	+	–	45	–	0	0	70	–	0	0	95	–	0	0
21	–	0	0	46	–	0	0	71	+	–	–	96	+	–	–
22	–	0	0	47	–	0	0	72	+	–	–	97	–	0	0
23	–	0	0	48	–	0	0	73	–	0	0	98	–	0	0
24	+	+	–	49	–	–	–	74	–	0	0	99	–	0	0
25	+	–	–	50	+	+	–	**75**	+	+	+	100	–	0	0

+, anti-invasive; –, not anti-invasive; 0, not tested.

days' incubation. In histological sections of these cultures only the remnants of the heart tissue could be discerned, as visualized by haemtoxylin–eosin staining and even more clearly by selective immunohistochemistry of the heart tissue (Fig. 2A, B).

Many compounds showed no inhibition of invasion, and histologically their cultures were similar to controls. Other compounds were anti-invasive at the concentrations 100 or/and 10 μM. Six out of the 100 compounds (**18**, **53**, **58**, **75**, **85** and **88**), however, inhibited invasion

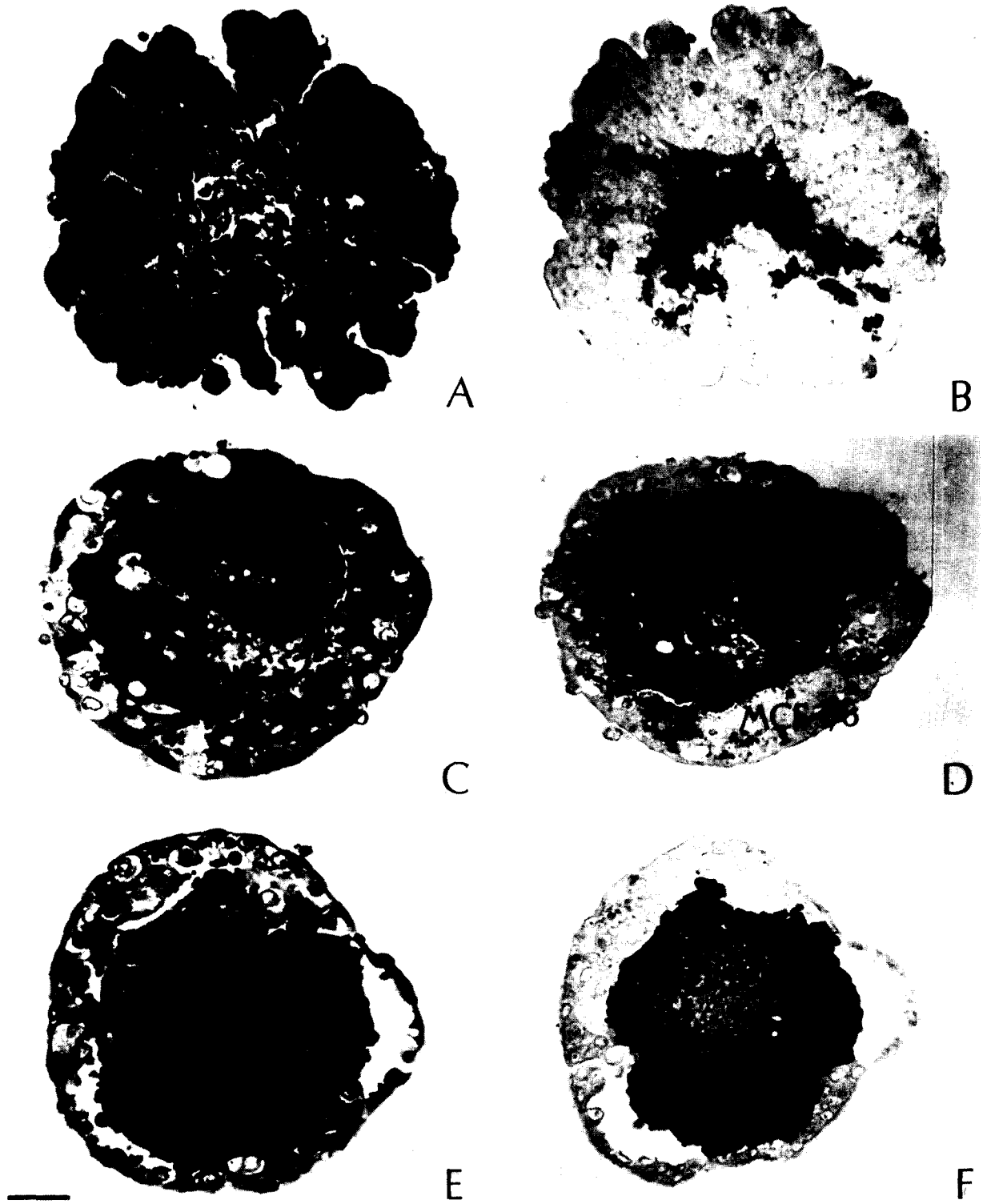


Figure 2. Light micrographs of sections from eight-day-old confronting cultures between precultured heart fragments (PHF) and MCF-7/6 cells. Solvent-treated (0.1% DMSO) confrontations (A and B), that show invasion of the MCF-7/6 cells, are compared with confrontations treated with 100 μM of the compounds **58** (C and D) and **75** (E and F), that show absence of invasion. The sections on the left panels were stained with haematoxylin–eosin; in the right panels, PHF antigens were revealed immunohistochemically and appear dark. Scale bar = 50 μM.

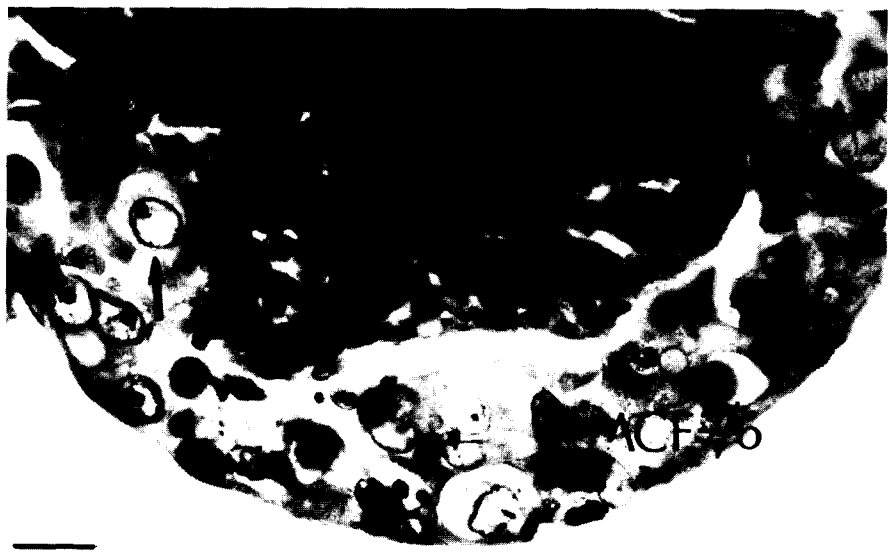


Figure 3. Histology of MCF-7/6 cells treated with 100 μM of compound **75** in confronting cultures with PHF for eight days. Note the pale and swollen nuclei of the MCF-7/6 cells (arrow) and the normal aspect of the heart cell nuclei (arrowhead). Haematoxylin–eosin staining. Scale bar = 20 μM.

of MCF-7/6 cells at a concentration as low as 1 μM. At 0.1 μM none of the compounds were active. As shown in Figures 2C–F, cultures treated with anti-invasive compounds showed an intact PHF surrounded by MCF-7/6 cells.

In cultures treated with 100 μM of the compounds **53**, **58**, or **75**, common histological features were observed. While the heart tissue was morphologically unaltered, the MCF-7/6 cells showed many abnormal nuclei ranging from small pycnotic to swollen vesicular (Fig. 3). In haemtoxylin–eosin stained sections, the cytoplasm was paler than in control cultures.

Growth

Both PHF and MCF-7/6 aggregates were cultured separately in the presence of 100 μM of the compounds **18**, **53**, **58**, **75**, **85** or **88**. After eight days' incubation the final volumes of the PHF were similar to or larger ($p < 0.005$ for the compounds **53** and **58**) than those of the solvent-treated control PHF (Fig. 4).

Histological analysis confirmed the absence of cytotoxicity signs, and showed that the increased volumes were to be ascribed to an increased cell mass, and not to accumulation of intercellular fluid. The latter phenomenon had been reported previously with the anti-invasive compound 3,7-dimethoxyflavone.[18] In contrast with PHF, the final volumes of the MCF-7/6 aggregates treated with the compound **53** or **58** were significantly ($p < 0.01$ for compound **53** and $p < 0.005$ for compound **58**) smaller than those of the solvent-treated aggregates (Fig. 4). Histological alterations in the treated MCF-7/6 cells were similar to those described above for the confronting cultures treated with the same

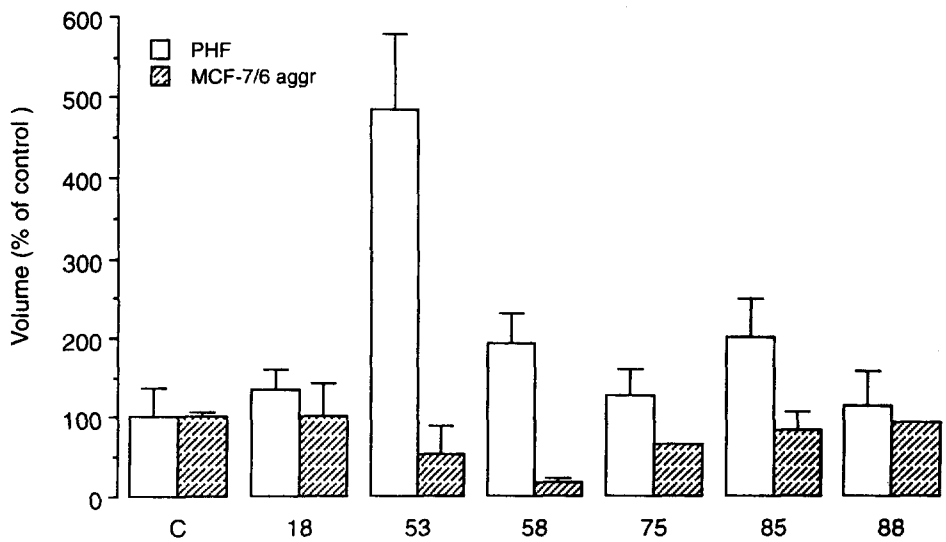


Figure 4. Effect of six anti-invasive compounds on growth of precultured heart fragments (open bars) and of MCF-7/6 aggregates (hatched bars) cultured in suspension for eight days, and treated with 100 μM of each compound. The effects of the compounds **18**, **53**, **58**, **75**, **85** and **88** on the final volumes of the cultures are presented as percentages of the final volumes of solvent-treated controls (C, mean ± standard deviation).

compounds. So, these data suggest that the compounds **53** and **58** provoke a selective growth inhibition on the MCF-7/6 cells, since the growth of PHF was not reduced.

Cytoxicity

The MTT assay for cytotoxicity was applied on MCF-7/6 cells treated with the anti-invasive compounds **18**, **53**, **58**, **75**, **85** and **88**. This assay, which measures the mitochondrial succinate dehydrogenase activity, showed significant ($p < 0.005$) decreases of MTT conversion in MCF-7/6 cells treated with 100 μM of the compounds **53**, **58** and **75** (Fig. 5A). In all other experiments no significant effects of the compounds on MTT conversion could be observed. In order to discriminate whether the effects on MTT conversion were due to a reduction of the number of cells (and thus of mitochondria) or to an activity decrease on the individual mitochondria, an estimation of the final cell number was done. The results of the sulforhodamine B assay, which measures the total amount of cellular protein in the cultures, are shown in Figure 5B. So, the reduced MTT conversion by MCF-7/6 cell cultures treated with 100 μM of the compounds **53**, **58** and **75**, could be explained essentially by a lower ($p < 0.005$) cell number.

Apoptosis

When compared with negative (omission of the TUNEL reagent) and positive (DNAse-treatment)

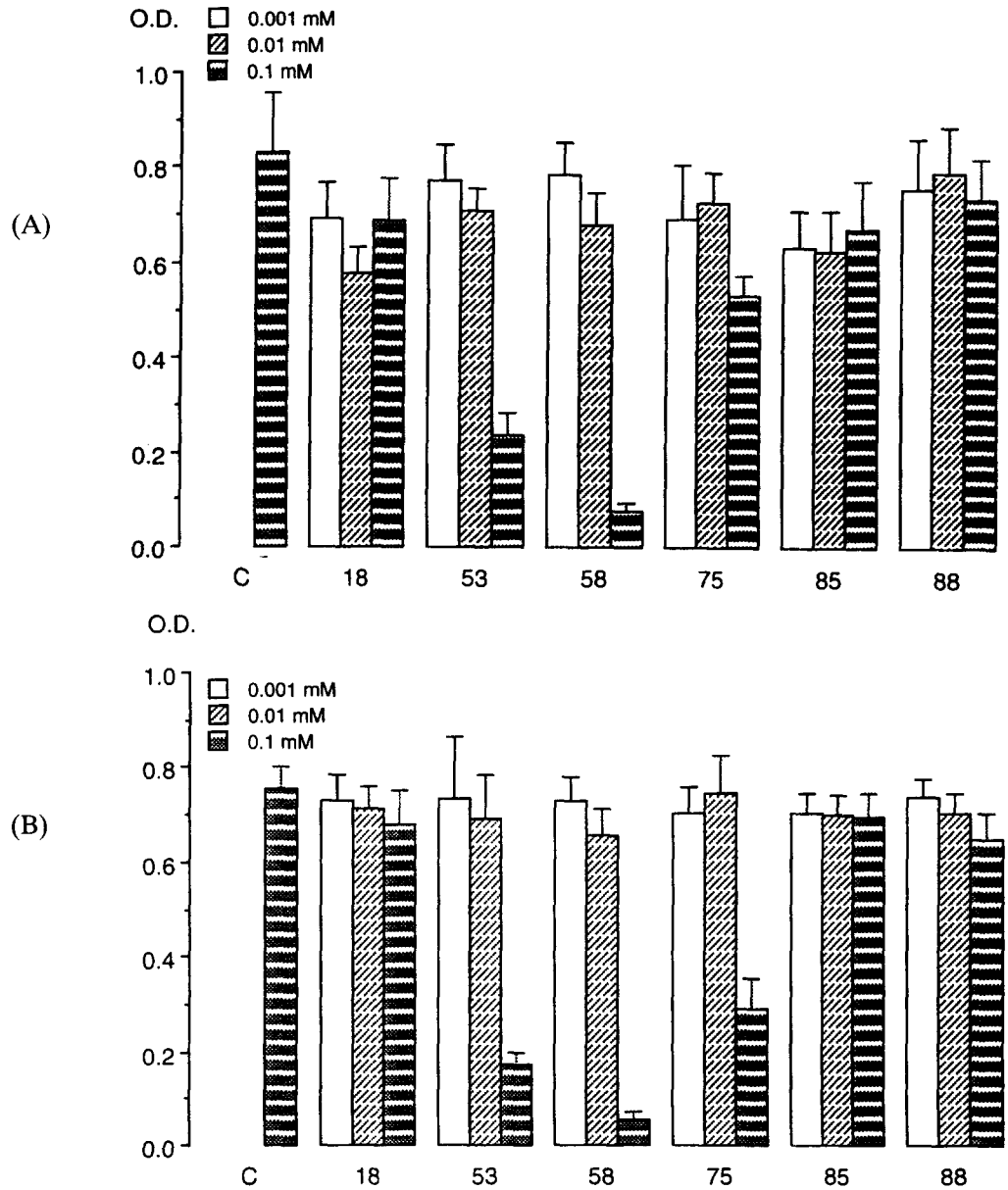


Figure 5. Effect of six anti-invasive compounds on MTT conversion (panel A) and sulforhodamine B staining (panel B) of MCF-7/6 cells grown in microtitre plates for eight days. The cells were treated with 0 (C), 1 (open bars), 10 (hatched bars) or 100 μM (dark bars) of each compound. The effects are read by optical density (OD) of the cultures (mean ± standard deviation).

control cells MCF-7/6 cells revealed no spontaneous DNA strand breaks on flow cytometry analysis. The same is true for cells treated with 100 μM of the compounds **18**, **53**, **58**, **85** and **88**. Only with MCF-7/6 cells treated with compound **75** was a small shift of the populations of labelled cells observed. Kolmogorov–Smirnov analysis revealed that this shift was statistically significant ($p \leq 0.001$) (data not shown). These data indicate that induction of apoptosis cannot be considered as the major action mechanism of the anti-invasive and growth-inhibiting compounds **53**, **58** and **75**.

Fast cell aggregation

Comparison of the particle size distribution profiles showed some spontaneous aggregation of solvent-treated MCF-7/6 cells within 30 min. This aggregation was calcium dependent, and could be inhibited by the E-cadherin neutralizing monoclonal antibody MB2. This indicated that E-cadherin was implicated in this aggregation. No effects of the compounds **18**, **53**, **58**, **75**, **85** and **88** at 100 μM could be observed on the MCF-7/6 cell aggregation profile, as shown by the Kolmogorov–Smirnov statistics (data not shown). So, the anti-invasive effect of these compounds could not be ascribed to an increased MCF-7/6 cell–cell adhesion.

Structure–activity relationship

In this investigation, 100 compounds were tested for anti-invasive activity out of which 44 compounds were shown to have marginal to profound activity and belong to different classes of polyphenolics and alkaloids: chalcones (14), flavones (11), isoflavanones (five), flavanones (two), alkaloids (six) and pyrazole derivatives (six).

Chalcones as a group of compounds have shown a higher activity compared to the other polyphenolics. Four chalcones out of 14 active ones (**53**, **58**, **85** and **88**) have shown activity at a concentration level as low as 1 μM. Three other chalcones (**50**, **51** and **67**) have shown activity up to a 10 μM concentration. It is further observed that chalcones having the prenyl group (acyclic or in the cyclic form) have shown higher activity compared to those lacking the prenyl group. Out of 14 active chalcones, eight contain such a group: **10**, **50**, **51**, **53**, **56**, **67**, **85** and **88**. The highest activity is found in the chalcones having the 2′,3′,4′- or 2′,4′,5′-substitution pattern, i.e. chalcones **53**, **58**, **85** and **88**.

Only one flavone, **18**, has shown activity at a concentration of 1 μM, while other flavones have shown marginal to moderate activity. The flavone **18** has a 3,7-dimethoxy substitution pattern and is similar to our previously reported compound, 3,7-dimethoxyflavone, which had shown strong anti-invasive activity against MCF-7/6 cells.[18] It can thus be concluded that 3-methoxyflavones having only one substituent in the A-ring, i.e. C-7 methoxy group, are of interest in combatting the invasion of cancer cells. This is further clear from the activity of the C-7 desmethyl analogue of **18**, i.e. 7-hydroxy-3,3′,4′,5′-tetramethoxyflavone (**20**) which showed activity only at 100 and 10 μM concentrations, and is inactive at 1 μM concentration. Similarly, isoflavanones having only one substituent in the A-ring, i.e. the C-7 methoxy function, show the highest anti-invasive activity. This is clear by comparing the isoflavanone **75** (having only one substituent, i.e. C-7 methoxy) which is active at 1 μM concentration, with other isoflavanones (**76**, **78**, **79** and **87**; having two substituents in the A-ring) which are marginally active.

The structure–activity correlation in other groups of compounds is not clearly discernible.

Discussion

Among 100 alkaloids and polyphenolics, six compounds inhibited the invasion of human MCF-7/6 mammary carcinoma cells in confronting cultures with embryonic chick heart fragments at a concentration of 1 μM. Theoretically the action mechanism of these compounds can be based either on a reduction of the cancer cells, invasiveness or on an increased resistance of the chick heart tissue towards invasion. A number of data suggest that the target(s) of the anti-invasive compounds reside(s) in the cancer cells. With compounds **53**, **58** and **75**, at a concentration of 100 μM, effects on the MCF-7/6 cells were evident: morphological signs of cytotoxicity, growth inhibition and a concomitant reduction of MTT conversion. Results from the TUNEL assay indicated that growth inhibition by the anti-invasive compounds could not be ascribed to induction of apoptosis. Furthermore, we investigated the influence of the anti-invasive compounds on cell–cell adhesion, since E-cadherin, a molecule implicated in this phenomenon is considered to be an invasion suppressor,[49] and since MCF-7/6 cells are sensitive to functional modulation of E-cadherin.[50] However, we did not find any evidence that the inhibition of MCF-7/6 invasion was the result of increased cell–cell adhesion, because none of the compounds increased fast aggregation of the cells. We cannot exclude that the anti-invasive compounds also act via the embryonic chick heart. Such a mechanism was suggested previously for 3,7-dimethoxyflavone, an anti-invasive flavonoid which increases growth of the heart fragments by creating large intercellular spaces.[18] With compound **53** the final volumes of the heart fragments were larger than those of solvent-treated fragments, but histological analysis showed that this increase was related to cell mass and not to the formation of extracellular material or fluid.

The structure–activity relationship revealed that anti-invasive compounds were frequently found among chalcones with a prenyl group. Prenylation by geranyl–geranyl or farnesyl moieties has been described as a post-translation modification of cytoplasmic proteins. These prenyl lipids serve as anchors for signaling molecules such as GTP-binding proteins at the plasma membrane.[51] We speculate that prenylated chalcones

may inhibit cell signaling by interference with membrane localization of prenylated proteins.

We are currently investigating the selective cytotoxicity of the anti-invasive compounds using a panel of human epithelioid cell lines. Preliminary results indicated that the effect is restricted to MCF-7 cell variants: both the invasive MCF-7/6 and the non-invasive MCF-7/AZ variant[52] were sensitive. No signs of cytotoxicity, however, were observed so far with other human mammary cell lines (SKBR-3, MDA-MB 231 and HBL-100), human Hu 456 bladder carcinoma, human Caco-2 colon carcinoma or with human PC-9 lung carcinoma. A more extensive collection of cell lines will have to be studied in order to determine the cytotoxicity restrictions of the anti-invasive compounds.

Another example of an anti-invasive treatment that acts via selective cytotoxicity is the combination of tumour necrosis factor (TNF) and interferon-gamma (IFN-γ). These polypeptides, which do not share any chemical relationship with alkaloids or with polyphenolics, were shown to inhibit invasion of murine B16–B16 melanoma cells in chick heart fragments in organotypic cultures.[53] Histological and growth analysis showed cytotoxic effects on the tumour cells, but not on the heart tissue. We feel that agents that inhibit tumour invasion by selective killing of the cancer cells should be considered as potential therapeutic drugs in cancer.

Experimental

Compound **1** was obtained as a white solid, mp 145 °C (literature value:[47] 145 °C). ^{1}H NMR (250 MHz, CD_3OD): δ 3.34 (2H, t, J = 7.4 Hz, H-5), 4.21 (3H, s, C-10 OCH_3), 4.29 (3H, s, C-9 OCH_3), 4.94 (2H, t, J = 6.4 Hz, H-6), 6.23 (2H, s, OCH_2O), 7.03 (1H, s, H-4), 7.74 (1H, s, H-1), 8.15–8.21 (2H, d, J = 9 Hz, H-11 and H-12); 8.78 (1H, s, H-13) and 9.85 (1H, s, H-8); ^{13}C NMR (62.5 MHz, CD_3OD): δ 28.21 (C-5), 57.21 and 57.70 (2 × OCH_3), 62.54 (C-6), 103.67 (OCH_2O), 106.55 (C-1), 109.38 (C-4), 121.51 (C-11), 122.23 (C-8a), 123.14 (C-1a), 124.48 (C-12), 128.21 (C-13), 132.32 (C-4a), 135.37 (C-12a), 146.38 (C-8), 149.97 (C-2 and C-10) and 152.04 (C-3 and C-9); EIMS, *m/z* (%): 353 $[MOH]^+$ (39), 337 $[M + 1]^+$ (94), 336 $[M]^+$ (78), 322 $[MOH–OCH_3]^+$ (37), 320 (28), 94 (18), 52 (34), 50 (100), 46 (24), 28 (38) and 18 (96).

Jatrorrhizine (**2**) was isolated as brown needles, mp 204 °C UV λ_{max}^{MeOH} nm: 224, 263 and 349; +$AlCl_3$/HCl: 224, 263, 348; +NaOMe: 215, 246, 387; IR ν_{max}^{Nujol} cm^{-1}: 3350, 1630, 1600, 1560, 1455, 1360, 1325, 1265, 1235, 1100, 1020, 960, 900 and 870; ^{1}H NMR (250 MHz, CD_3OD): δ 3.21 (2H, t, J = 6 Hz, H-5), 4.12 (3H, s, C-2 OCH_3), 4.18 (3H, s, C-10 OCH_3), 4.27 (3H, s, C-9 OCH_3), 5.03 (2H, t, J = 6.1 Hz, H-6), 6.95 (1H, s, H-4), 7.73 (1H, s, H-1), 8.07–8.15 (2H, d, J = 9 Hz, H-11 and H-12), 8.84 (1H, s, H-13) and 9.85 (1H, s, H-8); ^{13}C NMR (62.5 MHz, CD_3OD): δ 27.7 (C-5), 57.0, 57.4 and 57.7 (3s, 3 × OCH_3), 62.6 (C-6), 110.1 (C-1), 116.0 (C-4), 119.4 (C-8a), 121.0 (C-11), 123.2 (C-4a), 124.4 (C-12), 128.2 (C-13), 130.3 (C-1a), 135.81 (C-12a), 141.0 (C-13a), 142.0 (C-10), 145.8 (C-2), 146.2 (C-8), 149.8 (C-9) and 151.8 (C-3); EIMS, *m/z* (%): 339 $[MH]^+$ (51), 322 $[MH–CH_3]^+$ (100), 307 $[M–OCH_3]^+$ (27), 294 (20), 278 (18), 265 (10), 236 (6), 220 (4), 162 (10), 137 (38), 127 (8), 96 (4), 36 (6) and 18 (18).

Cells

MCF-7/6, a variant of the MCF-7 cell family, was obtained from Dr Henri Rochefort, Unité d'Endocrinologie Cellulaire et Moleculaire, Montpellier, France. MCF-7 cells were originally established from a pleural effusion of a breast adenocarcinoma patient.[54] These cells, whose identity was confirmed in our laboratory,[55] are both invasive in vitro[52] and in vivo.[56] MCF-7/6 cells were maintained in 25 cm^2 Falcon tissue culture flasks (Becton Dickinson Europe, Meylan, France) in a mixture of Dulbecco's modification of Earle's Medium and Ham F12 (50:50; Flow, Irvine, U.K.), supplemented with 0.05% glutamine (w/v), 250 IU/mL penicillin, 100 μg streptomycin, 2.5 μg/mL amphotericine B and 10% fetal bovine serum.

Assay for invasion

The assay consists of three-dimensional confrontations between tumour cells and normal tissue.[23] Fragments of nine-day-old embryonic chick heart were precultured and selected for a diameter of 0.4 mm. These precultured heart fragments (PHF) were confronted individually with an aggregate (diameter of 0.2 mm) of MCF-7/6 cells, first on top of semi-solid agar overnight to allow attachment, and subsequently in liquid medium for eight days in suspension culture. The cultures were then fixed individually in paraffin and serially sectioned for histological analysis. In order to evaluate the interaction between the MCF-7/6 cells and PHF, the sections were stained with haematoxylin–eosin or with an immunohistochemical technique to reveal chick heart antigens.

Treatments

All compounds were dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 10^{-1} M from which further dilutions in culture medium were made. For each compound, two confrontations were treated in suspension with 100 μM for eight days. If this concentration appeared to be anti-invasive or cytotoxic, lower concentrations (10, 1 and 0.1 μM) were applied. Control cultures were treated with solvent alone (DMSO at corresponding concentrations).

Assay for growth

Aggregates of MCF-7/6 cells and PHF were cultured separately in the same conditions as described for the

assay for invasion. Their larger (a) and smaller (b) diameters were measured with a microscope (× 25) at the start and after eight days of incubation. The volumes of the cultures were calculated in accordance with the formula $V = 0.4 \times a \times b^2$.[57] At least six cultures were measured for each treatment.

MTT assay for cytotoxicity

MCF-7/6 cells were cultured and treated in Nunclon microtitre plates (Nunc, Roskilde, Denmark) at an initial concentration of 1.5×10^4 cells/well for five days. Then the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for 2 h, and solubilized with DMSO.[58] The formazan reaction product was read at 490 nm with a V_{max} ELISA reader (Molecular Devices, Palo Alto, CA, U.S.A.). At least 12 cultures were analysed for each treatment.

Assay for cell number

Cultures of MCF-7/6 cells in Nunclon microtitre plates, parallel to the ones described for the MTT assay, were fixed after five days with 50% trichloroacetic acid in water at 2 °C for 1 h, and stained with 0.4% sulforhodamine B in 1% glacial acetic acid at room temperature for 30 min.[59] Protein-bound stain was solubilized and read at 490 nm with the V_{max} ELISA reader. The correlation with cell number was initially confirmed by cell counts using a Bruker counting chamber. At least 12 cultures were analysed for each treatment.

TUNEL assay for apoptosis

Labelling of DNA strand breaks usual for apoptosis with the terminal deoxynucleotidyl transferase-mediated dUPT nick end labelling (TUNEL) technique was performed using the 'In Situ Cell Death Detection Kit, Fluorescein' from Boehringer Mannheim (Mannheim, Germany). We cultured and treated 3×10^5 MCF-7/6 cells in 25 cm^2 Falcon tissue culture flasks for six days. Flow cytometry (FACSort, Becton Dickinson, Mountain View, CA, U.S.A.) on 2×10^6 trypsinized cells was used to detect DNA strand breaks. Cell nuclei treated with 1 μg/ml DNAse I were used as a positive control for apoptosis. All experiments were done at least twice.

Assay for fast cell aggregation

MCF-7/6 cells were treated for eight days, and detached from their plastic culture substratum by a technique which preserves the cell–cell adhesion molecule E-cadherin.[60] Cell aggregation was inferred from comparing the initial particle size distribution profile of the cell suspension with the profile after 30 min of incubation in a calcium-containing buffer. These profiles were obtained with a LS 200 Particle Size Analyzer (Coulter, Miami, FL, U.S.A.) in accordance with the Fraunhofer optical model (0.4–2000 μm).

Statistics

Student's *t*-test was used for statistical evaluation of the data, except for profile comparisons in the assays for apoptosis and fast cell aggregation, where the Kolmogorov–Smirnov method was used.

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